Universitat Autònoma de Barcelona

Genetic and molecular study of Brassinosteroid receptors in Sorghum bicolor

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CRAG CENTRE DE RECERCA EN AGRIGENÒMICA

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PhD Dissertation

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Abstract

Brassinosteroids have been proven to be of great importance for the development of plants but also in the adaptation of plants to different biotic and abiotic stresses. Our previous work in Arabidopsis showed that BRL3, a discretely expressed Brassinosteroid receptor, intervenes in the adaptative response of plants to drought, and BRL3 overexpressing plants were tolerant to drought. Therefore, we wanted to test whether this adaptative mechanism is conserved in one of the most important crops in arid and semiarid zones of the planet, sorghum.

In the first chapter, we identified the two Brassinosteroid receptor genes of sorghum. Their genetic sequence and the protein 3D structure of BRI1 was analyzed showing a high degree of conservation. Furthermore, we tested their functionality through heterologous complementation in Arabidopsis.

In the second chapter, a mutagenized collection was screened to isolate six sorghum brassinosteroid receptor mutants. The plants were backcrossed to eliminate background mutations and to obtain segregant populations for plant phenotyping. Furthermore, different lines were subjected to drought and elevated temperature stresses, showing that both receptors control abiotic stress responses.

The third chapter constitutes an effort to establish sorghum transformation protocol in our laboratory. Sorghum is known to be a recalcitrant specie for transformation. Thus, we established a collaboration with the laboratory of Prof. Godwin (Queensland Alliance for Agriculture And Food Innovation, The University of Queensland, Brisbane, Australia). The results obtained at CRAG for two years were compared with the results obtained during an international stay in QAAFI. The methodologies and the candidate sorghum transgenic plants will be a useful tool for further research.

Overall, the aim of the present PhD thesis is to contribute to the translational research from fundamental biology to agriculture. Towards this direction, we managed to set Sorghum research at CRAG, adapt protocols from Arabidopsis to Sorghum, and set conditions for drought and elevated temperature experiments with this resilient crop. Furthermore, the scarce genetic materials for brassinosteroid research in sorghum have been extended, providing valuable data and tools for future research in this resilient cereal to the upcoming environmental assaults.

Resumen

Los brasinosteroides son fitothormonas esenciales para el desarrollo de las plantas y su adaptación a diferentes tipos de estrés ambiental. Nuestro trabajo previo en Arabidopsis demostró que BRL3, uno de los receptores de brasinosteroides, interviene en la respuesta adaptativa de estas plantas a la sequía. Las plantas sobreexpresoras de BRL3 toleran mejor la sequía sin penalizar el rendimiento. Por lo tanto, queríamos comprobar si este mecanismo de adaptación está conservado en uno de los cultivos más importantes en las zonas áridas y semiáridas del planeta, el sorgo.

En el primer capítulo, identificamos los dos genes receptores de brasinosteroides en el genoma de *Sorghum bicolor.* Su secuencia genética y la estructura proteica 3D de BRI1 fue analizada mostrando un alto grado de conservación. Además, comprobamos que son funcionales mediante complementación heteróloga en Arabidopsis.

En el segundo capítulo, se identificaron varios alelos mutantes en los receptores de brasinosteroides a partir de una colección existente de mutantes. Las plantas fueron retrocruzadas dos veces, para eliminar mutaciones en el fondo genético y así obtener poblaciones segregantes para el fenotipado de las plantas. Asimismo, diferentes de estas líneas fueron sometidas a caracterización fisiológica, en ensayos de estrés por sequía y a temperaturas elevadas.

En el tercer capítulo se reportan los avances hacia el establecimiento de un protocolo de transformación de sorgo en nuestro laboratorio. El sorgo es conocido como una especie recalcitrante a transformación. Así pues, establecimos una colaboración con el laboratorio del Prof. Ian Godwin, en Australia. Los resultados obtenidos en el CRAG durante dos años diferentes fueron comparados con los resultados obtenidos durante mi estancia internacional en QAAFI (Australia). Obtuvimos plantas transgénicas de sorgo que serán útiles para investigaciones futuras.

En general, el objetivo de esta tesis doctoral es contribuir a la biología traslacional y aportar valor en la agricultura frente a los retos del cambio climático. En esa dirección, logramos establecer de manera pionera una línea de investigación en sorgo en el CRAG, adaptar y desarrollar nuevos protocolos de Arabidopsis a sorgo, y establecer condiciones para el fenotipado en condiciones de sequía y elevadas temperaturas. Además, el escaso material genético para la investigación de brasinosteroides en sorgo ha sido ampliado, proveyendo valiosas herramientas para futuras investigaciones en este cultivo tan resiliente a condiciones ambientales extremas.

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Abbreviations

Aa: Amino acid

ABA: Abscisic Acid

BAK1: BRI1 Associated Kinase-1

BC(#)F(#): Backcrossing number # Filial number #

BES1: BRI1 EMS-Suppressor 1

BIM1: BES1-Interacting MYC-Like 1

BIR3: BAK1-Interacting RLK

BKI1: BRI1 Kinase Inhibitor

BL: Brassinolide

BLSM: Block Substitution Matrix

BR(s): Brassinosteroid(s)

BR6OX: Brassinosteroid-6-Oxidase

BRAVO: Brassinosteroids at Vascular and Organizing center

BRI1; Brassinosteroid Insensitive 1

BRL: BRI1-LIKE

BSK(s): Brassinosteroid Signaling Kinase(s)

BZR1: Brassinazole Resistant 1

CBF: C-REPEAT/ DRE Binding Factor

CDG1: Constitutive Differential Growth 1

CIM: Callus Induction Media

CPD: Constitutive Photomorphogenic Dwarf

CRY1: Cryptochrome 1

DLT: Dwarf and Low Tillering

DWF11: Dwarf 11

DWF7: Dwarf 7

ECD: Extra Cellular Domain

EMS: Ethyl methanesulfonate

ER: Endoplasmic

FC: Field Capacity

FLC: Flowering Locus C

FLS2: Flagellin Sensitive 2

Fv/Fm; Variable Fluorescence / Maximal Fluorescence

GW5: Grain Width 5

ho: Homozygous

ht: Heterozygous

HY5: Elongated Hypocotyl 5

IBH: ILI1 Binding BHLH

ICE1: Inducer of CBF Expression

ID: Island Domain

ILI1: Increased Lamina Inclination 1

KD: Kinase Domain

LD: Long-Day

LIC: Leaf and Angle Increased Controller

LRR(s): Leucine Rich Repeat

NPQ: Non-Photochemical Quenching

OM: Osmotic Media

PAR: Photosynthetically Active Radiation

PI: Propidium Iodide

PIF4: Phytochrome Interacting Factor 4

PIF5: Phytochrome Interacting Factor 5

PP2A: Protein Phosphatase 2

PSII: Photosystem II

QC: Quiescent Center

RD26: Responsive to Disecation 26

REM: Regeneration Media

RGS1: Regulator of G protein Signaling

RLK(s): Receptor Like Kinase(s)

ROS: Reactive Oxygen Species

RWC: Relative water content

SLG: Slender Grain

TF(s): Transcription factor(s)

TMD: Trans-Membrane Domain

Wt: Wild-type

CHAPTER 1:

GENERAL INTRODUCTION

Brassinosteroid hormones in plants

Multicellular organisms use signaling molecules, called hormones, for intercellular communication to regulate their development, organize their growth and trigger adaptive responses (Kushiro et al., 2003). Steroids are a class of hormones important for normal growth, development, and differentiation in multicellular organisms (Friedrichsen & Chory, 2001). In plants, Brassinosteroids (BRs), comprise a group of polyhydroxylated steroid hormones essential for plant growth and development, since they regulate processes like cell elongation, cell division, and differentiation, reproduction, but also the adaptation of plants to environmental conditions (Nolan et al., 2020; Planas-Riverola et al., 2019). The most active BR is brassinolide (BL), which was first identified in rapeseed (*Brassica napus*) pollen as responsible for promoting the elongation of bean internodes (Grove et al., 1979; Mitchell et al., 1970).

BRs play a myriad of roles in the plant

The isolation of BR deficient and insensitive mutants in *Arabidopsis thaliana* (Arabidopsis) established BRs as important plant-growth regulators affecting cell elongation, division and differentiation (Clouse et al., 1993; Kauschmann et al., 1996; Mandava, 1988; Szekeres & Né, 1996). BR loss-of-function mutants showed multiple developmental defects like dwarfism, a dark-green color and de-etiolated growth in darkness. The exogenous application of BRs can rescue these phenotypes in BR-deficient mutants, affecting the biosynthetic enzymes of the hormone, while BR insensitive mutants and their growth defects are not reverted by treatments with BL, since they are defective in the BR signal transduction.

BR are major regulators of plant development

The first evidence regarding the implication of BRs in the cell differentiation processes was found in explanted mesophyll cells of *Zinnia elegans* and *Helianthus tuberosum,* where tracheid formation was promoted by exogenous application of BL at nanomolar concentrations (Clouse & Zurek, 1991; Iwasaki & Shibaoka, 1991). In Arabidopsis, several studies also revealed the importance of BRs in cell differentiation. The BR-deficient mutants *cpd* and *dwf7,* which mutations affects *Constituve Photomorphogenic Dwarf (CPD)* and *Dwarf 7* (*DW7)*,

genes involved in BR biosynthesis, exhibited an increase in the proportion of phloem over xylem cells, and *dwf7* showed a reduction in the number of vascular bundles (Choe et al., 1999; Szekeres & Né, 1996). In addition, the differentiation of tracheary elements (TE) was found to be promoted by BRs. More specifically, it was found that BRs levels reached a maximal concentration at the initiation of the TE differentiation process (Yamamoto et al., 2001). In Arabidopsis, BR-receptor mutants exhibit defective xylem differentiation (Caño-Delgado et al., 2004), and BR levels modulate the number of vascular bundles and together with local auxin concentration levels regulate the radial pattern of vascular bundle formation (Ibañes et al., 2009). Apart from vascular tissue, BRs are involved in the differentiation of other cell types. For example, BIN2, a central regulator of BR signaling, is responsible for the formation of stomata (Gudesblat et al., 2012; Kim et al., 2012). BRs also control cell fate determining the formation of root hairs in Arabidopsis roots (Cheng et al., 2014) and the differentiation of columella stem cells in the root tip of Arabidopsis (Lee et al., 2015).

The first BR-related mutant, *det2*, was found in a genetic screening for seedlings with de-etiolated phenotype, evidencing a role of BR in the control of light-induced responses. This dwarf mutant showed short hypocotyl and opened cotyledons grown in dark (Chory et al., 1991). De-etiolated 2 (*DET2)* gene encodes one of the essential enzymes involved in the biosynthesis of BRs (Fujioka et al., 1997). Another BR biosynthetic enzyme, *Dwarf 4* (DWF4), was also identified due to the phenotype of *dwf4*, displaying similar phenotypes to those of *det2*. Dark-grown *dwf4* mutants were unable to elongate the hypocotyls and cotyledons were partially opened. When grown with light, they also display a strong dwarf phenotype which is caused by the lack of cell elongation (Choe et al., 1998; Azpiroz et al., 1998).

Using the Arabidopsis primary root as a mode, our laboratory has showed that BRs control cell division in Arabidopsis root meristems (González-García et al., 2011), and the division of quiescent center (QC) cells of the stem cell niche. The QC, acts as stem cell reservoir, is regulated by the expression of Brassinosteroids At Vascular and Organizing center (*BRAVO)*, a target gene of BES1 and BZR1 (Vilarrasa-Blasi et al., 2014).

BRs also affect several processes in plant reproduction. BRs delay floral transition by regulating the activity of Flowering Locus C (FLC) (Li et al., 2018), while BES1 and BZR1, the two main TFs controlling BR target-gene expression, fine-tune environmental responses with other components of the flowering machinery (Jiang et al., 2015; Wang et al., 2019; Zhang et al., 2013). BRs were also found to regulate seed size and shape through transcriptional regulation by BZR1. In homozygosity, *bri1* and other BR deficient mutants showed less elongated seeds, but the seed width of those mutants was increased in heterozygosity in comparison to the *Wt* (Jiang et al., 2013). Furthermore, male sterility defects are found in most

of the BR-defective mutants, with short filament and decreased pollen grain number (Ye et al., 2010).

Studies in several crops have proved that the exogenous application of BRs enhance resistance to pathogenic infections (Hussain et al., 2020; Nakashita et al., 2002). OsBSK1-2 has been proposed to be a major player in rice immunity (Wang et al., 2017). In Arabidopsis, BRs have been proposed to transcriptionally control the trade-off between growth and immunity (Lozano-Durán et al., 2013). BAK1, co-receptor for the signaling of BRs is also a co-receptor of Flagellin-Sensitive 2 (FLS2), which is responsible of binding flg22, a 22 amino acid peptide found in different pathogens and when detected, triggers immunity responses in plants (Chinchilla et al., 2007). Furthermore, the availability of BAK1 seems to be critical for its different co-receptors. The binding of BRs to BRI1-BAK1 heterodimer could compete for the activation of immune signaling pathways (Belkhadir et al., 2012; Ortiz-Morea et al., 2020).

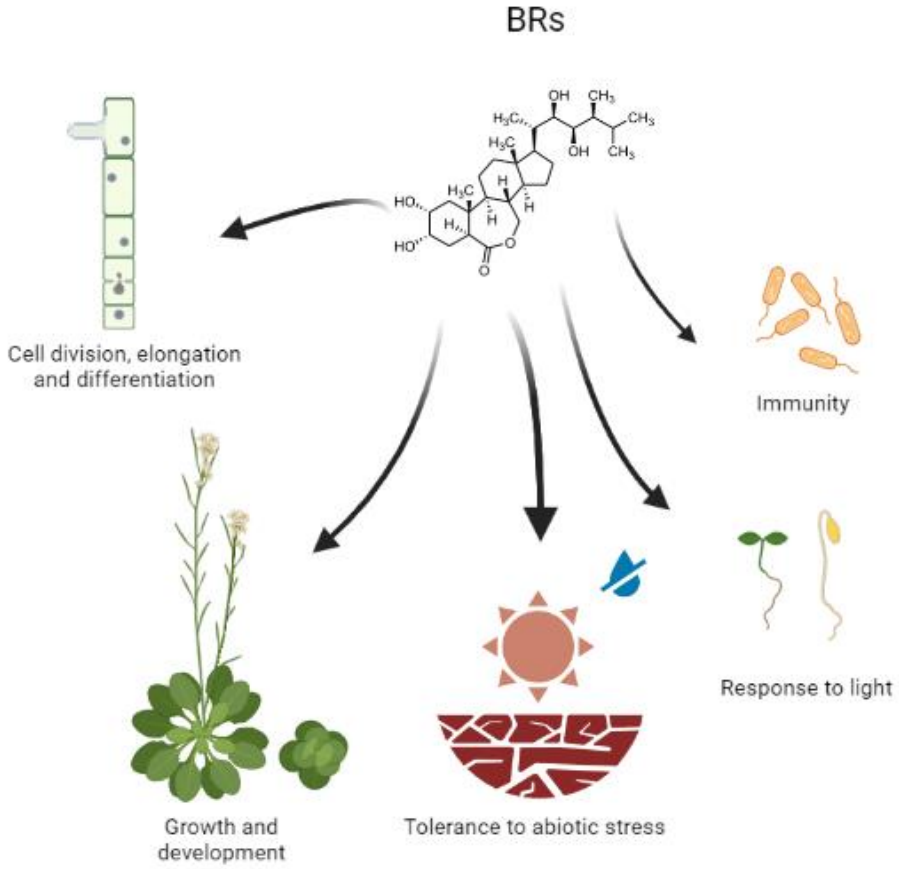


Figure 2. Functions of BRs

A molecule of BL is represented. BRs are growth and development regulators controlling cell division, elongation and differentiation. They also control responses to light and to biotic and abiotic stress conditions.

The role of BRs in adaptive responses

BRs play important roles in the signal transduction of environmental cues regulating plant adaptation to the changing light and temperature. Several photoreceptors bind BES1 and BZR1 to regulate photomorphogenesis during seedling development. The interaction between BRs and PIF4, an integrator of light and temperature inputs (Zhao & Bao, 2021), has been already described. BZR1 and BES1 bind Phytochrome Interacting Factor 4 (PIF4) in the nucleus to regulate the transcription of different genes and enhance BR biosynthesis at dawn (Martínez et al., 2018; Oh et al., 2012), while in the cytoplasm BIN2 phosphorylates PIF4 and Phytochrome Interacting Factor 5 (PIF5) and leads them to the proteasome for degradation. Therefore, the inactivation of BIN2 by BRs signaling stabilizes PIF4 which contributes to the hypocotyl elongation inhibition in presence of light (Bernardo-García et al., 2014). De-phosphorylated BZR1 interacts with Elongated Hypocotyl 5 (HY5), which also plays an important role in light signaling regulating cotyledon growth in darkness (Li & He, 2016). BZR1 DNA binding activity is regulated by Cryptochrome 1 (CRY1), which together with BIN2 phosphorylates BZR1 (He et al., 2019). BES1 is also affected by several photoreceptors, like UV-B Resistance 8 (UVR8), CRY1 and Cryptochrome 2 (CRY2). They all compete for BES1-Interacting MYC-like 1 (BIM1), partner of BES1 in transcription regulation of BR target genes (Liang et al., 2018; Wang et al., 2018; Yin et al., 2005). Finally, the activation of Phytochrome B (PHYB) by red light represses BR signaling through direct interaction with BES1 (Wu et al., 2019).

Under elevated temperatures, BES1 and BZR1 accumulate and promote thermomorphogenic growth together with PIF4 (Ibañez et al., 2018; Martínez et al., 2018). The accumulation of these two TFs promotes the expression of PIF4, thus increasing BR biosynthesis. At receptor level, elevated temperatures decrease BRI1 levels by ubiquitination, endocytosis, and finally degradation, which negatively affects BR signaling and promotes root growth (Martins et al., 2015, 2017). Under low temperatures, BES1 and BZR1 also accumulate in their unphosphorylated forms and promote the expression of C-Repeat/DRE Binding Factor 1 (CBF1) and C-Repeat/DRE Binding Factor 2 (CBF2) regulating cold-stress responses (H. Li et al., 2017). BIN2, can phosphorylate Inducer of CBF Expression 1 (ICE1) during prolonged cold treatment, which promotes the degradation of CBF1. CESTA (CES), a positive regulator of BR signaling, also activates CBF genes in response to low temperature. Furthermore, CESTA dephosphorylation and sumoylation in response to BRs activates cold-responsive genes from a CBF independent pathway (Eremina et al., 2016; Poppenberger et al., 2011).

The BR signaling pathway

The main BR receptor, Brassinosteroid Insensitive 1 (BRI1), was the first BR signaling component identified (Clouse et al., 1996; J. Li & Chory, 1997). It is expressed ubiquitously and, unlike animal steroids which are usually perceived in the nucleus, BRI1 is localized at the plasma membrane (Friedrichsen et al., 2000). Since then, BRI1 signaling pathway have been intensively studied.

In absence of BRs, BRI1 and the co-receptor Brassinosteroid Associated Kinase 1 (BAK1) (Nam & Li, 2002) are impeded to heterodimerize by several factors, such as Brassinosteorid Kinase Inhibitor 1 (BKI1) and BAK1 Interactor Receptor-like Kinase 3 (BIR3) (Hohmann et al., 2018; Wang & Chory, 2006). The cytosolic kinase Brassinosteroid Insensitive 2 (BIN2), when phosphorylated, negatively regulates BR signaling by phosphorylating downstream BRI1 EMS Suppressor 1 (BES1) and Brassinazole-Resistant 1 (BZR1) (Nam & Li, 2002), the two main TFs controlling the expression of BR responsive genes (He et al., 2005; Yin et al., 2002). As a result, the phosphorylation of BES1 and BZR1 leads to their cytoplasmic retention and degradation causing their inactivation (Gampala et al., 2007; Peng et al., 2008a). When BR signaling complex is activated through the perception of BL by BRI1 receptor (Hothorn et al., 2011), BRI1 and BAK1 interact, leading to the dissociation of BKI1 and BIR3 from the complex, allowing the phosphorylation of the receptors and triggering a series of signaling events (Russinova et al., 2004). Constitutive Differential Growth 1(CDG1) and Brassinosteroid-Signaling Kinases (BSKs) cytoplasmic kinases phosphorylate BRI1 Suppressor 1 (BSU1) (Kim et al., 2009; Tang et al., 2008), which promotes BR signaling by inhibiting BIN2 through proteasomal degradation (P. Peng et al., 2008). Subsequently, BES1 and BZR1 get dephosphorylated by Protein Phosphatase 2A (PP2A) proteins leading to their activation and move to the nucleus (Tang et al., 2011). BES1 and BZR1 dephosphorylated forms can function with other TFs and cofactors (Li et al., 2018; Oh et al., 2014; Yin et al., 2005) to regulate the expression of multiple genes by binding to BRRE, E-BOXES or G-BOXES DNA motifs (He et al., 2005; Sun et al., 2010; Yu et al., 2011). The tight transcriptional regulation of BRs allows plants to finely tune developmental processes and adaptative responses integrated with other hormones and environmental inputs. (Li et al., 2018; Zhao & Bao, 2021). Figure 1 summarizes the BR signaling pathway, showing the most representative effectors and events that take place in absence or presence of the BR hormone BL.

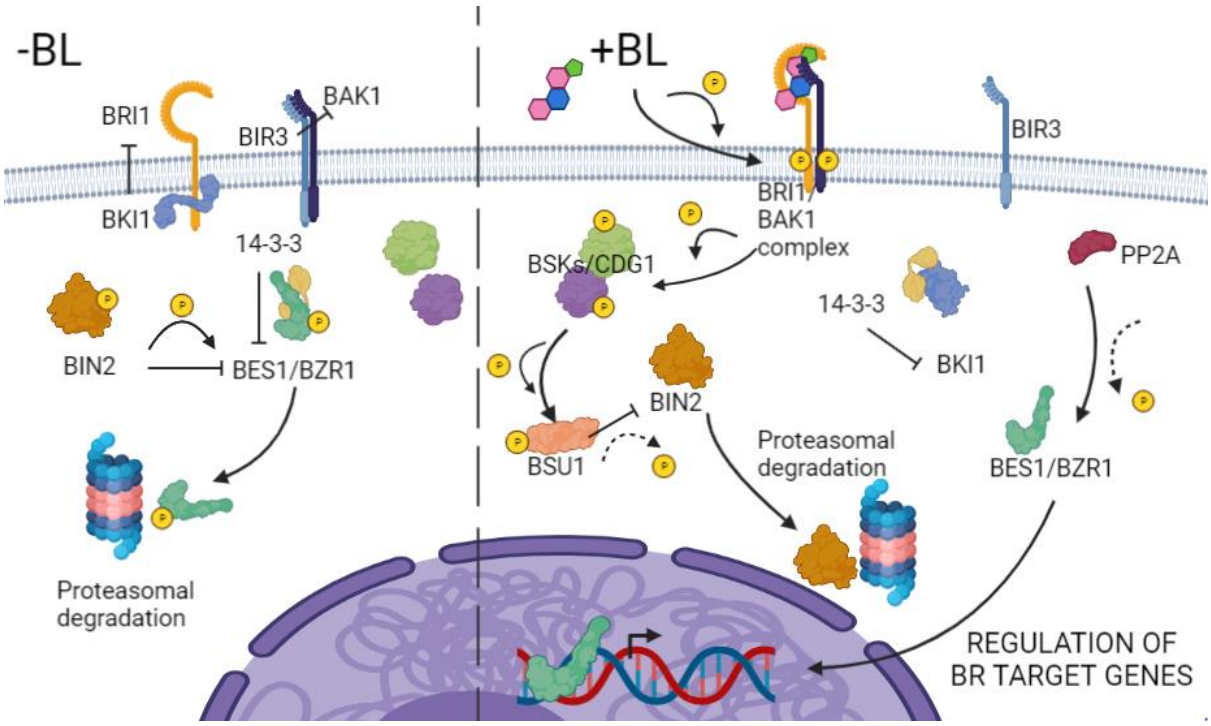


Figure 1. Schematic representation of BR signaling pathway.

In absence of BL, BRI1 kinase domain is inhibited by BKI1 and BIR3 binds BAK1 preventing the interaction of BRI1 and BAK1. Phosphorylated BIN2 inhibits BES1 and BZR1 by phosphorylation, and 14-3-3 proteins promote the degradation of both TFs. When BL is bound to BRI1, and BAK1 binds the complex, BIR3 and BKI1 are dissociated and BRI1 and BAK 1 heterodimer is phosphorylated. This triggers the phosphorylation of BSKs and CDG1 proteins, which leads to the dephosphorylation of BIN2 by BSU1, promoting BIN2 degradation. The dephosphorylation of BES1 and BZR1 by PP2A allows them to act by regulating gene transcription in the nucleus. The illustration was made with Biorender (biorender.com).

The BRI1-like family of BR receptors

In Arabidopsis, there are members of the BRI1-like family of receptor kinases: the first identified receptor BRI1 and three additional homologous proteins, the BRL1, BRL2 and BRL3. From those, only BRI1, BRL1 and BRL3, can bind BL and trigger the BR signaling cascade. BRL1 and BRL3 were shown to have a restricted vascular expression pattern and act with BRI1 regulating vascular differentiation (Caño-Delgado et al., 2004). Confocal microscopy analysis of the receptors´ translational fusions to GFP revealed that BRL1 and BRL3 are expressed along all the vasculature of the plant and BRI1 shows to be ubiquitously expressed. In the meristematic zone of the roots, the BRL1 and BRL3 vascular expression is interrupted

and recovered in the vascular initial cells by the diffusion from the QC, where they show an intense expression. On the other hand, BRI1 shows a reduction of expression in the QC (Figure 3, adapted from Fàbregas et al., 2013). Due to the discrete localization of the BRL1 and BRL3 proteins and the lack of any evident phenotype of single mutants upon normal conditions (Caño-Delgado et al., 2004), the role of BRLs remained unclear for a long time. It was first shown that the triple BR receptor mutant *bri1brl1brl3* enhanced the dwarf phenotype of the single *bri1* mutant, and that the expression of both BRL1 and BRL3 driven by *BRI1* promoter, but not *BRL2*, could restore the dwarfism of *bri1* mutants (Caño-Delgado et al., 2004).

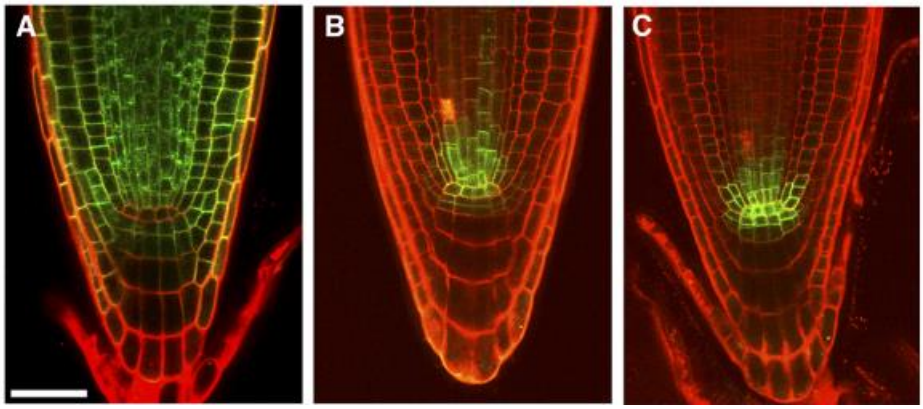


Figure 3. Native expression pattern of BRI1-like receptors in the Arabidopsis root apex. Adapted from Fàbregas et al., 2013. Expression patterns of BRI1 (A), BRL3 (B), and BRL1 (C) in Arabidopsis root tips driven by their respective native promoters. BRI1 expression is reduced in the QC, whereas BRL1 and BRL3 are enriched in these cells.

The *in vivo* interaction of BRL3 with BAK1 and BRL1, as well as the homodimerization of BRL3 proteins was demonstrated in Fàbregas et al., 2013, together with the list of proteins co-precipitating with BRL3. The lack of components of the BRL3 signalosome led to developmental defects in the root growth and insensitivity to BL promoting QC cells divisions (Fàbregas et al., 2013). Similarly, the developmental defects in the vasculature of bri1 mutant are accentuated when combined with *brl1brl3* mutants (Holzwart et al., 2018; Kang et al., 2017). Moreover, Regulator of G-Protein Signaling 1 (RGS1) cooperates specifically with BRL3 in glucose sensing and ROS production under flg22 treatment (Tunc-Ozdemir et al., 2017; Tunc-Ozdemir & Jones, 2017). It is also known that BR signaling regulates the spatial expression of *BRL3* in the meristematic provascular and QC cells by binding BES1 to a BRRE present in the promoter of *BRL3* (Salazar-Henao et al., 2016). Another example of the specificity of the different BR receptors with its partners is the inability of BRL1 to bind BKI1,

an inhibitor of BRI1 (Jaillais et al., 2011). The interaction of BRL1 and BRL3 with specific partners will be key to determine its functional specificity (Lozano-Elena & Caño-Delgado, 2019).

Spatiotemporal understanding of the BR pathway

Several studies have proven that BRs need to be transported in short distances. BRs are synthetized in the endoplasmic reticulum, and they are perceived in neighboring cells to control cell proliferation and elongation (Lozano-Elena et al., 2018; Vukašinović & Russinova, 2018). Normal root growth is tightly controlled by the BR concentration in each tissue (Vukašinović et al., 2021). It has been proven that the expression of BRI1 only in the epidermis is able to control leaf expansion, as well as the root meristem size of Arabidopsis (Hacham et al., 2011; Savaldi-Goldstein et al., 2007). Meanwhile, expressing BRI1 specifically in the phloem can restore the root length phenotype of null BR receptor plants (Kang et al., 2017).b Furthermore, BRs trigger the division of QC cells by controlling the expression of BRAVO (Betegón-Putze et al., 2021; Vilarrasa-Blasi et al., 2014). Besides the different functions of BRs in each tissue and the control of BRs biosynthesis, BRLs constitute another example of tissue specificity in BR signaling. They have a discrete localization pattern in the vascular tissues and stem cell niche, where they control adaptation to different conditions (Fàbregas et al., 2018; Lozano-Elena et al., 2022).

The role of BRs in plant adaptation to environmental stresses

Plants need to adapt to the changing environmental conditions to thrive. When the conditions are not favorable for growth, the ability of each plant to tolerate the stress can impact on its survival or, in case of crops, also the yield (Claeys & Inzé, 2013). Plants can respond in different ways to changes in the availability of water, soil salinity or extreme temperatures (H. Zhang et al., 2022). Abscisic acid (ABA) is the most studied hormone for the plant stress responses (Cutler et al., 2010), and BRs were found to play an antagonistic role to ABA (Zhang et al., 2009).Their interaction takes place in the nucleus by the differential regulation of BES1, BZR1 and other ABA-induced TFs (Cai et al., 2014; Ryu et al., 2014; Yang et al., 2016), but also in the cytoplasm through BIN2 (Li et al., 2020; H. Wang et al., 2018). BRs also have a great impact on the survival of plants under freezing temperatures. It is known that BIN2 regulates the stability of ICE1 in response to lower temperatures (Keyi et al., 2019) and *bes1-*

D and *bzr1-D* Arabidopsis mutants, with constitutive BR signaling activation, showed freezing tolerance (H. Li et al., 2017). BES1 and BZR1 are also regulated in elevated temperatures (Ibañes et al., 2018; Martínez et al., 2018), and BR application induce the expression of heat-shock proteins protecting the translational machinery (Dhaubhadel et al., 2002). A transcriptomic analysis of BR application under abiotic stress showed that BRs function under salinity and high temperature control multiple cellular processes (Divi et al., 2016).

Treatments with BRs have shown to alleviate stress symptoms during unfavorable conditions like high-salinity, drought, extreme temperatures or even pathogen infection in several crops and Arabidopsis (Ali et al., 2013; Anjum et al., 2011; Jiang et al., 2013; Kagale et al., 2007; Yuan et al., 2010). However, the implication of BRs in stress tolerance is quite complex. The activation of BES1 under drought conditions has been shown to be detrimental for plant survival, and *bri1* mutants, that cannot activate BES1, are more tolerant to the stress than *Wt* plants (Nolan et al., 2017). The expression of some WRKY TFs induced by BR application, represses the transcription of dehydration-inducible genes and the triple mutant of these TFs showed enhanced resilience to drought (Chen et al., 2017). The transcription of BR-regulated genes under stress conditions is also regulated by the interaction of BES1 with other TFs like Responsive to Desiccation 26 (RD26) or TINY (Xie et al., 2019; Ye et al., 2017). Despite the application of BRs promotes the tolerance to stress*,* molecular data indicates that the activation of the canonical BR signaling pathway is detrimental for plant tolerance to drought.

Our contribution to a better understanding of the role of BRs in drought, demonstrated that BRL3 overexpressing plants are more resistant to drought due to an enhanced accumulation of sugars to the roots among other factors. First, we showed that mutants affected in the complexes formed between BR receptors and the co-receptor *BAK1* have reduced sensitivity to osmotic stress. While BRL3 overexpressing plants showed increased hydrotropic responses, plants with defective BR signaling responded less. Experiments performed to quantify the sensitivity to osmotic stress showed that overall, BRL3 contributes in sensing osmotic imbalances. Roots of BRL3 overexpressing plants showed increased cell death under sorbitol treatment while BRs insensitive mutants behaved opposite. Other physiological parameters were measured under water withholding conditions to understand the mechanisms that make BRL3 overexpression and BR deficient plants resistant to drought. First, BR insensitive plants showed increased tolerance to 12 days of water withholding, which could be explained because BR-insensitive plants are smaller, consequently showing a reduction in transpiration and therefore, avoiding drought. Furthermore, the measurements of photosynthetic efficiency and relative water content at similar stress levels demonstrated that BR insensitive plants performed better than *Wt* plants. BRL3 overexpressing plants, showed

also enhanced tolerance to drought in the different tested parameters, including survival. This result cannot be attributed to a smaller size and the differences in transpiration were not significant. The integration of the metabolomic and transcriptomic experiments during a time course of drought stress suggested that BRL3 promotes the accumulation of osmoprotectant metabolites in the roots by controlling the expression different enzymes and TFs such as RD26, an inhibitor of BES1 under drought conditions, and (Fàbregas et al., 2018; Lozano-Elena et al., 2022). Therefore, the signaling by BRL3 could activate plant cell-specific mechanisms under drought stress.

Sorghum, a resilient crop

Sorghum bicolor (sorghum) is a versatile crop: its grain is a source of protein for human and animal consumption, and it is used in the brewing industry to produce beer and other liquors. Moreover, it has a high biomass production which serves as forage and for biofuel production. Its popularity in occidental countries is increasing as a gluten-free alternative to wheat and as a source of antioxidant and other beneficial compounds for human health (Aruna & Visarada, 2018), but sorghum domestication started between 5000 and 3000 years ago in Africa (Taylor, 2018). It is the fifth most cultivated cereal globally (Statista, 2022), it feeds over 500 million people and is a subsistence crop in the arid and semi-arid regions of the world, where other crops cannot grow. In these areas, it has been adapted to unpredictable weather with high temperatures, limited rainfall, and poor soils. The highly efficient C4 photosynthesis, together with a deep root system, and other adaptations to drought such as wax production or leaf rolling to prevent evaporation, makes sorghum a stress-resilient crop.

Sorghum is a perennial cereal grass from the family Poaceae, Panicoid sub-family, being a close relative of sugarcane and corn, from the Andropogonae tribe (Swigoňová et al., 2004). It takes around 4 months in most of the cultivars to complete their cycle and they bear hermaphrodite flowers that are usually self-pollinated. Sorghum plants can reach up to 4 meters tall for some landraces, but most grain sorghum cultivars were bred incorporating multiple dwarfing genes during the “Green Revolution" restricting their size to approximately 1.5 meters (Xin et al., 2021). Sorghum has a relatively small genome of ~732 Mbp (Deschamps et al., 2018) compared to its close relative *Zea mays* (2.4Gb). Moreover, it is diploid, and has a low gene redundancy (Paterson et al., 2009). It has also been established as a genetic model for C4 photosynthesis bioenergy crop (Mullet et al., 2014) and it grows a prominent primary root, suitable for confocal microscopy, which make it useful for studies

involving root development (Blasco-Escámez David, 2017; Rico and Blasco-Escamez, unpublished). The genetic and anatomical advantages, together with its inherent ability to overcome drought and heat stresses, made sorghum a good model to challenge BR signaling and its role upon abiotic stress responses.

BR signaling in sorghum and other crops

As mentioned above, BR application is beneficial for plants against different kind of stresses, but the use of BRs in agriculture is not limited to stress. Other economically important traits, such as fruit size, ripening, quality and yield are also improved by BRs application in multiple species (Ali, 2017; Coll et al., 2015; Gomes, 2011; Vriet et al., 2012). It is likely that many BRs signaling, and biosynthetic components are conserved in most vascular plants, and different mutants or transgenic lines reducing BR have been described in different crop species like pea, tomato, corn, wheat, potato or soy. Consistently to Arabidopsis, all of them presented dwarf phenotypes or reduced plant size, and in the most severe mutations, also sterility (Bishop et al., 1999; Koka et al., 2000; Liu et al., 2007; Nomura et al., 2003; Fang et al. 2020 Peng et al., 2016, Huang et al., 2021).

There have been efforts to characterize BR signaling pathway in rice in which the key modules of the signaling, like BR receptors and BAK1, BIN2, or BES1 and BZR1 are conserved (Gao et al., 2019; Tong & Chu, 2018; Yamamuro et al., 2000). Although some of the components from Arabidopsis BR signaling like BSU1, or PP2A are not found in rice and maize or vice versa, like Grain Width 5 (GW5), Dwarf and Low Tillering (DLT) , Leaf and Tiller Angle Increased Controller (LIC) or Taihu Dwarf 1 (TUD1) (Liu et al., 2017; C. Zhang et al., 2014). BRI1-like gene expression was studied in the mild *d61-4* allele of *BRI1* in rice, which had a strong dwarf phenotype in the shoots but no in the roots. Conversely to *OsBRL1* and *OsBRL3* transcripts, *OsBRI1* is strongly expressed in shoots and not in the roots, while in the *d61-4* background *OsBRLs* transcripts were strongly upregulated in the roots. *OsBRLs* were proposed to alleviate the absence of BRI1 in the roots (Nakamura et al., 2006). In *Brachypodium distachyon* and *Solanum* lycopersicum, BR signaling pathways have also been studied. Some studies showed partially conserved pathways, even though the heterologous complementation of *bri1-5* mutant did not manage to recover its dwarf phenotype. The BR biosynthetic pathway is similarly conserved, since enzymes like DWARF4, DET2 or BR6OX are conserved (Corvalán & Choe, 2017; Hong et al., 2002; Makarevitch et al., 2012; Xia et al.,

2021) but others like DWARF11 from rice and maize, have not been found in Arabidopsis (Sun et al., 2021).

Multiple studies have demonstrated that plants with altered BR pathways can improve yield and biomass production by regulating seed size, plant architecture in crops like maize or rice (Sakamoto et al., 2006; Sun et al., 2021; Sun et al., 2015) and the formation of wood and fibers in poplar or cotton (Jiang et al., 2021; Jin et al., 2017; Yang et al., 2014). In tomato, BRI1 overexpressing plants showed increased seed germination and vegetative growth, ethylene production and accumulation of carotenoids and better yield and quality attributes (Nie et al., 2017). Root architecture and lateral root formation have also been shown to be tightly controlled by BRs in wheat. In this context, BR treatments showed a reduction in root diameter due to a reduction in the growth of the vasculature, while brassinazole (BRZ), a potent inhibitor of BL biosynthesis, increased it. Furthermore, lateral root formation was promoted by BRs, while BRZ induced the emergence of the lateral root buds (Hou et al., 2019). Interestingly, a role for BR controlling sex determination was found in a DET2 homolog maize mutant, *na1* (Hartwig et al., 2011). The role of BRs in the epigenetic control of several monocot crops has also been studied and suggested potential roles of histone modifications in BR signaling (Zheng et al., 2021).

The tolerance to drought promoted by BR application is not always translated in the same way by enhancing BR signaling. In maize, drought-tolerant genotypes have higher endogenous levels of BR than drought-sensitive genotypes (Tůmová et al., 2018), and ZmBSK1 has been shown to phosphorylate an essential regulator of plant tolerance to drought. In *Brachypodium* the downregulation of BRI1 led to a dwarf phenotype with enhanced drought tolerance (Feng et al., 2015). Contrasting with the results obtained in Arabidopsis and other species, in which plants overexpressing BR receptors are more tolerant to drought (Fàbregas et al., 2018; Xiang et al., 2021), BRI1 overexpressing plants in tomato are more susceptible (Nie et al., 2019) while plants with constitutive BES1/BZR1 signaling show enhanced tolerance to salt stress (Jia et al., 2021). In *Triticum aestivum*, BRI1 promotes tolerance to the combined stresses of high light intensity and elevated temperatures. Two knock-down mutants *Tabri1a1* and *Tabri1d1* presented increased sensitivity to high light and elevated temperatures.

Despite some beneficial aspects found in BR altered lines, to our knowledge only two BR signaling mutants are widely used in agriculture. In barley, the *uzu* line presented a semi-dwarf phenotype as a result of a single-amino acid substitution in the kinase domain of BRI1 and has been extensively used for plant breeding in Japan (Chono et al., 2003). The second is the *dw1* mutant gene from sorghum, present in the BTx623 elite inbred line, which was the first reference genome available for sorghum (Yamaguchi et al., 2016). DW1 was found to be a

positive regulator of BR by establishing the localization of BIN2 in the nucleus and inhibiting its transportation to the cytoplasm (Hirano et al., 2017).

The knowledge about BRs in sorghum is scarce. However, many components of the sorghum BR pathway have been identified, and allelic variation in 16 BR-related genes alter plant architecture parameters that are interesting for agriculture, like plant and panicle size, leaf angle or flowering time (Mantilla-Perez et al., 2014). A more recent study also supported the fact that leaf angle is regulated by genes like *Slender Grain (SLG)*, *Increased Lamina Inclination* (*ILI1)* or Ili1 Binding BHLH 1 (*IBH)* (Zhang et al., 2009; Zhi et al., 2022), which have been probed to also control leaf angle in rice through BR signaling (Z. Feng et al., 2016; Zhang et al., 2012). Similarly, *Triticum aestivum bri1* mutants are more compact than Wt plants by exhibiting erected leaves. The effects of application of BRs in sorghum roots also showed that BL controls root growth and promotes QC divisions at high BL concentrations, and it had a similar effect than in wheat, by controlling vascular differentiation (Blasco-Escámez et al., 2017)

OBJECTIVES

The general objective of this PhD thesis was to study the role of brassinosteroid receptors in sorghum and investigate if their manipulation regulates adaptation responses to abiotic stresses such as elevated temperatures or drought.

In particular, the following specific objectives have been accomplished:

1. Identification and validation of brassinosteroid receptors in sorghum.

2. Isolation and characterization of brassinosteroid receptor mutant from sorghum from an existing EMS mutant collection.

3. Physiological analysis of sorghum BR-receptors mutants to drought and elevated temperature.

4. Set up sorghum transformation methods towards the generation of transgenic sorghum plants with increased expression of brassinosteroid receptor *BRL1/*3.

CHAPTER 2:

IDENTIFICATION OF SORGHUM BRASSINOSTEROID RECEPTORS

Introduction

BR hormones and their physiological responses have been identified, apart from vascular plants, also in algae and non-seed plants (Stirk et al., 2013; Yokota et al., 2017). However, BR receptors appeared in the common ancestor of angiosperms and gymnosperms with the presence of the characteristic Island Domain (ID), which is responsible of the binding of the hormone (Ferreira-Guerra et al., 2020; Wang & Mao, 2014). BR receptors are part of the protein superfamily Receptor Like Kinases (RLKs) which consists of an extracellular domain (ECD), a single-pass trans-membrane domain that anchors it to the plasma membrane, a juxta membrane domain, and a cytosolic kinase domain (KD) for signal transduction (Gou et al., 2010; Shiu & Bleecker, 2001). RLKs can be classified by their extracellular N-terminal domain or ECD (Kajava, 1998). Leucin-Rich Repeats-RLKs (LRR-RLKs) are the most abundant type of RLKs (Shiu & Bleecker, 2001)) and they are characterized by a consensus motif of 20-30 amino acids including the sequence LxxLxLxxNxL which is highly repeated in their ECD. These domains allow the interaction with other proteins or several ligands (Matsushima et al., 2007). As mentioned before, what characterizes BR receptors is the interruption of the LRR tandem repeats by the ID, which creates a surface pocket that folds back inside the LRR super-helix for the binding of BR hormones and consequently trigger BR signaling (Hohmann et al., 2017; Hothorn et al., 2011). *bri1* mutants or transgenic lines affecting the expression of BRI1 have been reported in Arabidopsis and many other different species such as barley, maize, tomato, pea, or rice (Chono et al., 2003; Kir et al., 2015; Koka et al., 2000; Li & Chory, 1997; Nomura et al., 2003; Yamamuro et al., 2000). The misexpression of *bri1* leads to plants with dwarf-phenotype, dark green leaves with altered morphology, short petioles and, for the most dramatic mutations, male sterility. Regarding the other two BR receptors of Arabidopsis, BRL1 and BRL3, mutants of these receptors have been shown to regulate vascular differentiation and root growth (Caño-Delgado et al., 2004; Fàbregas et al., 2013). More recently it has been discovered that brl3 mutants show a defective response to elevated temperatures (Gupta et al, unpublished data).

Sorghum conserves 3 members of the BRI1 family

To explore the conservation of BR early signaling steps in sorghum, we employed a combination of bioinformatic and physiological approaches. We identified 3 orthologs that allowed us to build a phylogenetic tree and a 3D model of sorghum BR receptors, showing

conservation of key elements in the sequence of the proteins. These results were helpful in selecting the genes to study in sorghum since it is important to know the degree of redundancy in sorghum.

Phylogenetic analysis of sorghum BR receptors

The protein sequences of Arabidopsis BRI1-family members were used to identify homologous proteins in sorghum using BLAST (https://blast.ncbi.nlm.nih.gov/). A phylogenetic tree was generated after multiple alignment the sequences of the BRI1-family members in Arabidopsis and the orthologs in different crops as *Solanum lycopersicum*, *Oryza sativa*, *Zea Mays* and *Sorghum bicolor* (Figure 4). FLS2 was used as outgroup control, since it is a member of LRR-RLK protein family but has a completely different function as receptor of flg22 peptide. The phylogenetic tree showed conservation of 3 different clades in all the species tested, one for BRI1, another for BRL2 and the last for BRL1/3. First, a diversification of the BRI1 family occurred between BRI1 and BRL2, and later BRI1 diverged to BRLs. For BRLs, there has been a duplication in species like *Zea mays*, *Oryza Sativa* and Arabidopsis*;* but in *Sorghum bicolor* and *Solanum lycopersicum* we were only able to find one BRL1/3, named SbBRL1.

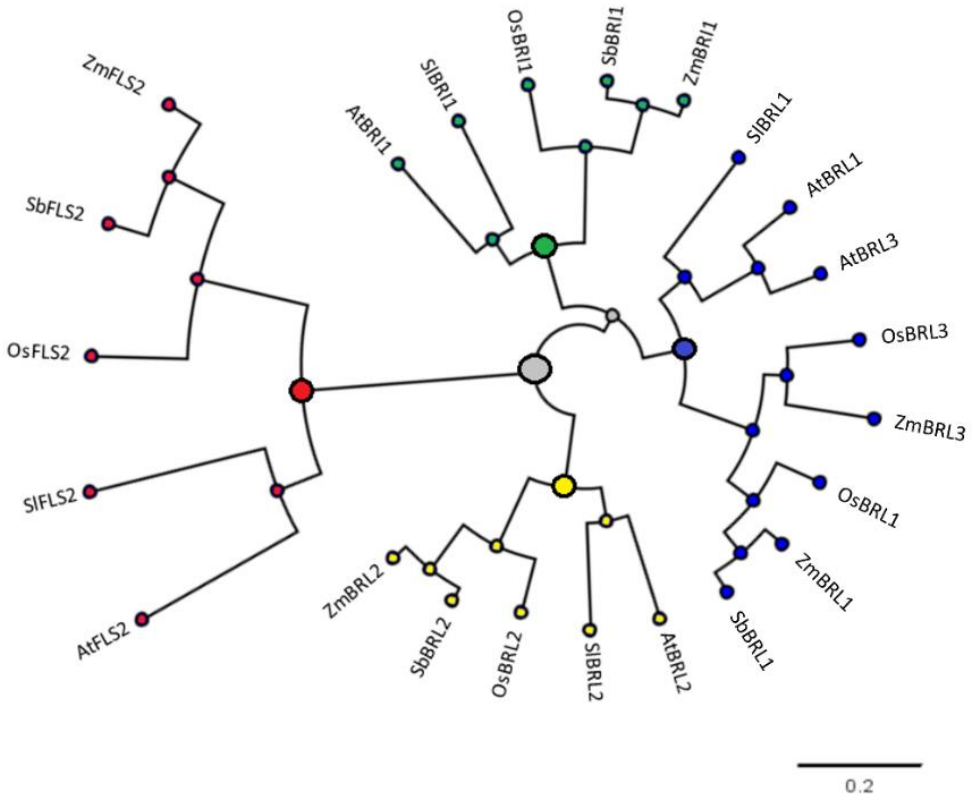


Figure 4. Phylogenetic tree of BR in different species

Brassinosteroid receptors protein sequences from different species were retrieved from BLAST. The species used are *Arabidopsis thaliana (At*), *Solanum lycopersicum (Sl)*, *Oryza sativa (Os)*, *Zea mays (Zm)* and *Sorghum bicolor (Sb).* FLS2, another LRR-RLK was used as an outgroup. Genious Prime ® software was used to build the circular phylogenetic tree. The nodes of the different genes have been colored: Blue, BRI1; orange, BRL1/3; and yellow, BRL2.

3D structure of sorghum BR receptors is conserved

The 3D structure of AtBRI1 was available by X-ray crystallography (Hothorn et al., 2011), where it was shown that it has an helicoidal structure in the extracellular domain with an aperture in the center caused by the ID. We aligned and built a homology model of the extracellular domain of sorghum BRI1 receptor based on the available Arabidopsis crystal (performed by Dr. Fidel Lozano-Elena). It shows a similar structure with conservation of the ID, which binds BRs (Figure 5).

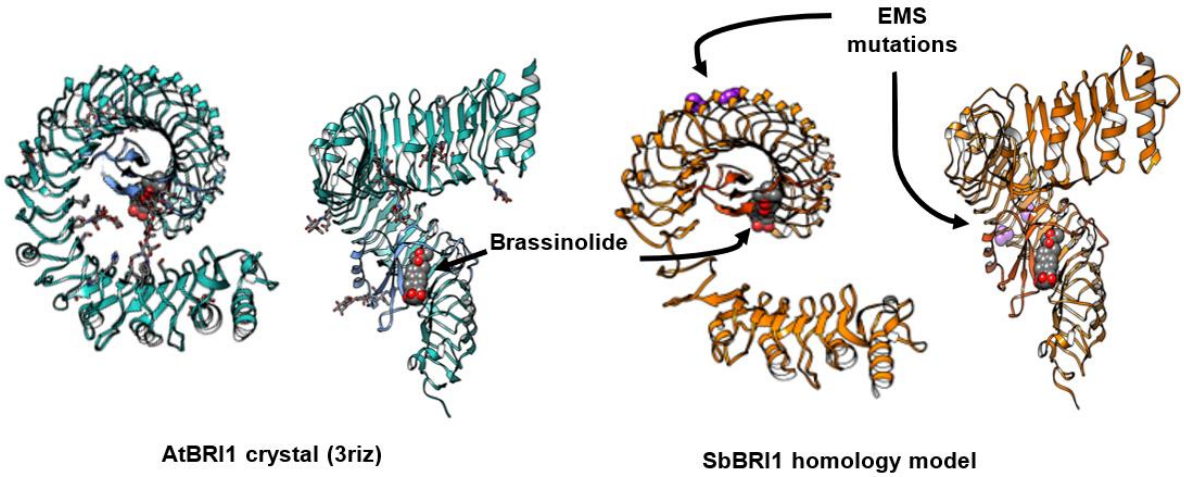


Figure 5. 3D structures of AtBRI1 and SbBRI1 extracellular domains

On the left, 3D structures of AtBRI1 obtained by X-ray crystallography, from different perspectives. On the right, the 3D structure homology model built based on the structure of AtBRI1 and sequence of SbBRI1. In both images, a BL molecule (grey and red) is represented on the binding site, in purple are displayed the amino acid changes of the *bri1* mutant plants obtained from the EMS collection.

The sequence similarity search in Arabidopsis, shows that there are 3 homologs for BRI1: BRI1-LIKE 1, 2 and 3. In Sorghum, we identified 3 orthologs for AtBRI1, namely SbBRI1, SbBRL1 and SbBRL2. The percentages of similarity between Arabidopsis and sorghum BRI1-

like proteins identified are displayed in Table 1. Among these comparisons and considering the number of identical sites, BRL2 is the protein that shows higher percentage of conservation (58%) between sorghum and Arabidopsis. SbBRL1 and Arabidopsis BRLs are also highly conserved, SbBRL1 shows a slightly higher conservation percentage with AtBRL3 (55.8%) than with AtBRL1 (54.6%), even though the number of identical sites shows to be higher for the comparison of SbBRL1 with AtBRL1 than with AtBRL3. AtBRI1 shows 53.3% of identical sites with SbBRI1. The BLSM62 algorithm for the calculation of probability of substitution of amino acids, shows that the divergence of the similarity of the protein sequences was likely to happen by specific nucleotide substitutions and that they evolved from a common ancestor.

Table 1. Protein sequence comparison analysis of BR in Arabidopsis and Sorghum orthologs.

	AtBRI1-SbBRI1	**AtBRL3-SbBRL1**	**AtBRL1-SbBRL1**	**AtBRL2-SbBRL2**
Alignment length	1,211 aa	1,228 aa	1,229 aa	1,158 aa
Identical sites	640 (53.3%)	654 (55.8%)	668 (54.6%)	664 (58.0%)
Pairwise Positive (BLSM62)	68,7%	69,0%	68,0%	72,9%

Conservation of the BR binding site

As shown within the 3D structure of BRI1, the ID of sorghum BRI1 receptor is likely to create a pocket for the potential binding of BR, essential for the signal transduction. To confirm the conservation of this motif in the rest of the BRI1 family members, the ID amino acid sequence was compared among the different receptors found in different crops. The detailed analysis of that region revealed that the ID region is highly conserved in the beforementioned species (Figure 6).

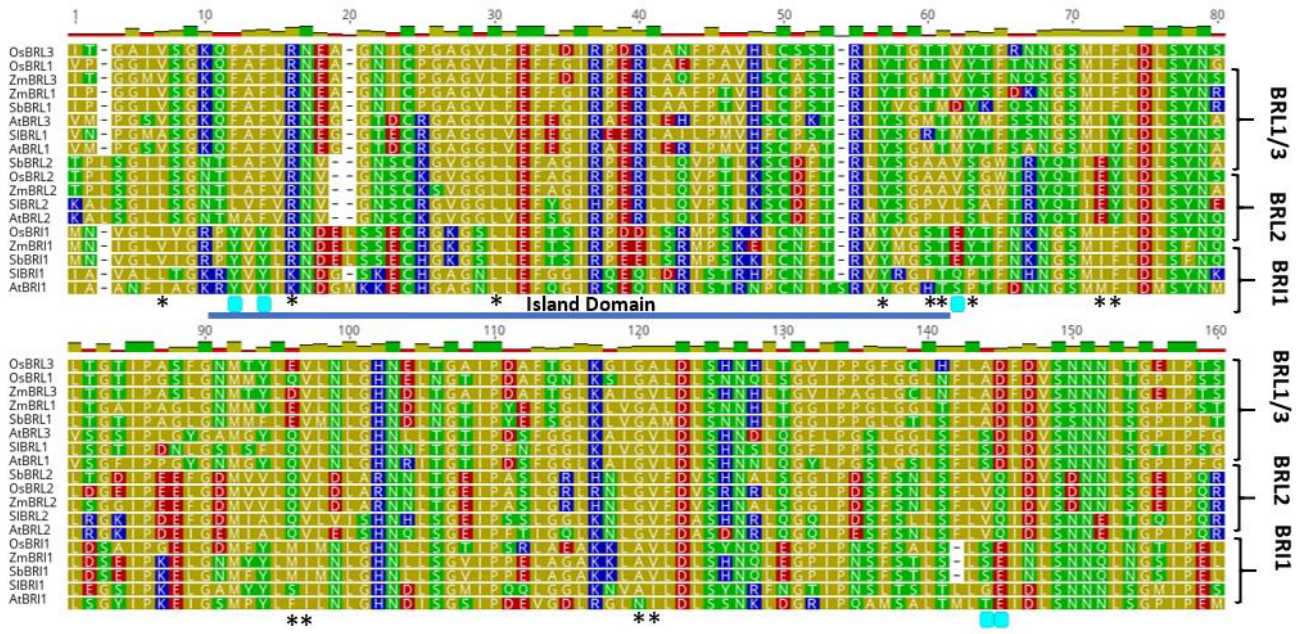


Figure 6. Alignment of the protein sequences of BR receptors in the binding domain.

The mean pairwise identity of the residues among the sequences is plotted in different colors on the top of the chart (Green: 100%; yellow: 99-31%; red: <30%). Apolar amino acids are plotted in yellow, polar non-charged in green, positively charged in blue and negatively charged in red. Light blue annotations on the bottom denote residues from AtBRI1 that form hydrogen bonds BL hormone (Aldukhi et al., 2020), asterisks denote amino acids that make direct contact with BL in AtBRI1 protein (Hothorn et al., 2011). The ID of BRI1 is underlined in blue. Genious Prime ® software was used for the alignment of the sequences.

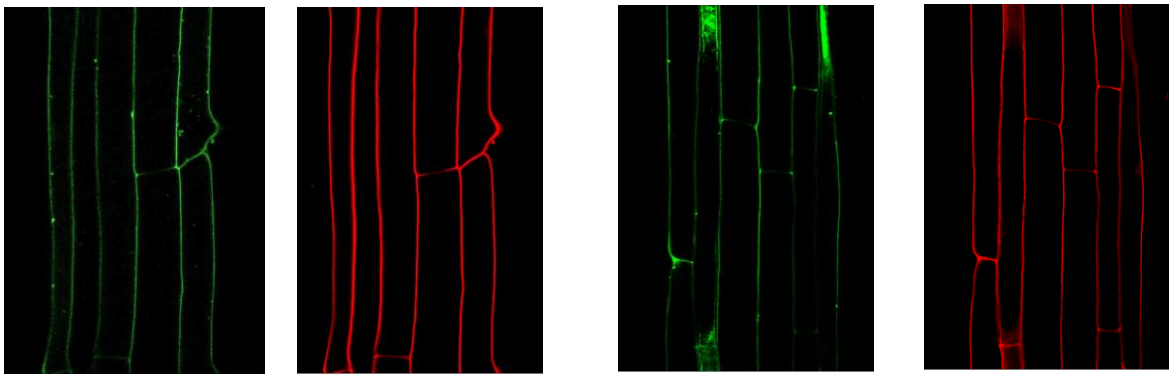
The analysis of the alignment of the ID of different species, shows for most of them either absolute conservation of many residues for all the receptors of the family or replacement with amino acids that have similar physio-chemical properties. Interestingly, the amino acids that are important for the protein-hormone interaction in BRI1 are not always conserved among Arabidopsis and the other species but are conserved among the monocotyledonous species like rice or maize, in which BRI1 have been demonstrated to be functional (Kir et al., 2015; Nakamura et al., 2006). The high conservation of the ID of Arabidopsis BRI1 and BRLs have been discussed by (She et al., 2013) confirming previous works that claim that from the 4 members of BRI1 family all can bind BL except for BRL2 (Kinoshita et al., 2005). BRL2 shows important amino acid changes in the sites of contact with the hormone. For this reason, its ortholog in sorghum, SbBRL2, was not further studied in this thesis.

Heterologous complementation of sorghum BR receptors in Arabidopsis recapitulates native receptor functions

To confirm that the candidate genes of sorghum BR receptors are functional we designed a heterologous expression system driving the overexpression by CaMV 35s promoter of sorghum BR receptor CDS sequences in Arabidopsis BR receptor mutants *bri1-301* and *brl3-2*. The *bri1-301* mutant is a weak allele of BRI1, since bri1 knock-out mutants are sterile, which would complicate the analysis of the transgenic lines. *bri1-301* has been reported to have a stronger phenotype when grown in elevated temperatures (Lv et al., 2018; Zhang et al., 2018). In the case of *brl3-2*, is a knock-out line which have been reported to have developmental and vascular defects when combined with other mutations such as *bri-301*, *brl1-1* and *bak1-3* (Fàbregas et al., 2013; Kang et al., 2017), and more recently it has been discovered that the single mutant have altered thermomorphogenic responses. The physiological characterization of these lines was performed by analysing the protein localization, plant size, sensitivity to BL and thermomorphogenic responses.

Sorghum BR receptors are membrane localized

As previously mentioned, BR receptors have a TMD which anchors them to the plasma membrane. To reveal the localization of sorghum BR receptors in the plant, T3 homozygous Arabidopsis transgenic plants were grown for 6 days in long day (LD) conditions. Confocal microscopy performed in the root differentiated zone, confirmed that sorghum BR are also membrane localized as shown from the propidium iodide (PI) staining which stains the lignin in the cell walls (Figure 7).



35s::SbBRI1-GFP/PI 35s::SbBRL1-GFP/PI

Figure 7. Plasma membrane localization of sorghum BRI1-like receptors.

Confocal microscopy images of root epidermal cells of 6-day-old *bri1-301* plants transformed with either 35s::SbBRI1-GFP or 35s::SbBRL1-GFP. In green, each BR receptor fused to eGFP tag and in red a counterstaining of propidium iodide staining cell wall.

Sorghum BR receptors complement bri1 dwarf phenotype

Multiple studies have shown that *bri1* mutations in Arabidopsis lead to dwarf phenotypes (Kinoshita et al., 2005; Li & Chory, 1997; Wang et al., 2002) while studies in *brl1* and *brl3* Arabidopsis mutants have no apparent phenotype in plant size for single or double mutants (Fàbregas et al., 2013, 2018). Despite *bri1-301* is known to be a weak allele of *bri1*, *bri1-301* mutant is dwarf, with defective in petiole elongation and shows rounded leaves (Caño-Delgado et al., 2004; Li, Kang, Wang, Nam, et al., 2010; Xu et al., 2008). The complementation of *bri1* mutants with both sorghum BR receptors reverted the dwarf phenotype of *bri1* in three-week old plants (Figure 8).

Col-0 | ***bri1-301*** | ***bri1-301* 35s:SbBRI1-GFP** | ***bri1-301* 35s:SbBRL1-GFP**

Figure 8. Complementation of the rosette phenotype of bri1-301 mutants in Arabidopsis
Image of 25-day old plants. Col-0 Wt, bri1-301 and complementation lines with SbBRI1 and SbBRL1 in bri1-301 background were grown in LD conditions at 22ºC with 16h photoperiod. bri1-301 mutant plants are dwarf and the lines overexpressing BR in this mutant background complement this phenotype.

Sorghum BRI1-like receptors rescue the BR-insensitivity phenotypes of *bri1-301* mutants of Arabidopsis

When applied exogenously, 4nM BL application can be inhibitory for root length but an increase in hypocotyl elongation, and *bri1* mutants remain insensitive to BL application (González-García et al., 2011; Clouse et al., 1996).

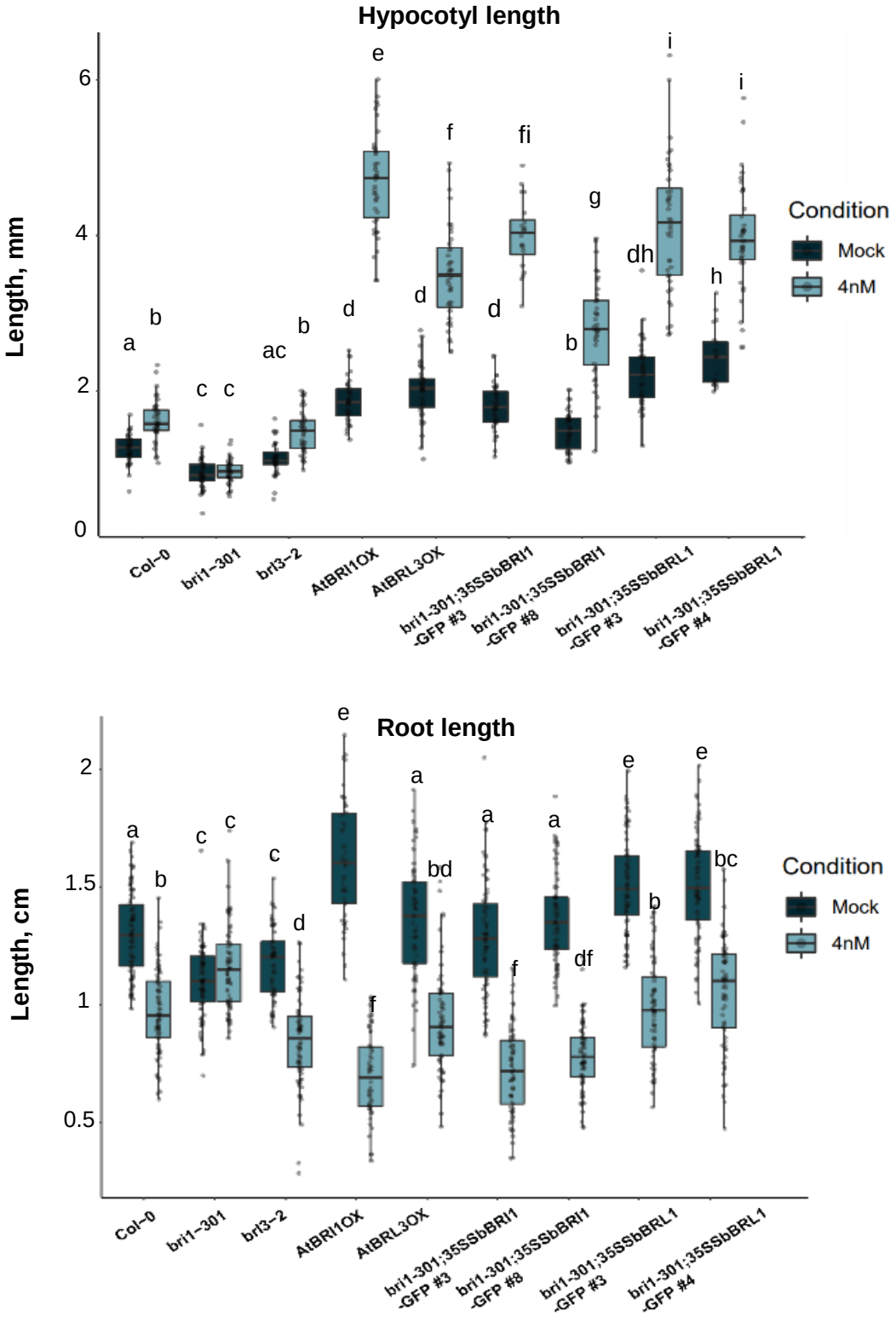


Figure 9. BR sensitivity assays.

Seeds were germinated in ½ MS media supplemented with 4nM BL or mock and grew vertically for 7 days before hypocotyl (A) and root length (B) were measured. Statistics: one-way ANOVA plus a Tukey's HSD were used to detect significant differences (p-value< 0.05). 3 independent biological replicates consisting of 40 seedlings each, were measured.

We treated *bri1-301* mutants complemented with sorghum BR receptors with BL to test whether sorghum receptors can restore the insensitivity of *bri1-301* mutants to the hormone treatment (Figure 9). The results showed that both SbBRI1 and SbBRL1 are recovering the BL insensitivity of *bri1-301* mutants. Moreover, in the absence of the hormone, the complemented lines are also showing longer hypocotyls and roots in comparison to the mutants, reinforcing that sorghum BR receptors are involved in plant growth, restoring the dwarf phenotypes observed in the Arabidopsis *bri1-301* mutant.

As mentioned before, the complementation of *bri1-301* mutants with both sorghum BR receptors showed an increment in hypocotyl length in both mock and BL treatments. Interestingly, SbBRL1 overexpression caused a higher elongation of the hypocotyl in comparison to other sorghum and Arabidopsis BR receptor overexpression. However, upon BL treatment, the growth promoting effect of BL in hypocotyls was higher for Arabidopsis mutant lines complemented with SbBRL1 than SbBRI1, without reaching *AtBRI1* overexpressor levels. Regarding the root length, *SbBRL1* showed a stronger growth promoting effect than *SbBRI1* in the elongation of the roots, while upon BL treatment, *SbBRI1* shortened the roots in a similar fashion to *AtBRI1* overexpression, while *AtBRL3* and *SbBRL1* overexpressor lines rescued the insensitivity of *bri1-301* mutants without causing an incremented response compared to *Wt* plants.

Sorghum BR receptors respond to elevated temperatures

BRs have a role in thermomorphogenesis (Praat et al., 2021). While Wt plants elongate their hypocotyls in elevated temperatures, *bri1-301* mutants are not able to elongate, and brl3-2 are defective in the thermomorphogenic response (Ibañez et al., 2018, Gupta et al., unpublished data). In contrast, overexpression of Arabidopsis BRI1 and BRL3 receptor promotes the elongation of the hypocotyl and AtBRL3 overexpressors reached the highest levels of elongation upon elevated temperatures. In this case *AtBRL3* overexpression had the strongest effect in this thermomorphogenic response and the Arabidopsis BR mutant lines

complemented with BR receptors was slightly increased compared to *Wt* seedlings. Interestingly, these complementation experiments failed to completely restored the sensitivity of *brl3-2* mutant to elevated temperatures.

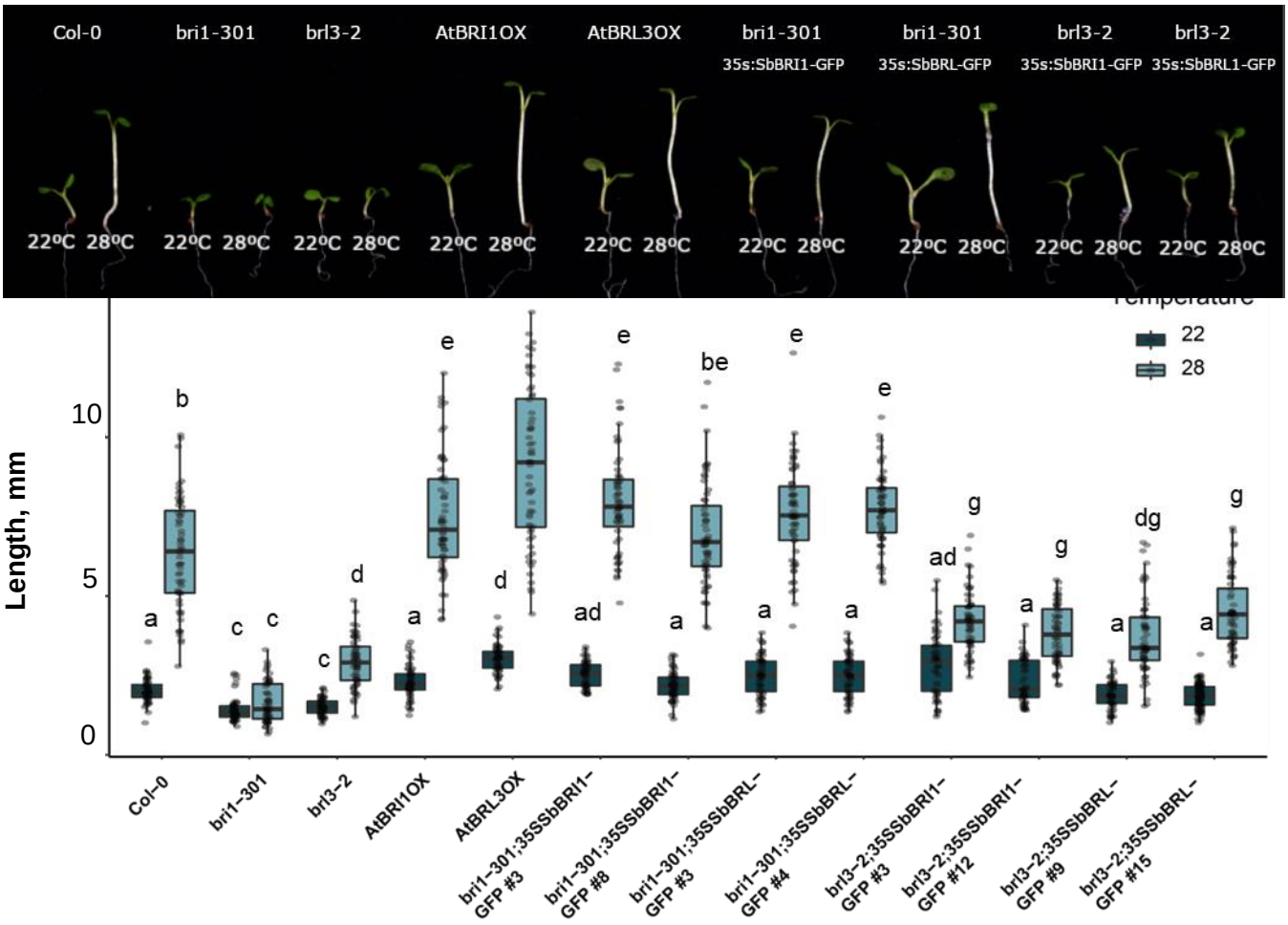


Figure 10. Thermomorphogenesis assay for hypocotyl length.

Seeds were germinated in ½ MS media at 22ºC in LD conditions for 24h. The control group was kept growing at 22ºC and the treated group was placed in a controlled growth chamber at 28ºC. Hypocotyl length was measured 5 days after germination. Statistics: one-way ANOVA plus a Tukey's HSD were used to detect significant differences (p-value< 0.05). 3 independent biological replicates consisting of at least 40 seedlings each, were measured.

From all the experiments performed, we concluded that Sorghum BR receptors are functionally binding BRs in Arabidopsis. Despite the role of AtBRL3 is not fully complemented by sorghum BR receptors, since the hypocotyl elongation did not reach the Wt levels, both

sorghum receptors complement the dwarf phenotype of *bri1-301* and its insensitivity to BL and elevated temperatures. This suggests a deep conservation of BRI1 function in different species, while Arabidopsis BRL3 may have a more specialized role.

CHAPTER 3:

CHARACTERIZATION OF SORGHUM BRASSINOSTEROID RECEPTOR MUTANTS

Introduction

To investigate the role of BR receptors in sorghum, we used the mutagenized collection published by Dr. Zhanguo Xin in (Jiao et al., 2016), whose team sequenced the genome of 256 mutagenized lines and identified >1.8 million of mutations covering >95% of sorghum genes in the elite inbred line BTx623. Ethyl Methanesulphonate (EMS) mutagenesis has been widely used in agriculture and research for generation of genetic variability and screening of new mutations with potential interest in agriculture (Jung, 2021). It can induce missense mutations, or less frequently, premature stop codons by causing GC-to-AT transitions with great efficiency in random locations. Studies trying to identify the function of a specific gene with plants originated by any kind of untargeted mutagenesis must consider background mutations of these lines.

In our case of study, with a frequency between 223 and 314 to compare the effects of a single mutation compared to their non-mutagenized parental line, each mutant should be ideally backcrossed 7-8 times depending on their number of mutations, expecting a mendelian segregation. due to time limitations, we performed the physiological characterization of the EMS-BR receptor mutants comparing the backcrossed plants having or lacking the mutation in the CDS (mutagenized mutant vs mutagenized Wt).

As mentioned in chapter 2, BR-receptor mutants have been identified in several species playing roles in plant growth and adaptation to stress conditions, therefore our efforts were concentrated on the analysis of plant architecture, yield, and tolerance to stress.

Isolation and characterization of sorghum BR mutants from a TILLING collection

Gramene database (https://ensembl.gramene.org) was retrieved for identification of BR receptor mutants. M4 seed pools from 10 different M3 panicles previously sequenced in batch were available for distribution in USDA. A total of 12 new alleles carrying missense mutations in the BR receptors CDS were identified by whole genome sequencing but we were only able to isolate 6 of these mutants due to non-available seeds stocks, poor germination, or the absence of the described SNPs in our batch. In Table 2, the mutagenized lines used in this work are listed. The seed pool name obtained from the original resource (USDA) was

conserved, and in this work, it is used to name a line that is segregating wild-type and mutant individuals for BR. receptors (i.e., ARS87 could segregate *Wt-87* or *bri1-87*).

Table 2. Summary of the sorghum mutants used reported in this PhD thesis.

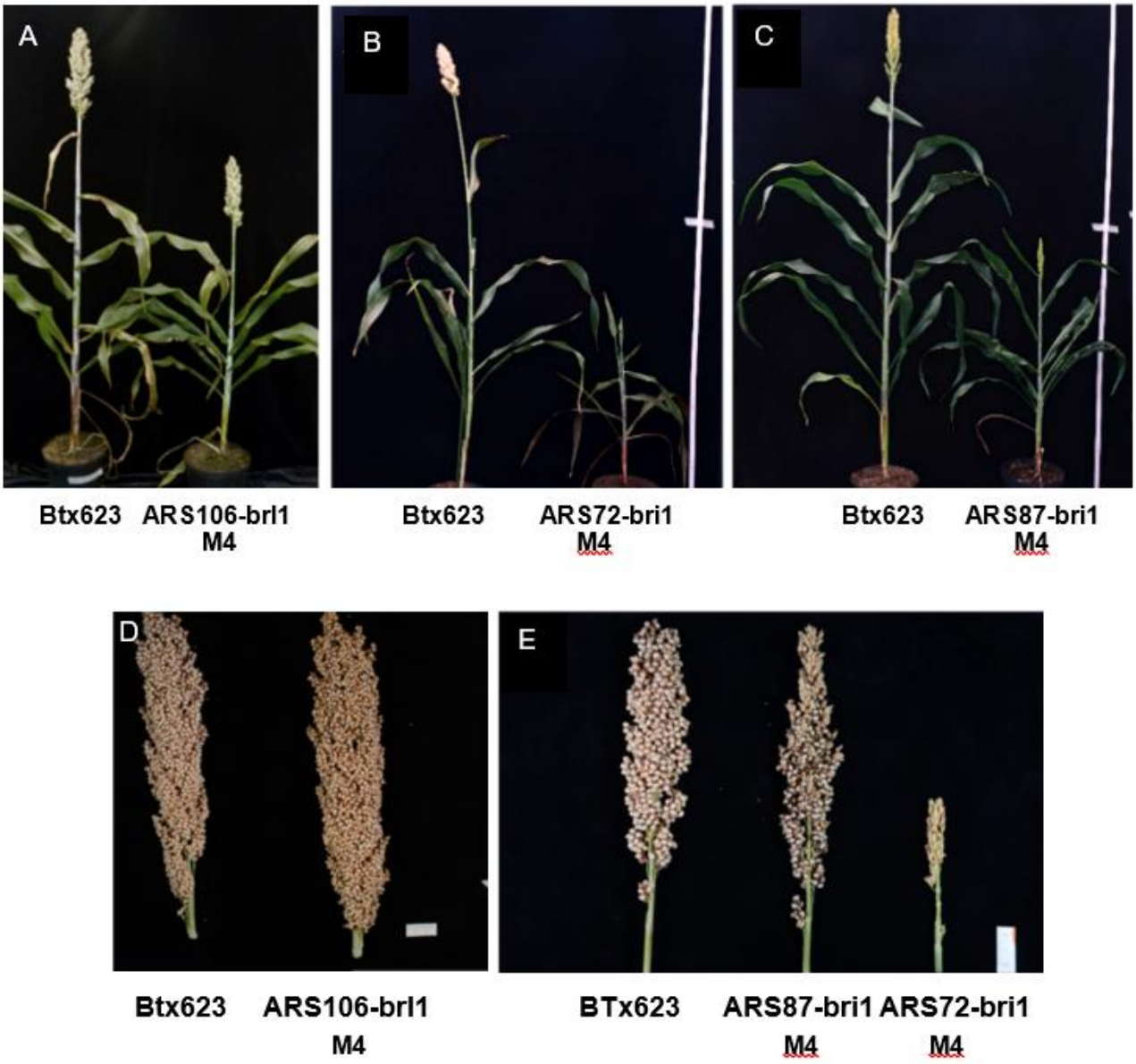
Seed pool	Gene	Allele	Status	Mutation	SNP position	Mutated genes in M3	Current expected mutated genes
ARS87	BRI1	*bri1-87*	BC1F4	V403M	LRR	223	112
ARS72	BRI1	*bri1-72*	BC2F4	P407L	LRR	291	73
ARS106	BRL1	*brl1-106*	BC2F4	S1151L	Kinase	243	61
ARS3	BRL1	*brl1-3*	BC1F4	G392R	LRR	314	207
ARS121	BRL1	*brl1-121*	BC1F4	P471L	LRR	276	138
ARS95	BRL1	*brl1-95*	BC1F3	S411L	LRR	313	157

In Table 2, the seed pool column represents the name given by Jiao et al. to the pool of M3 plants which were sequenced. Gene column shows the mutagenized BR receptor. Allele column is the name given to each of our mutants, namely in concordance with the seed pool. Status refers to the backcross generation reached when finishing the thesis. The mutation column shows the amino acid change in the sequence. SNP position indicates the protein domain mutated. Mutated genes show the number of genes that were mutated damaging the synonymous coding of the proteins in every M3 line.

M4 mutagenized plants showed severe defects in plant size and fertility

The abovementioned M4 seed pools were germinated and M4 mutant plants were genotyped for the described mutations by PCR amplification and capillary sequencing or CAPS if any restriction enzyme was available (see Table 8, Material and methods). Several mutant individuals were obtained from each line in homozygosis and heterozygosis. The phenotypes of the identified M4 homozygous mutant plants presented developmental defects in plant

height, and fertility. Sorghum *brl1* mutants showed reduced plant height and reduced internode elongation but in general, a normal development. Both sorghum *bri1* mutants, *bri1-72,* and *bri1-87*, displayed more dramatic phenotypes: plants were much shorter in plant size, and both had fertility defects. *bri1-72* mutant was completely sterile, and *bri1-87* was partially sterile due to defective elongation of the stamen filament (Figure 12). The phenotypes observed for bri1 sorghum mutants resembled Arabidopsis *bri1* knockouts with severe dwarfism and male sterility (Clouse et al., 1996; J. Li & Chory, 1997).



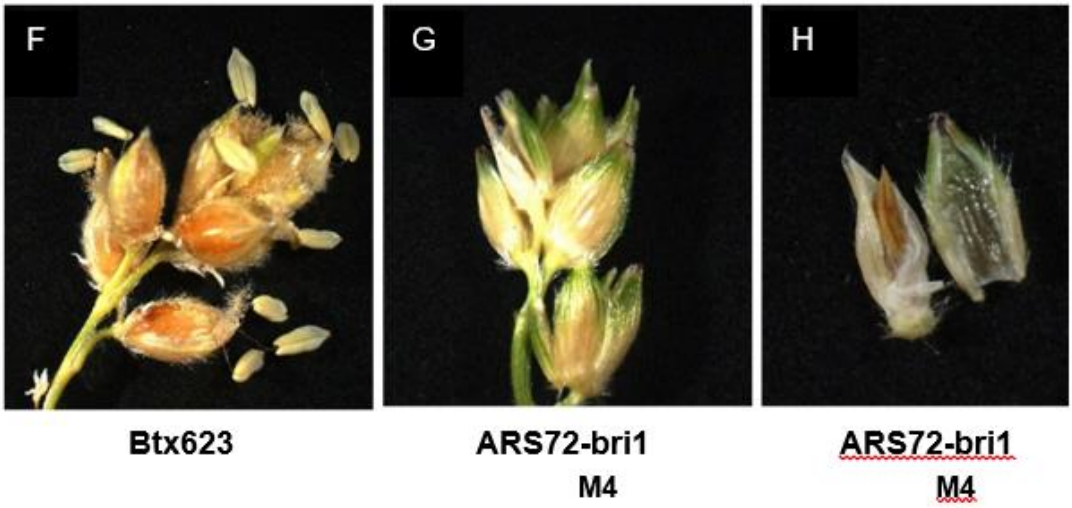


Figure 11. M4 sorghum mutant phenotypes.

A, B and C: Images of adult sorghum plants grown at CRAG greenhouses. At the left Wt BTx623 and at the right M4 brl1-106 (A), bri1-72 (B) or bri1-87 (C). D and E: Detail of the panicles. at the left Wt BTx623 and at the right M4 brl1-106 (D), or Wt BTx623, *bri1-87* and *bri1-72* from left to right (E). F, G and H: Detail of immature spikelets. Wt (F), and *bri1-72* mutant (G and H), anthers are emerged in Wt plants but not in *bri1-72* mutant.

Phenotypical analysis of sorghum BR mutant lines

The phenotypical analysis of the segregation BC1F2 of Wt and *bri1* mutant lines revealed that some of the phenotypes we previously observed were not caused by the mutations in the BR genes. Further phenotyping for plant architecture, yield, sensitivity to BL, and thermomorphogenic responses were analysed.

Plant size and grain yield analysis

Regarding the plant size of BR mutants, both BR signaling, and biosynthetic deficient mutants have been reported to affect multiple phenotypic traits in sorghum and other species. In tomato or rice, BR-mutants showed dwarf phenotypes with curly leaves and practically inexistent internodes (Bajwa et al., 2013; Tong et al., 2014). Also, in maize, different transgenic lines with downregulated expression of *ZmBRI1* showed that *BRI1* controls plant size and internode length (Kir et al., 2015). The widely used barley variety *uzu, bri1* mutant, shows a reduced plant size and erected leaves. An association mapping study in sorghum reflected that BRs

controls multiple plant architecture traits such as plant height, panicle size, or leaf angle (Mantilla Perez et al., 2014). Phenotypical characterization was carried out showing that from *bri1-72, brl1-106* and *brl1-3* mutants did not show any significant difference when comparing to their respective *Wt* siblings. However, *bri1-87* mutants have a significant difference in plant height), being shorter than their *Wt* siblings, suggesting that the role of BRI1 in sorghum is conserved and is regulating the overall plant development.

Nevertheless, there is a big variation in the plant size with segregating dwarf phenotypes independent of BR receptor mutations (Figure 6D). Indicating that there is a need of further backcrossing to elucidate the penetrance of the isolated mutations.

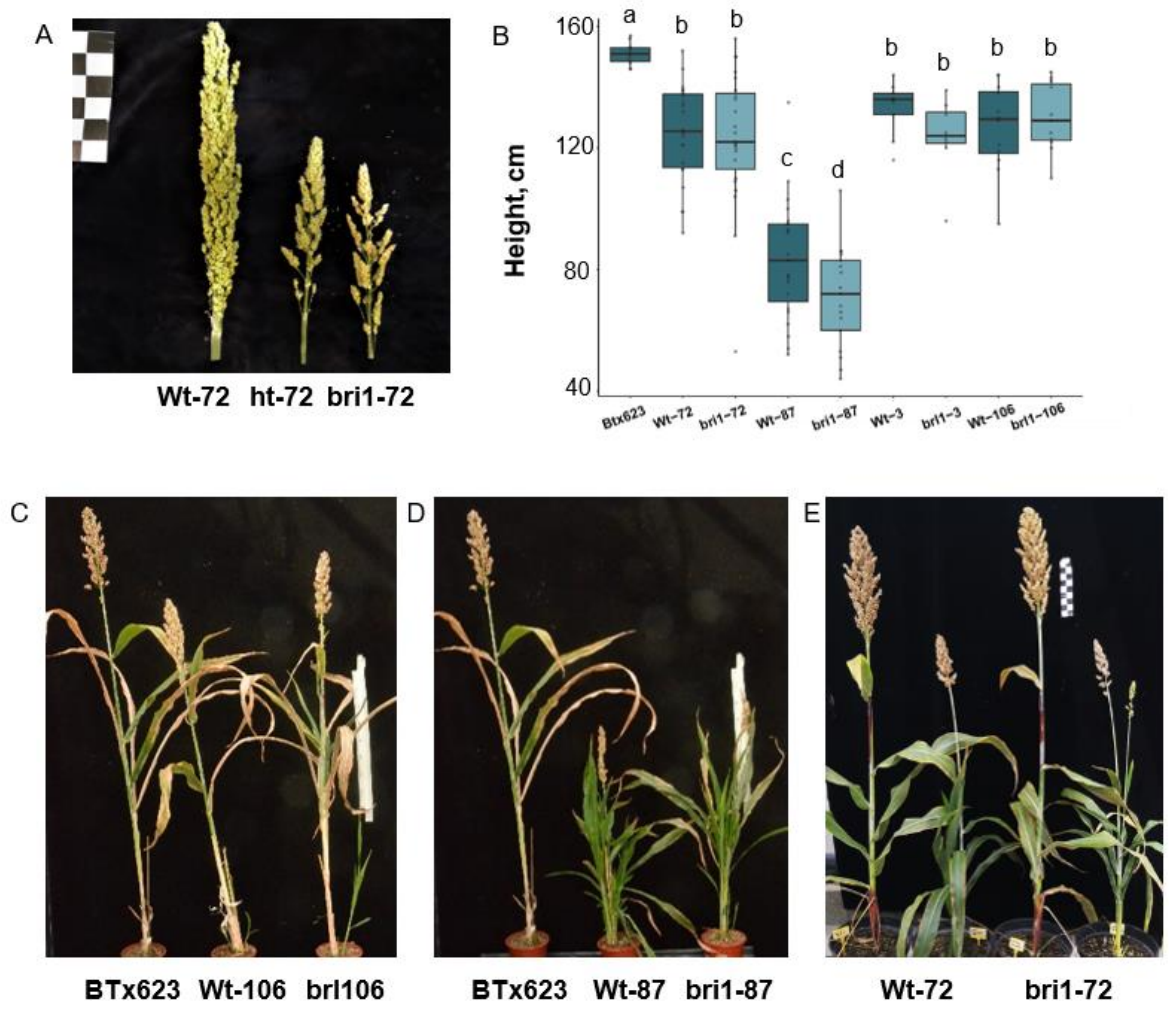


Figure 13. Phenotypes of backcrosses lines for adult sorghum plants of BR-receptor mutants.

A: Segregation of sterility phenotype. From left to right: Wt, heterozygous and *bri1-72* sterile panicles from ARS72BC1F2 segregant population. B: Boxplot representation of plant size for BTX623 parental line, BR receptor mutants and their respective *Wt* siblings in sorghum lines. ARS106 and ARS72 were

backcrossed twice and ARS3 and ARS87 were backcrossed once. Individuals were analyzed from 3 different parental lines. Statistics: One-way ANOVA with Tukey HSD test was performed to detect significant differences in plant height (p-value<0.05). 3 independent biological replicates consisting of at least 10 plants each, were measured. C, D and F: Images of sorghum mature plants. From left to right: BTx623, Wt-106 and *brl1-106* BC2F2 (C); 2 *Wt-72* and 2 *bri-72* mutants (D); and BTX623, wt-87 and *bri1-87*.

The sterility phenotypes observed in both M4 *bri1* mutant plants was segregating independently of *bri1* mutations. Sterile plants were found in *Wt*, heterozygous and *bri1* homozygous plants (6A). Suggesting that the sterility was not due to the identified mutation in the SbBRI1 CDS. However, a difference in grain yield of both bri1 mutant alleles was found. bri1 mutants were producing smaller panicles and less grain than their respective Wt siblings (Figures 13 and 14). Although the grain production of *Wt-87* was severely affected in comparison with other *Wt* lines, we found strong indications suggesting that Sb*BRI1* is implied in grain production.

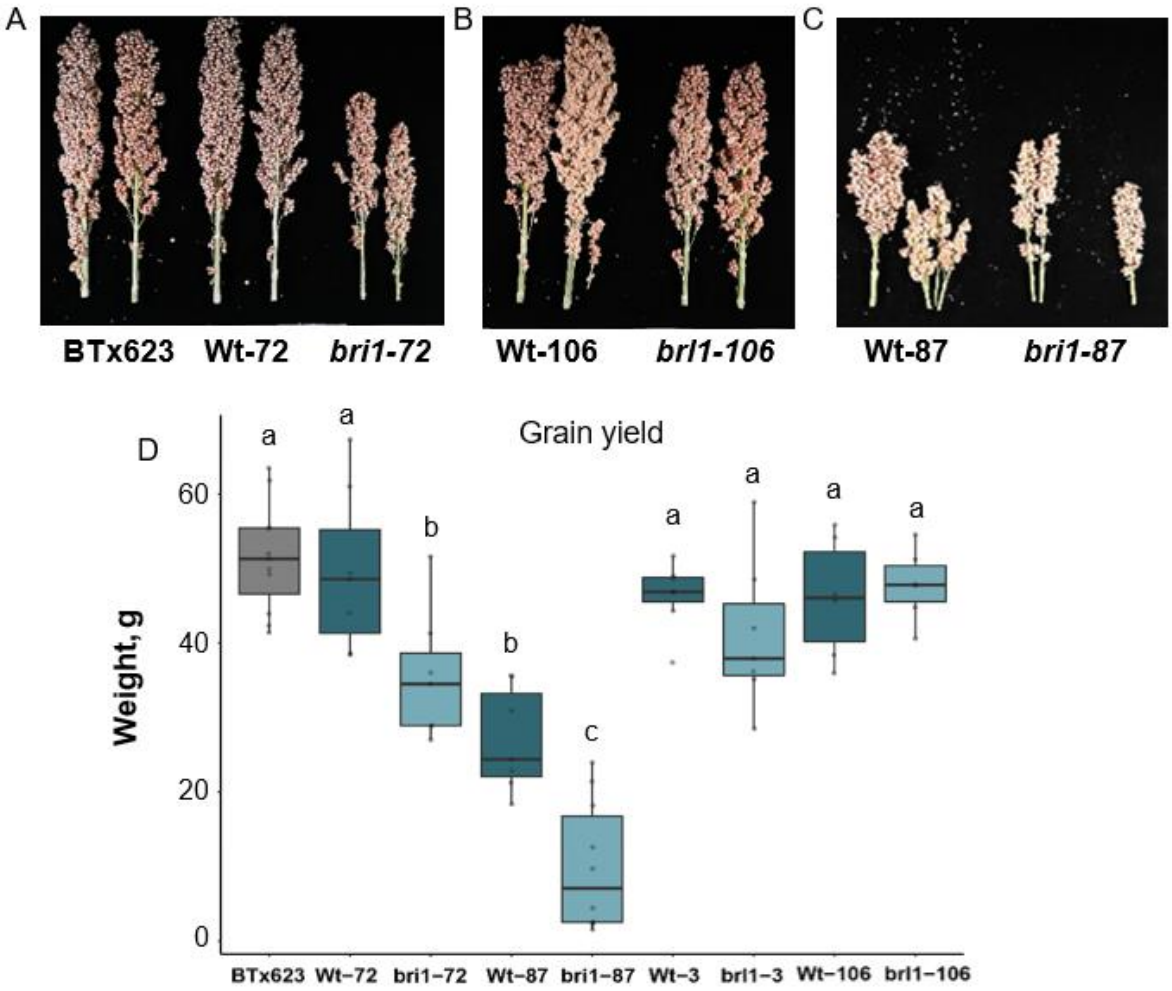


Figure 14. Grain yield phenotypes of BR receptor mutants in sorghum.
A, B and C: Images of mature sorghum panicles. From left to right: 2 panicles of BTx623, 2 *Wt-106* and 2 *brl1-106* (A), 2 Wt-72 plants and 2 *bri1-72* mutant panicles (B) and *Wt-87* and *bri1-87* panicles, these lines presented multi-tillering phenotype and several small panicles were produced by some plants (C). D: Boxplot graph of the grain yield of sorghum BTx623 parental line, BR receptor mutants and their respective *Wt* siblings. F2 individuals were analyzed from 3 different backcrossed F1 lines. ARS106 and ARS72 were backcrossed twice and ARS3 and ARS87 once. Statistics: One-way ANOVA with Tukey HSD test was performed to detect significant differences in the grain yield of each genotype(p-value> 0,05).

BL sensitivity of sorghum BR mutant lines

To further elucidate whether our BR mutants are affected in BR signaling we performed two physiological assays with some of the lines. The lamina joint bending assays (Maeda, 1965; Takeno & Pharis, 1982) consist in applying different hormones or herbicides to the plant in the lamina joint and observe the response of the plant in the inclination of the lamina. It has been used in several studies for identification of BR molecules and mutants in distinct species including sorghum (Hirano et al., 2017; Wada et al., 1981). Wild-type plants respond to BL by increasing the growth of the cells in the treated area, while BR signaling mutants are insensitive to the treatment Fujioka et al., 1999.; Noguchi et al., 1997). The growth of the cells implies an increased angle of the lamina compared to the rest of the leaf sheath. Our preliminary experiment did not show any response to BL in Wt BTx623 plantlets. From research in literature, we concluded that BTx623 cultivar carries the *dw1* mutant allele (Yamaguchi et al., 2016), which affects the signaling of BRs, and is not responding to BL by bending the lamina joint (Hirano et al., 2017).b) As reported for species like Arabidopsis, tomato, other cereals, and another sorghum cultivar (Blasco-Escámez et al., 2017; Chono et al., 2003; González-García et al., 2011; Koka et al., 2000), *Sorghum* BTx623 roots are shorter when treated with 40nM BL (Figure 15-A). To further analyze if the identified BR receptor mutant plants are sensitive to BL, we treated seedlings for 7 days in ½ MS media with increasing concentrations of BL (Figure 15B). Low BL concentrations (0.4 nM) did show a trend in slightly reducing the root length although no significant differences were detected in any of the genotypes. In higher BL concentrations, (4nM and 40nM BL) significant differences were detected, but the root length was decreased in a comparable manner for all the lines, even though for lines *Wt-72* and *bri1-87* significant differences were only found in the highest concentration (40 nM BL). The average root length of BTx623 non-mutagenized control, similarly to the plant height phenotyping, higher than the rest of backcrossed lines. The slight

insensitivity observed for *Wt-72* and *bri-87* lines may be due to background mutations, therefore analysis of the different lines upon additional backcrossing will be required to clarify these potential phenotypes.

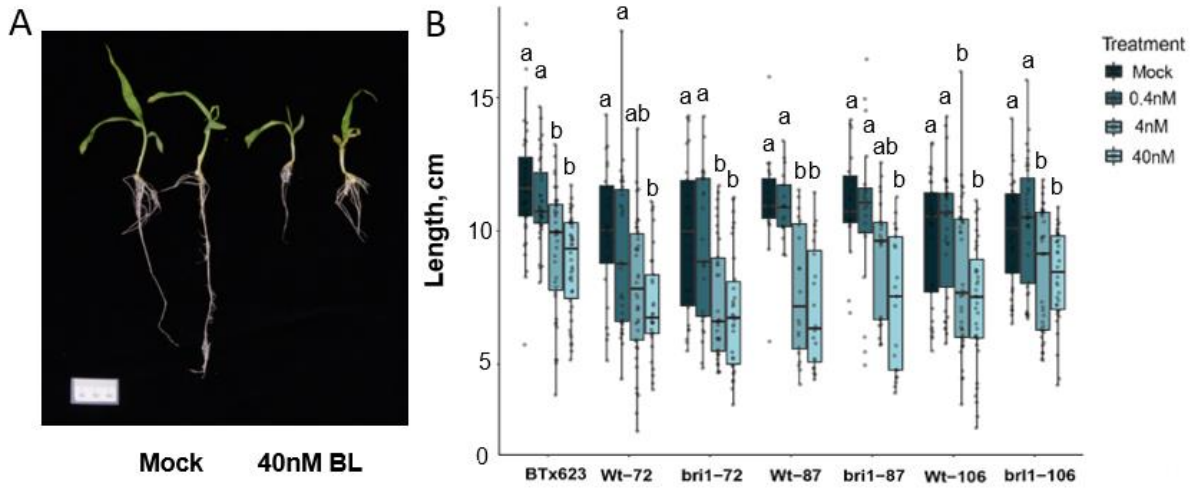


Figure 15. BR-sensitivity assays measuring root length phenotypes of sorghum mutants.

A: BTx623 Wt 7-day old sorghum seedlings grown in ½ MS agar media with mock (left) or 40nM BL treatment (right). B: Boxplot representing the primary root length after 7 days of BL treatment in different concentrations (0 (Mock), 0.4, 4 and 40nM BL). 3 biological replicates of the experiment were performed using BTx623 non-mutagenized parental line and backcrossed F3 homozygous mutant lines and F3 Wt relatives for the studied mutations as controls. Statistics: One-way ANOVA with Tukey HSD test was performed to detect significant differences among the treatments of each genotype (p-value> 0.05). 3 independent replicates consisting of at least 15 seedling each were measured.

Sensitivity to elevated temperatures of BR mutant lines

Next, we wanted to evaluate whether the sorghum BR receptor mutants were involved in the adaptation to elevated temperatures as happening in Arabidopsis. Since the optimal growth conditions for sorghum vary between 28ºC and 32ºC we performed several experiments at different temperatures and different sowing depths. The final experiment designed to assess the growth of sorghum mesocotyls in elevated temperature, consisted in sowing sorghum seeds at 15cm of depth and expose them to continuous temperature treatment at 28ºC (control group) or 38ºC (elevated temperature). The mesocotyl length was measured after 1 week. The mesocotyl is the organ responsible for seed emergence from the soil during germination,

it elongates in dark allowing the coleoptile to reach the light and expand the leaf sheath. Three biological replicates of the experiment were performed with different BTx623 and Wt as control group, and mutant seeds from three different BC2F3 panicles for *bri1-72* and *brl1-106* and BC1F3 *brl1-121*. The results showed that *bri1* and *brl1* mutant seedlings are hypersensitive to elevated temperatures. *bri1-72, brl1-106* and *brl121* elongate less the mesocotyl than their respective control backcrossed seedlings only in elevated temperatures (Figure 15). Together with our Arabidopsis data (Gupta et al., under revision), this represents the first studies showing a phenotype for single *brl* mutants.

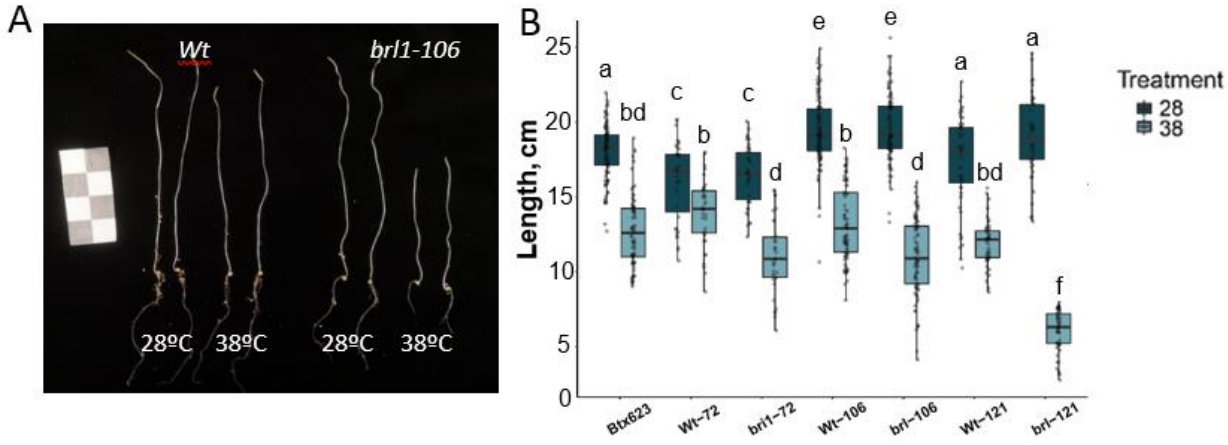


Figure 16. Hypersensitivity of sorghum BR mutant seedlings to elevated temperatures

(A) Image of 7-day-old *Wt-106* and *brl1-106* seedlings grown 15cm underground at different temperatures. (B) Boxplots represent the mesocotyl length of the different sorghum lines. Statistics: One-way ANOVA with Tukey HSD test was performed to detect significant differences in mesocotyl length among the conditions (p-value> 0,05).

Responses to drought of BR mutant plants

As mentioned previously, BR deficient mutants in Arabidopsis show enhanced tolerance to drought. bri1 mutants consume less water, respond less to osmotic treatments, and keep increased relative water content (RWC) and photosynthetic activity than wild-type plants (Fàbregas et al., 2018). In order to investigate if sorghum BR receptors are involved in the adaptation to drought stress, we adapted some of these experiments with sorghum seedlings. 8-day old sorghum plantlets were subjected to water withdrawal while keeping also watered control plants. To monitor the water status of the plants of the experiment and ensure that all the genotypes were analyzed in the same stress level, the field capacity (FC) of the soil, or in other words, the grams of water contained in a pot, was calculated as water-saturated soil

weight minus the dry soil weight. Pots were weighted daily, and the water content of the pot was calculated as the percentage of *(current weight - dry weight of the soil) / field capacity*. The water content of the pot, or FC percentage was monitored during the drought period (Figure 16). These results showed that *bri1-72* and *brl-106* BC2F4 mutants consume less water and took longer to reach critical FC percentages than their respective wild-type lines.

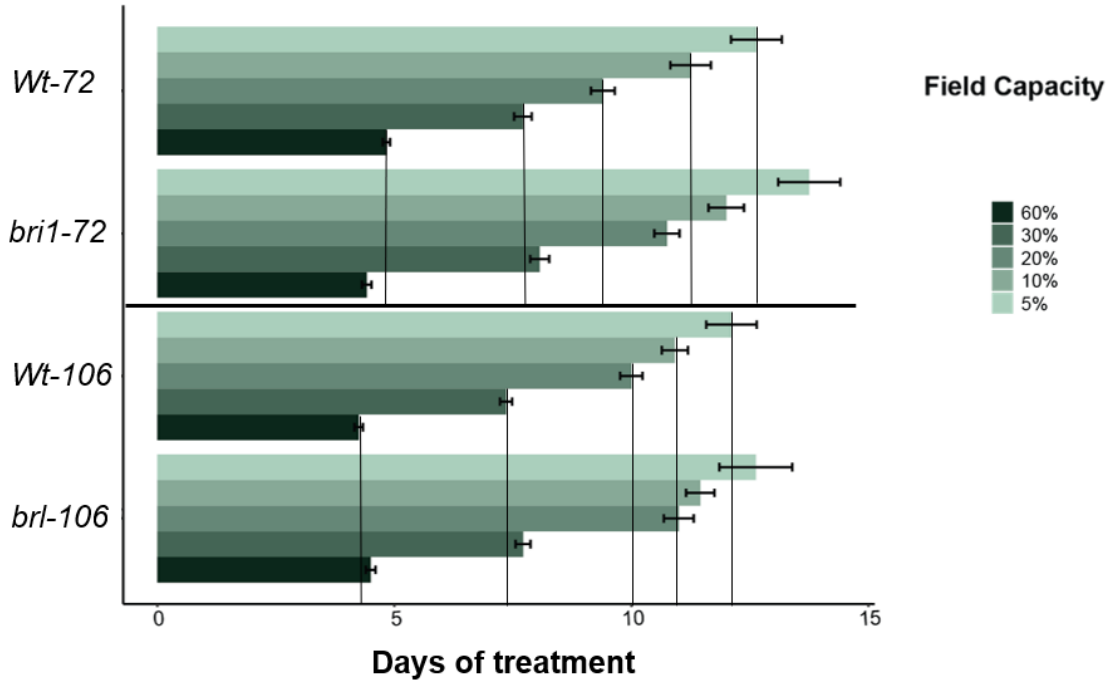


Figure 17. Physiological analysis of plant adaption to drought stress indicated by days necessary to reach percentage of field capacity (FC).

Bars represent the days needed for each genotype to consume the 60, 30, 20, 10 and 5% of the water available in each pot. 3 independent biological replicates consisting of at least 8 pots. were performed.

Plants subjected to drought were collected for analysis at different FC percentages (80, 30, 10 and 5%) for phenotypic analysis. Chlorophyll fluorescence, one of the most used methods to determine stress levels in plants, is based in Photosynthetically Active Radiation (PAR) measurements, the changes of fluorescence when the reaction centers of photosystem II (PSII) are opened or closed can be measured, indicating several photosynthetic parameters. Fv/Fm which is the ratio of the fluorescence values of the chlorophyll at a variable light intensity split by the value when all reaction centers are open is an indicator of the stress level. For most plants grown in optimal conditions Fv/Fm ratio is the range of 0.79-0.84, below this threshold plants are considered stressed (Baker & Oxborough, 2004; Guimarães et al., 2022).

As shown in Figure 18 *bri1-72* mutants maintain a higher Fv/Fm ratio at 5% FC. Non-photochemical quenching (NPQ) is a protective mechanism for plants in which they dissipate the excess light energy in heat (Mü et al., 2001; Ruban & Wilson, 2021). In Figure 18, it is shown that well-watered plants show almost no NPQ while plants under drought have a clear increment in the value of NPQ, while it is notable that *Wt* plants have a bigger area of the leaf producing NPQ. Taken together, these results show that Sbbri1 plants are more resistant to drought.

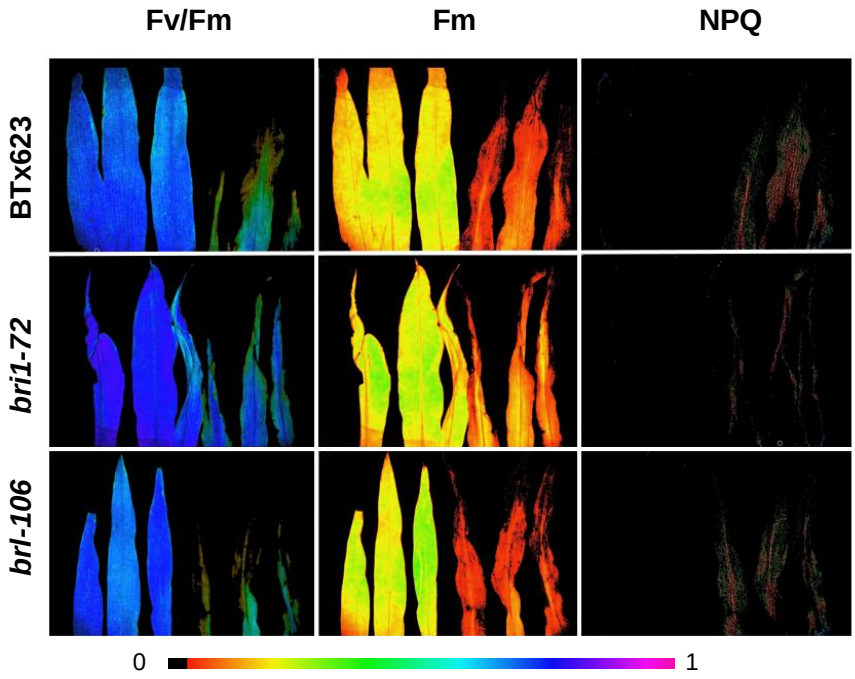


Figure 18. Visual representation of Fv/Fm, Fm and NPQ in *Wt* compared to the BR mutant leaves in control and drought conditions.

Three leaves of watered control plants on the left and 3 of plants in drought on the right were imaged in every image. Fv/Fm ratios are represented in color scale: intense blue value of 0.8 and changes to green, yellow, red or black for values of 0. Fm is presented to contrast values of 0 in the Fv/Fm images. NPQ scale color is black if 0 and the value representation is increased in green and red.

Photosystem II efficiency (YII) measurements by PAR, are based in the amount of reaction centers which are opened or closed after a light pulse excitation and acclimation to actinic light was also measured (Figure18) (Genty et al., 1989). The results of both measurements, Fv/Fm and PSII efficiency, are generally in concordance. In water scarcity conditions *bri1* and *brl1* mutants showed a better performance. Despite in well-watered conditions (80% FC) *bri1* and *brl1* mutants showed a lower PSII activity, and in the case of *bri1* also a lower Fv/Fm, at 30

and 10% FC *brl1-106* mutant showed a better performance in both measurements. *bri-72* mutant showed a better Fv/Fm ratio and PSII efficiency in 5% FC.

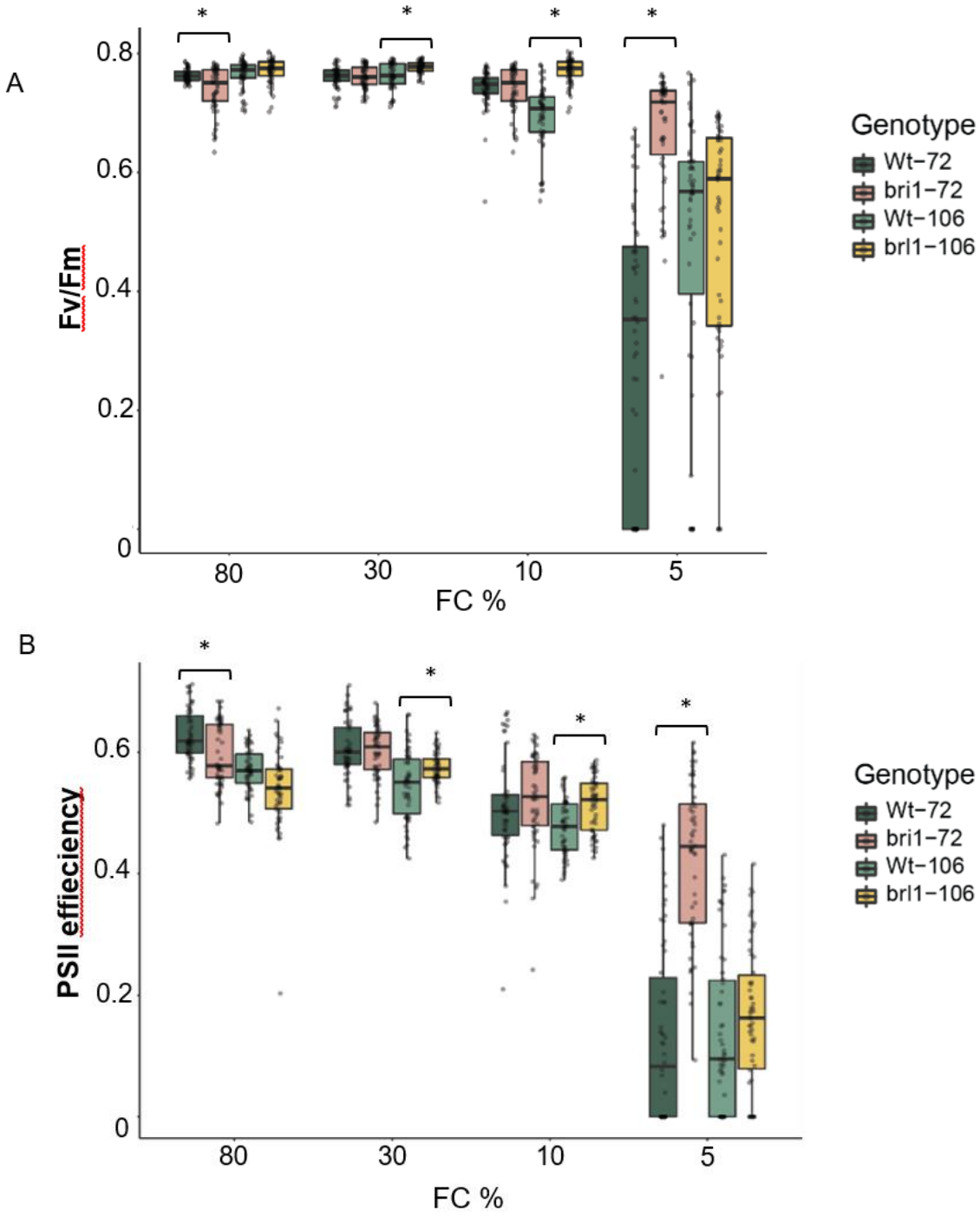


Figure 19. Fv/Fm and PSII efficiency upon drought conditions indicates the stress levels and the photosynthetic activity of the plants subjected to different FC.

(A) Boxplot represents the maximum potential quantum yield of PSII of plants at different FC %. (B) Boxplot represents the PSII efficiency of plants at different FC%. Statistics: One-way ANOVA with Tukey HSD test was performed to detect significant differences in Fv/Fm or PSII efficiency among the

genotypes (p-value> 0.05). Three independent biological replicates were performed. The measurements were performed in six different spots along the fully expanded youngest leave. 3 plants were measured per replicate in every time point.

To study the possible causes of these phenotypes, plant size and leaf width of plantlets in control conditions and subjected to drought treatment was measured 8 days after the initiation of treatment (Figure 19). While plantlet height was affected upon drought treatment similarly in all the genotypes, the leaf width was not. The only significant difference observed was that the leaf width of *brl1-106* plantlets was increased in both watered and drought conditions, which would not support the hypothesis of having a better tolerance to drought due to smaller size and thus lower transpiration.

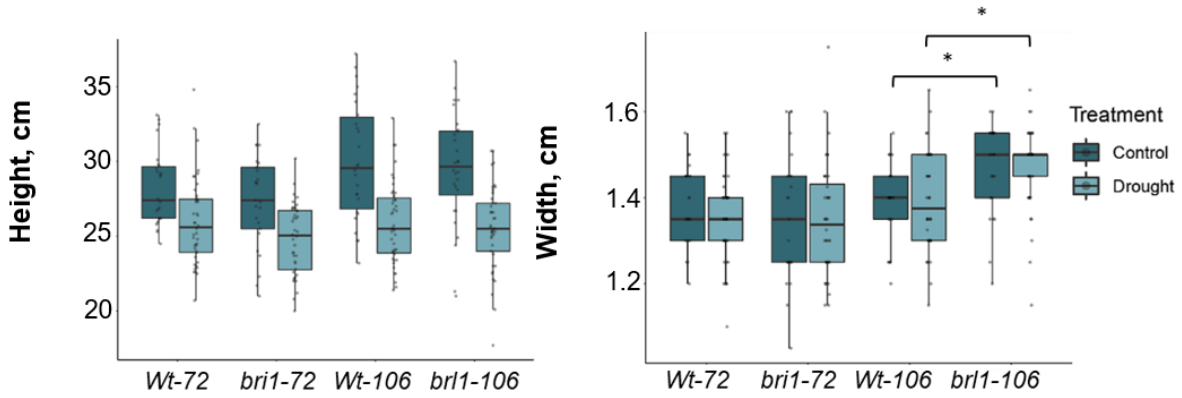


Figure 20. Plant size of seedlings subjected to drought or water treatment show differences in plant height and leaf width.

Eight-day-old plantlets subjected to eight more days of drought or water treatment were measured for plant size and leaf width. Statistics: No significant differences were found after T-test analysis comparing Wt and mutant siblings in each treatment.

We also calculated the relative water content of the 2nd youngest fully expanded leave by the formula (Fresh Weight - Dry Weight) / (Turgid Weight - Dry Weight). Despite the results showed that in average *bri1-72* plants can keep a better water status in the plant in extreme drought conditions, no significant differences were found.

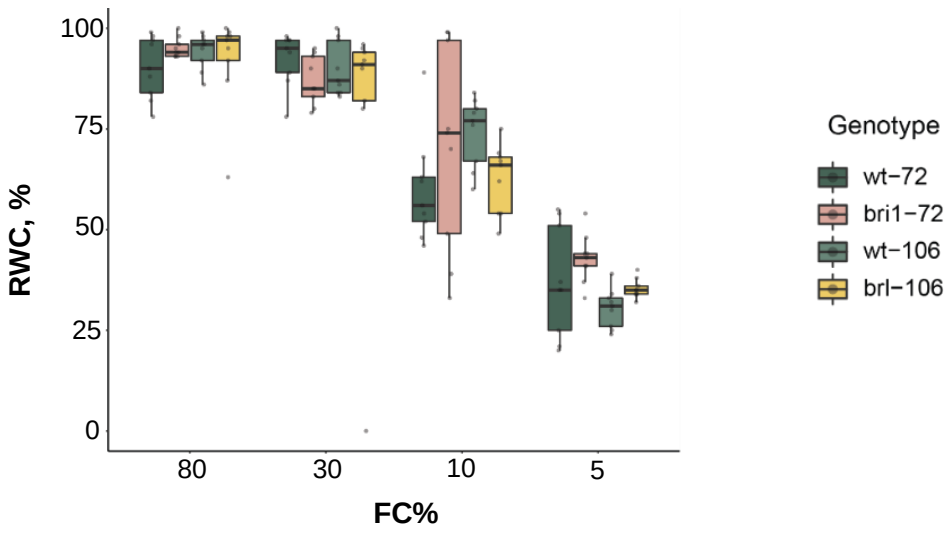


Figure 21. Relative water content of leaves at different FC % revealing the impact of drought in the water status of Sorghum *Wt* mutants leaves.

Boxplot represents the RWC % values of plants in well-watered and drought conditions. No significant differences were found after T-test analysis comparing *Wt* and mutant siblings in each treatment.

Summarizing the results of the assay, we observed that both sorghum BR mutants consume less water than their respective controls. *bri1-72* mutant plants were less stressed and were able to perform photosynthetically better than their *Wt* relatives along the drought period.

CHAPTER 4:

SORGHUM TRANSFORMATION

Introduction

Since the first fully transgenic plant was published in 1984 using Agrobacterium (de Block' et al., 1984; Somssich, 2019), plant genetic engineering has become a valuable tool in plant biology and crop breeding. It can help to understand different processes in plant biology research, but it can also improve interesting traits for agriculture, like durability of the fruits or yield in Flavr Savr tomatoes, but also confer new ones, like plague resistance in Bt crops. Transgenic plants have been shown to improve the yield and farmers profit reducing the general use of pesticide (Klümper & Qaim, 2014). Moreover, the discovery of the CRISPR cas9 system to edit plant genomes opened an opportunity to plant breeders in many countries to use plants obtained using transformation methods.

Sorghum is considered a recalcitrant species for genetic transformation due to accumulation of phenolic compounds, low regeneration frequency and loss of regeneration potential during the tissue culture (Liu et al., 2015; Nguyen et al., 2007). Transformation efficiencies of this crop are much lower than in other cereals like maize: 50% (Ishida et al., 2007), wheat: 40-90% (Ishida et al., 2015) or rice: 50-90% (Hiei & Komari, 2008). Since the first sorghum transformation reported (Casas et al., 1993), with a transformation efficiency varying between 0.08 and 0.3% using immature embryos and particle bombardment, other protocols using *Agrobacterium* improved the efficiency (Gurel et al., 2012; Z.-Y. Zhao et al., 2000). Different explants like immature inflorescences, shoot tips or leaf whorls have been used as an attempt to ease the labor and/or the costs of embryo isolation for transformation (Casas et al., 1997; O'Kennedy et al., 2006; Silva et al., 2020). One the most efficient protocol published up to date is based on particle delivery transformation of immature embryos, reaching an efficiency of 20,7% (Liu & Godwin, 2012).

Several publications describing sorghum transformation based on particle shoot delivery have been reported by Prof. Ian Godwin's laboratory (Liu et al., 2012, 2014, 2015, 2019). After several unsuccessful attempts of obtaining transgenic plants at CRAG following the protocol of Ian Godwin laboratory, a collaboration was established and Dr. Guoquan Liu bombarded calli with constructs overexpressing SbBRI1 and SbBRL1 under ZmUbi promoter. Furthermore, I performed a 4 month stay in their laboratory in Brisbane bombarding with constructs for CRISPR gene editing of SbBRI1 and SbBRL, and constructs containing promoter of these 2 genes fused to GUS/GFP, all of them generated by Dr. Damiano Martignago at CRAG.

In this chapter I will describe the adaptation of their protocol to CRAG comparing the results of 2 different transformation campaigns in Barcelona with the ones performed in Prof. Ian Godwin laboratory in QAAFI, Australia.

Protocol for biolistic transformation of sorghum

The protocol and the media composition are based on several publications from Dr. Guoquan Liu and Prof. Ian Godwin, with some contributions from my personal experience in my stay in their laboratory in QAAFI, Brisbane, Australia.

The stable transformation of different organisms by DNA coated particles is based on the delivery of DNA inside the cells and presumably, the ability of these particles to produce double strand breaks (DSBs) in the DNA of the cells by direct contact and the ability of these cells to survive and repair the DNA damage integrating the T-DNA of interest (Köhler et al., 1989; Krysiak et al., 1999; Nakayama et al., 1998). The whole duration of the transformation can take more than one year considering the time needed to get explants in the proper stage and the generation of T2 seeds after placing them on soil. The overview of the different steps for biolistic transformation of sorghum is presented in Figure 19. Every step for the transformation and in vitro regeneration of transformant plants will be comprehensively described in the next sections of chapter.

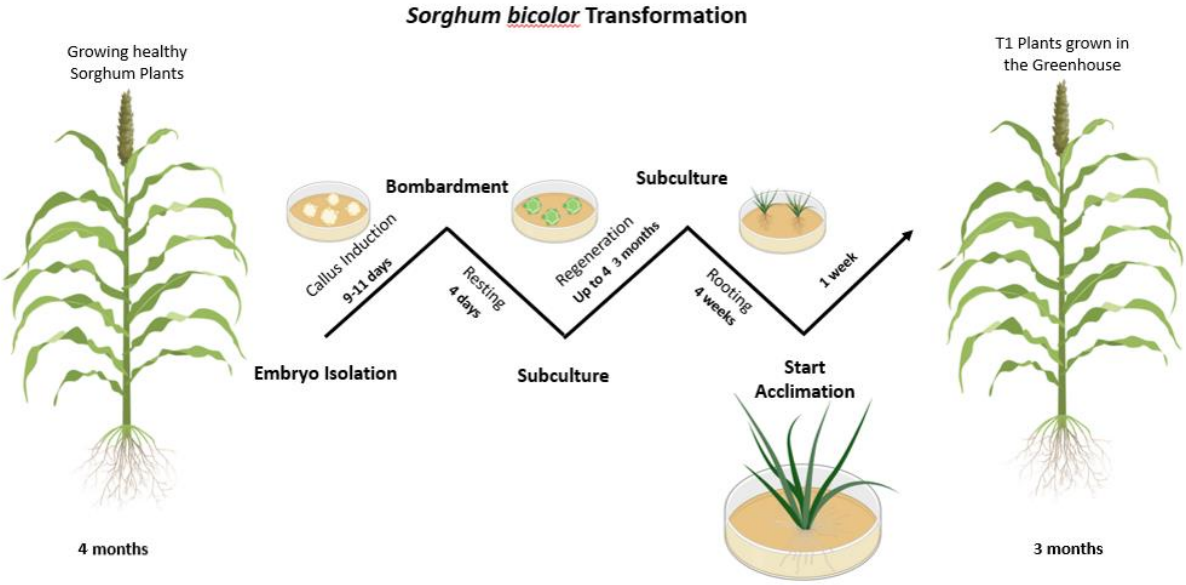


Figure 22. Schematic representation of the timeline for sorghum transformation

The first step for a successful transformation of sorghum is the obtention of immature embryos, for which, a constant supply of plants in the proper stage is needed. The approximate time for the obtention of immature seeds is 4 months. After embryo isolation and 9 to 11 days of callus induction, takes place the bombardment of gold particles with coated with the DNA of interest. Plants are placed back in callus induction media for the recovery after the bombardment and then moved to selective regeneration media. Once the surviving plantlets reach a proper size at an average time of 3 months later, they can be moved to selective rooting media. Resistant plants develop a sufficient root system to be place on soil after 4 weeks in rooting media and acclimation.

Strategy for transformation vector design

We wanted to test if altering BR receptor levels could affect the adaptative response of the plants to stresses. Therefore, constructs with GFP fusion to sorghum BR receptors driven by ZmUBI promoter were generated, as well as vectors expressing gRNAs for editing SbBRI1 and SbBRL1 under SbU6 promoter.

The strategy used by the laboratory of Pr. Ian Godwin was to co-bombard two vectors at the same time. For the generation of overexpressor lines, one of the vectors contained the gene of interest and the expressed the gene for resistance to kanamycin and geneticin, *nptii* driven by ZmUBI promoter. In the case of CRISPR editions, the vector driving the expression of the gRNA contained also the *nptII* gene and was co-bombarded with a vector driving zCas9 under the expression of ZmUBI.

Growing healthy plants

The starting material for sorghum transformation is proven to be critical for a good regeneration efficiency. The stage and health of the material affects the ability of sorghum calli to grow and produce embryogenic tissue. Sorghum as a C4 plant tolerant to extreme high temperatures, has also big photosynthetic potential which demands high-light intensities. The optimal temperature is between 28ºC and 32ºC and sorghum completes the cycle faster in Long-Day conditions (16h/day). A common problem of sorghum growing in greenhouses is calcium deficiency which is linked to low photosynthetic activity (Murtadha et al., 1998; Q. Wang et al., 2019). The most typical symptom is leaf splitting perpendicularly to the lateral vein of leaves. It has been reported that spraying the leaves twice a week with a 0.1M solution of $CaNO_3$ improves the symptoms (Liu et al., 2014). In our experience the substrate used was

crucial to alleviate these symptoms (Figure 20). 25L pots were used for every 3 plants, previously germinated in smaller pots. Plants grown in substrate 1 and 2 displayed the reported calcium deficiency symptoms but plants grown in substrate 3 did not show the symptoms. The substrate 3 mix, rich in calcium, used for growing our sorghum plants can be found in Table 3.

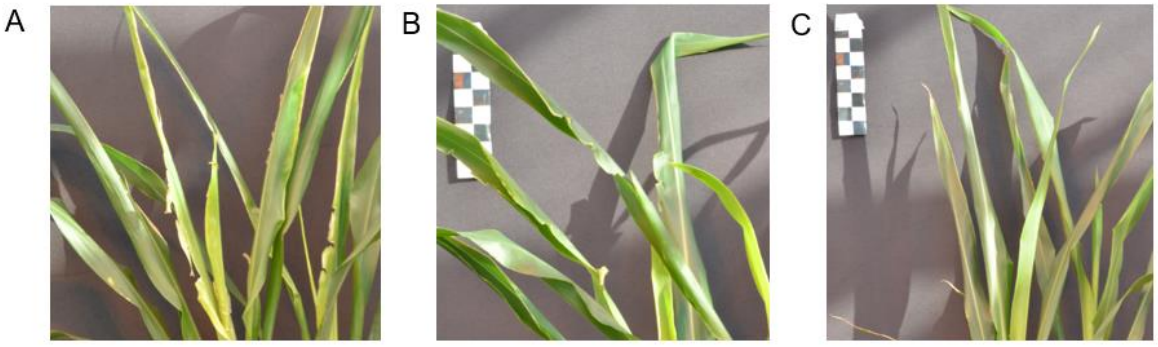


Figure 23. Calcium deficiency symptoms using different substrates

Image of the leaves of 6-week-old plants grown in different substrates. (A) substrate 1, (B) substrate 2, (C) substrate 3. Calcium deficiency leads to leaves split perpendicularly to the leaf vasculature, these symptoms are alleviated in substrate 3.

Table 3. Composition and fertilization for the different substrates used

	Substrate mix	Fertilizer stock	Quantity	Application
Substrate 1	1/3 m3 of peat moss	Nitrogen	232 PPM	Fertigation once per week
		Phosphorus	68 PPM	
	1/3 m3Vermiculite	Potassium	395 PPM	
		Calcium	172 PPM	
	66g Micromax ®	Magnesium	30 PPM	
		Sulfur	39 PPM	
	Peat moss	Iron	5,4 PPM	
		Boron	0,26 PPM	

Substrate 2		Manganese	1,4 PPM	
		Zinc	0,28 PPM	
		Molybdenum	0,12 PPM	
		Copper	0,112 PPM	
Substrate 3	1/3 m^3 of sand	Calcium Nitrate	2kg	Mixed in the substrate
		Potassium Sulphate	1kg	
	1/6 m^3 of compressed peat	Superphosphate	9,33kg	
		Dolomite	10kg	
		Hydrated lime	6kg	
	4 kg of stock fertilizer mix	Gypsum	3kg	
		Micromax®	1,2kg	

Medias for tissue culture

Tissue culture and media preparation are laborious and time-consuming tasks. The *in vitro* subculture of sorghum can take up to 4 months and several kinds of media are needed in the different stages of the transformation. All the different types of media were based in Murashige & Skoog salts supplemented with Gamborg B5 vitamins, 3% sucrose and 8g/L agar. The pH was adjusted to 5.6-5.8 with KOH. Due to the high percentage of sucrose, it is recommended to autoclave at 121ºC for only 16 minutes to avoid the browning of the media. The first of the medias to be used is the callus induction media (CIM) where the immature embryo explants were grown in 2,4-D supplemented media until the formation of embryogenic calli. The osmotic media (OM) allows the dehydration of the calli, increasing the nucleus exposure to the bombarded particles and facilitating the survival of the cells to the impact of the gold particles by avoiding plasmolysis (Vain et al., 1993). The regeneration media (REM) is used for the regeneration of embryogenic calli into plantlets and the rooting media (ROM) helps to the development of a proper root system prior to move the transgenic plantlets to soil. A set of different hormones and other ingredients were used in each media (Table 4) and the stock solution with some technical information for every hormone can be found in Table 5. In

addition, Geneticin (G418) was used as selective agent at a concentration of 30mg/L and must be added to REM and ROM after autoclaved for the selection of transgenic calli or plantlets. The recommended size of the petri dish to warrant a better nutrition of the calli and less intoxication from the released phenolic compounds is 90mm diameter and 25mm tall. For OM shorter petri dishes can be used.

Table 4. Ingredients for the preparation of the different medias used in tissue culture

Media	**CIM**	**OM**	**REM***	**ROM***
Full Name	**Callus Induction Media**	**Osmotic Media**	**Regeneration Media**	**Rooting media**
2,4-D	1 mg/L	-	-	-
BAP**	-	-	1 mg/L	-
IAA**	-	-	1 mg/L	1 mg/L
IBA**	-	-	-	1 mg/L
NAA**	-	-	-	1 mg/L
CuSO4**	1µM	-	1µM	1µM
Mannitol	-	0,2M	-	-
Sorbitol	-	0,2M	-	-
KH2PO4	1g/L	-	-	-
L-Proline	1g/L	-	-	-
L-Asparagine	1g/L	-	-	-
Storage	1 month	3 months	2 months	3 months

All the medias are based on MS media supplemented with Gamborg B5 vitamins, 3% of sucrose and 8g/L of agar. pH 5.6-5.8

*For the selection of transgenic material Geneticin 30 mg/mL were added.

**Add after autoclaving at a temperature below 55ºC.

Table 5. Technical information for the hormone stocks used in tissue culture

Stock solution	2,4-D	BAP	IAA	IBA	NAA
Full name	2,4-Dichlorophenoxy-acetic acid	6-Benzylamino-purine	Indole-acetic acid	Indolebutyric acid	α-Naphtalene acetic acid
Molecular weight (g/mol)	221	225,3	175,2	203,2	186,2
Powder storage	RT	RT	--8 ºC	2-8 ºc	RT
Solution storage	0-5º C	0-5º C	0-5º C	0-5º C	0-5º C
Solvent	EtOH	1M KOH	EtOH	EtOH	1M NaOH
Diluent	Water	Water	Water	Water	Water
Sterilization	Co-autoclave	Filter	Filter	Filter	Filter
Concentration (mg/mL)	1	1	1	1	1

Immature embryo isolation and callus induction

As mentioned previously the starting material is critical for a successful callus induction and higher transformation efficiencies. Therefore, the developmental stage of the seeds is important, and the plants must be monitored for collection in a proper maturity stage. The immature seeds must be collected 10-15 days after pollination. The panicle of sorghum develops from the top to the bottom showing different developmental stages among the different parts of the panicle. Seeds for embryo isolation must be smooth and green with a width of 3mm, the embryos should measure between 1 and 2 mm in length and the scutellum must be plain and smooth (Figure 24). A single healthy panicle of Tx430 can produce almost 1000 seeds and the seeds can be collected in different days collecting first the upper ones. Callus induction efficiency in sorghum varies depending on the maturity status of the seeds, the media used, the vigor of the plant and the personal skill to not damage the embryos. Still, in good conditions, from 50 to 70% of the IE will develop embryogenic calli. The average transformation efficiency is 1-5%. Therefore, in good conditions, the recommended minimum

number of embryos to isolate to obtain enough transformant plants is 600. After separating the seeds from the panicle, the immature seeds were surface sterilized and placed in a petri dish inside a sterile laminar flow cabinet, allowing to air-dry the seeds. Immature embryos were explanted with tweezers and a scalpel or lancet. The embryos should be placed on CIM media with the scutellum facing upwards and the axis facing the media. Immature embryos are kept for 9-11 days in dark at 28ºC on CIM media until the day of bombardment. Fungal and bacterial contaminations are usual when isolating embryos from plants growing in field or big glasshouses, moreover, embryos growing in CIM media release phenolic compounds (Belide et al., 2017) that are undesirable for the survival of the calli. For these two reasons is recommended to keep a low number of embryos per plate with a maximum of 20 and subculture the calli 5-7 days after isolation due to the accumulation of a purple pigmentation in the media that is detrimental for the induction of embryogenic calli.

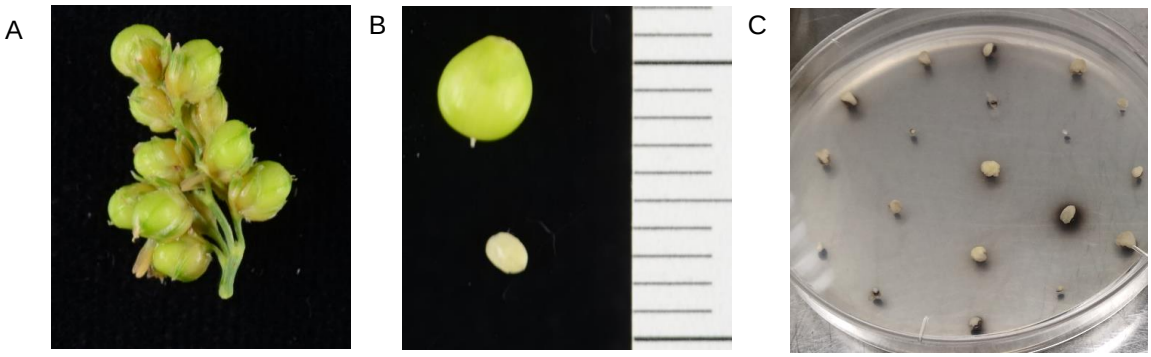


Figure 24. Embryo isolation and subculture

(A) Spikelet of sorghum immature seeds in the moment of collection for IE isolation. (B) Immature seed and embryo explant. (C) Immature embryos 5 days after callus induction, when purple pigmentation appears in the medium it is recommended to subculture them to new CIM.

In our experience, the callus induction varied from different campaigns of transformation during 2019 and 2020 at CRAG, and in the experiments performed in 2019 in QAAFI. Despite using the same media composition, calli grown at CRAG in 2020 were more white and bigger than in 2019. Calli from plants growing in Australia were much bigger and more yellow than those grown at CRAG.

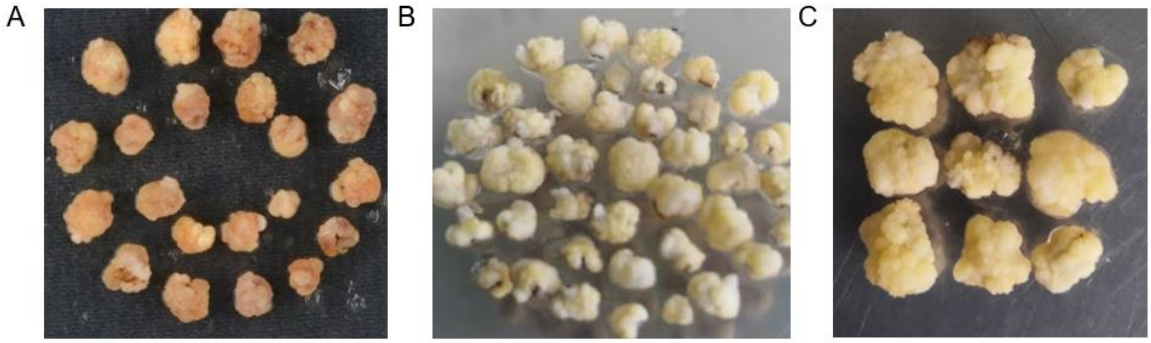


Figure 25. Calli in different campaigns

Calli grew for 10 days in CIM (A) 2019 at CRAG, (B) 2020 at CRAG or (C) 2019 at QAAFI.

Gold particle preparation

Several materials can be used for transformation by microprojectile bombardment, but gold is the most used due to its low cytotoxicity (Krysiak et al., 1999). Before coating the gold particles with DNA, the particles must be cleaned from impurities and aliquoted. Every aliquot described in this protocol can be used for six bombardments and they can be stored up to 6 months at -20ºC. First, 50 mg of gold (0.6 µm diameter) were aliquoted in a 1.5mL microcentrifuge tube. 1 mL of 100% ethanol was added to the tube and vortexed thoroughly for 5 minutes. The gold particles were allowed to settle for 15 minutes. The gold was pelleted at 15.500 rcf in a centrifuge for 10 seconds and ethanol was removed before washing the gold particles 3 times with 1mL of sterilized water by vortexing for 1 minute, standing for 1 minute, and finally centrifugation at 15.500 rcf for 10 seconds. Supernatant was removed and the gold particles were resuspended in 1mL of sterilized 50% glycerol as a final step. The concentration of gold particles is 50mg/mL. Frequent vortex is necessary to keep a stable concentration of gold particles while preparing 50µl aliquots into 2mL tubes.

DNA coating of gold particles

The gold particles are the microcarriers of the foreign DNA and they are responsible for the delivery of DNA inside the cells. The following protocol optimizes the precipitation of DNA molecules onto the gold particles, and it has to be done just before the bombardment, while the calli are being treated in OM First, all the frozen reagents were thawed. 50 µl of $CaCl_2$ 2.5

M and 20 µl of spermidine 0.1M freshly prepared were added simultaneously to the gold tube, the lid was closed, and the mixture was vortexed immediately during 1-2minutes. $CaCl_2$ and spermidine are essential for the precipitation of the DNA and their absence result in great losses of DNA in the washes. The mixture was let to precipitate for 5 minutes on ice and then vortexed until no clumps were visible. Since the system used for transformation was based on co-bombardment of a vector driving the resistance to geneticin (pUKN-NPTII) and another vector driving the expression of the gene of interest, 10 µg from 2 different plasmids were added to the gold suspension at a concentration of 1µg/µl and the mixture was vortexed for 1-2 minutes and placed on ice for 10 minutes. The gold particles and DNA were pelleted for 6 seconds in a benchtop microcentrifuge. It is important that all the gold particles are precipitated but they should be resuspended easily without formation of clumps. The pellet was washed with 130 µl of 70% ethanol, vortexed for 1-2, placed on ice for 5 minutes and pelleted for 6 seconds. The supernatant was removed again and resuspended in the final volume of 35 µl of ethanol 100%. The final volume of every tube will be used for 3 bombardments, so the number of aliquots to prepare should be calculated depending on the number of plates of the experiment.

Osmotic treatment and bombardment

After 9-11 days in CIM, we selected white and globular calli (Figure 25) and placed 7-9 of them on the middle of an OM plate for 3h and bombarded them. The bombardment device used was a PSD1000/He™ from Biorad® placed in a sterile laminar flow hood. The instructions for using this device can be found in the manual provided by the manufacturer. All the components and surfaces of the device were sprayed with 70% ethanol for sterilization and the ethanol was let to evaporate. Macrocarriers, macrocarrier holders and stop screens were sterilized by soaking in 70% ethanol. The rupture disks were soaked in isopropanol for a few seconds as recommended by the manufacturer. The macrocarriers were placed into the holders and the borders were pressed until they were well accommodated in the bottom of the holder, ensuring a straight dispersal of the gold particles. 5 µL of the DNA coated gold particles were placed on the middle of the macrocarrier and the ethanol was let to evaporate. For the assembly, first place a 900psi rupture disk into the retaining cap and tight it to the helium valve. Place a stop screen and a holder with the macrocarrier and the gold particles into the microcarrier launch assembly and tight the retaining ring. Open and place the petri dish with the calli on the plate shelf, close the door, apply vacuum until the manometer reaches 27 inHg and trigger the release of the helium until the rupture disk breaks dispersing the gold particles.

Open the valve for the vacuum and place the petri dish sealed in dark. 3-4h after the bombardment move the embryos to a new CIM plate and let recover for 3-4 days in dark. Keep several non-bombarded plates for future controls.

Optimization of the bombardment

To check that the coating of the gold particles was successful but also to optimize the conditions of the bombardment, we used a vector containing GUS under the expression of PvUbi promoter, proven to work in sorghum (Mann et al., 2012), to check the bombarded area and the differences in the GUS expression on the calli depending on the different bombardment parameters. First, we set up the amount of gold per bombardment by visually comparing the amount of GUS staining in bombarded embryos with 5 or 10 µl of the gold mixture, 0,36mg or 0,72mg of gold respectively. We clearly saw an increase in the stained area in the calli bombarded with the higher concentration of gold. This is a high amount of gold compared to other biolistic transformation protocols like maize or wheat (Ismagul et al., 2018; O'Kennedy et al., 2011), but very similar to the protocols from Ian Godwin, despite they used a different bombardment system.

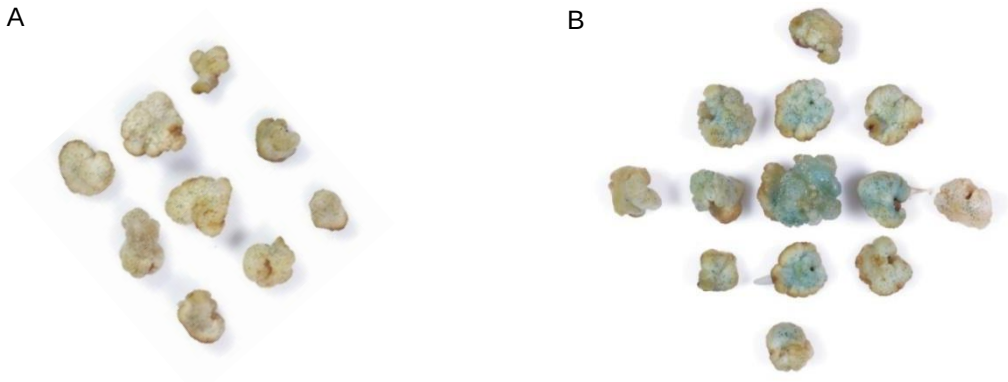


Figure 26. Transient GUS expression at different gold concentrations

Tx430 calli 9-day after CIM treatment bombarded with 0,36mg (A) or 0,72mg of 0,6µm gold particles and a vector containing GUS under PvUBI promoter.

Then, we designed an experiment to optimize other parameters like the distance of the target and the amount of calli that can be bombarded per shot. We evaluated the area of impact of the gold particles and the intensity of GUS expression depending on the position of the calli.

We placed the calli covering a circle of 3cm diameter, and after bombardment at different target distances (1.5, 3, 6 and 9 cm), scored the pixels showing GUS expression of the calli placed in concentric circles of 1,5, 2,5 or 3 cm (R1, R2 or R3). The results showed that the area covered by the bombardment does not change depending on the distance of the target, the maximum staining was found in calli placed in the central 1,5cm diameter circle, but the bombarded area covered up to 2,5 cm of diameter. Data from calli shot at 1.5cm was discarded because they were severely damaged.

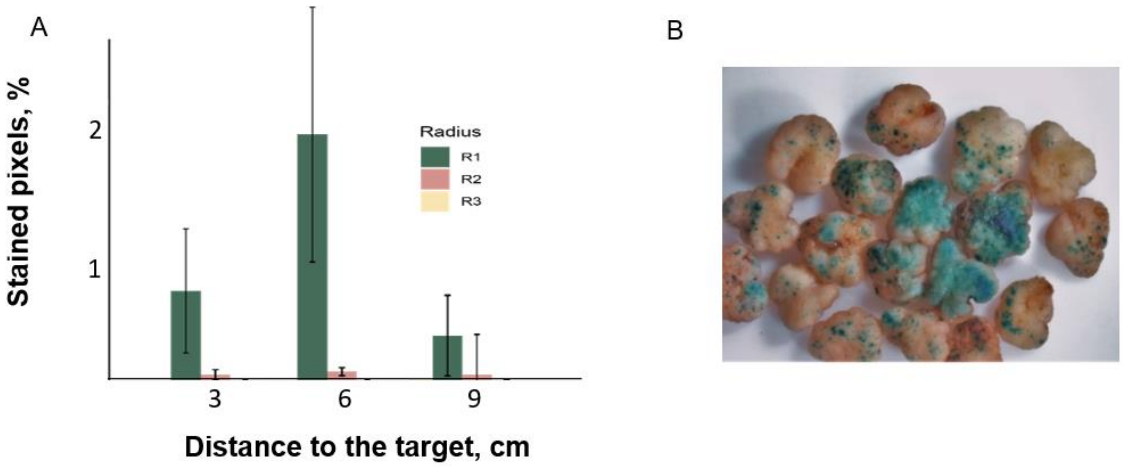


Figure 27. Optimization of target distance in bombardment.

(A) Graph showing the total pixel number of GUS expression per calli after bombardment with pANIC6B vector, at different target distances (3, 6 and 9cm) and the position of the calli the position from the center R1 to the outer part R3 of the circle.

Regeneration

After 3-4 days of recovery in CIM, the calli were placed in REM containing geneticin and the plates in an *in vitro* growth plant cabinet at 28ºC and 100 µmol s^{-1} m^{-2} of light (16 h/day). Several non-bombarded calli were placed in REM with and without geneticin as controls. The calli will have to be subcultured every 2 weeks on a new REM plate and this can be extended for 4 months. In the first subculture most of the calli were still alive, non-bombarded controls too. Necrotic black calli were discarded. For the second subculture, most of the calli should start to get green. Only calli that were green and those still presenting the white and globular embryogenic tissue were subcultured. In the following subcultures, most of the calli were necrotic, the necrotic tissue was carefully removed without damaging the calli. Once the regenerating plantlets reach a size of 2-3 cm, they can be easily separated from the rest of

the calli. Transformant plantlets can propagate themselves, every plantlet obtained from the same callus is considered a clone. When the plantlets are 6-8 cm long and they are propagating into more plantlets they can be subcultured into rooting media.

The rates of regeneration varied between the different transformation experiments, but also among the different campaigns of transformations carried during 2019 and 2020 at CRAG and 2020 in Ian Godwin's laboratory in Australia. In the first campaign of transformation at CRAG in 2019 very few regenerants were obtained. During the stay in Ian Godwin laboratory in QAAFI, most of the selected calli produced green tissue or still presented white globular parts during the first 2 weeks, and in the next subcultures onto regeneration media some of them were darker or the plantlets where browning due to the antibiotic selection. In the second campaign at CRAG, after the experience obtained in sorghum transformation in Ian Godwin laboratory, the results in regeneration improved notably compared to the first campaign. Real transformant calli can produce many plantlets from a single calli (Figure 28).

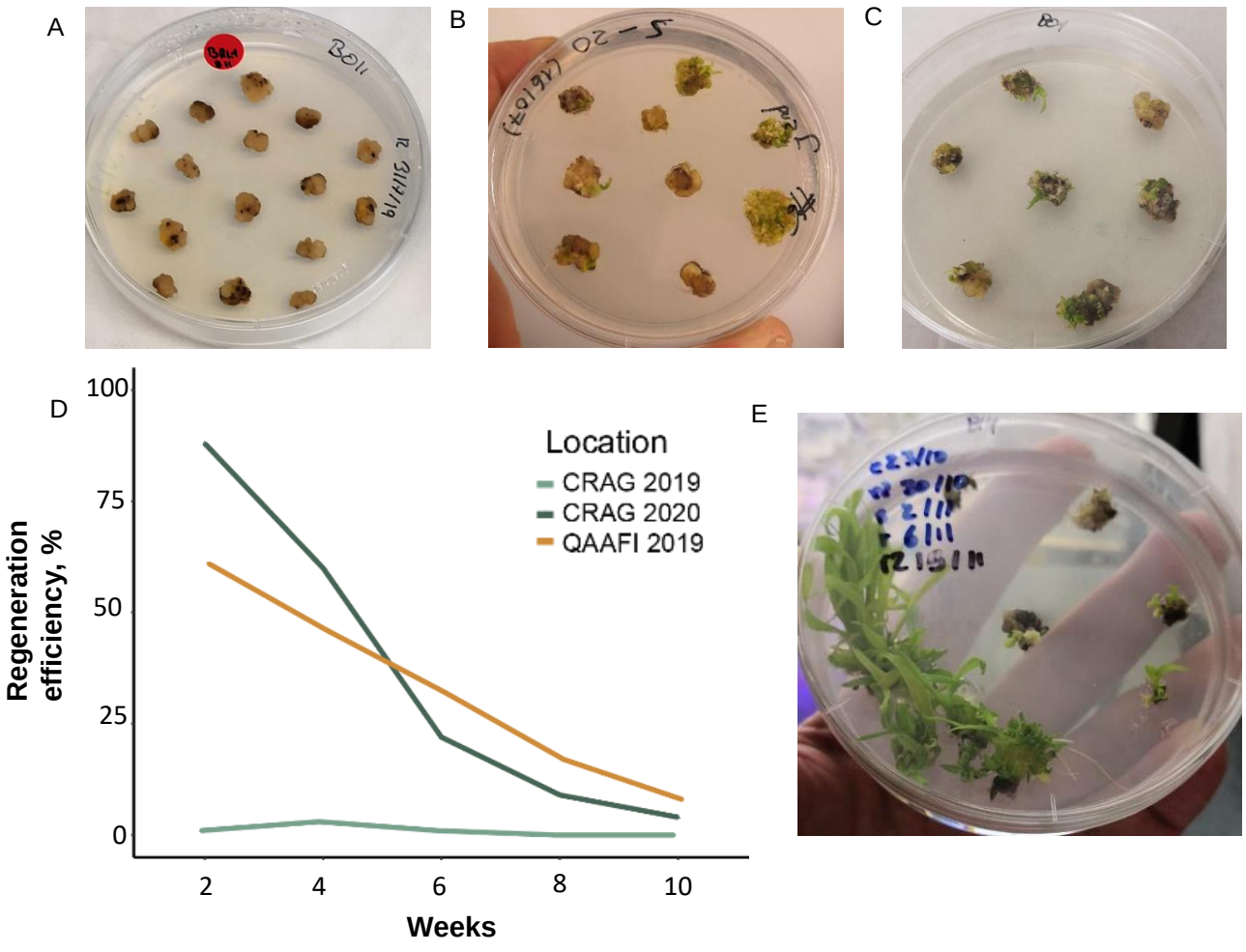


Figure 28. Regeneration and selection of transformant calli

A, B and C: Calli after 2 weeks in regenerative selective media in 2019 at CRAG (A), 2020 at CRAG (B) or 2019 at QAAFI (C). (D) Average regeneration percentages of the different transformation campaigns during time. (E) Image of putative transformant callus and plantlets in the left and left bottom border and necrotic/regenerating calli in the rest of a petri dish.

Rooting and acclimation

When the plantlets reach 2-3 cm long, the main shoots were separated from the rest of the calli with a blade and placed in rooting media, the rest of the plantlets can be kept in regeneration media as a backup. 2 weeks later, transformant plantlets should have white roots. Once the root system is developed, the transformant plantlets were acclimated by opening the petri dish with tap water on top of the media to prevent dehydration. 1 week later, the plantlets were carefully removed from the petri dish, the excess agar was cleaned with water, and they were transferred to pots in standard greenhouse conditions for sorghum.

During the first campaign of transformation in Barcelona, the few regenerant calli did not grow enough to be placed in rooting media. In Australia a total of 10 calli produced several plantlets able to root in selective media. In the second transformation campaign in Barcelona, many plantlets were placed in selective rooting media after reaching a proper size in selective regeneration media. Even though plantlets placed in non-selective rooting media as control produced roots, in selective media those would not generate roots.

A

B

Figure 29. Images of sorghum rooting plantlets

In the left, rooting plantlet opened for acclimation. In the right, once the plantlet has a good root system, the excess of agar is removed with water prior to plant it in soil.

Identification of transgenic T1 plants

To confirm the obtention of transgenic plants, T2 seeds sent from Ian Godwin to our laboratory were placed in a solution of 30mg/L of geneticin for four days. *Wt* seedlings do not develop a sufficient root system and the primary root is necrotic while transgenic plants show white and lateral roots. (Figure 29).

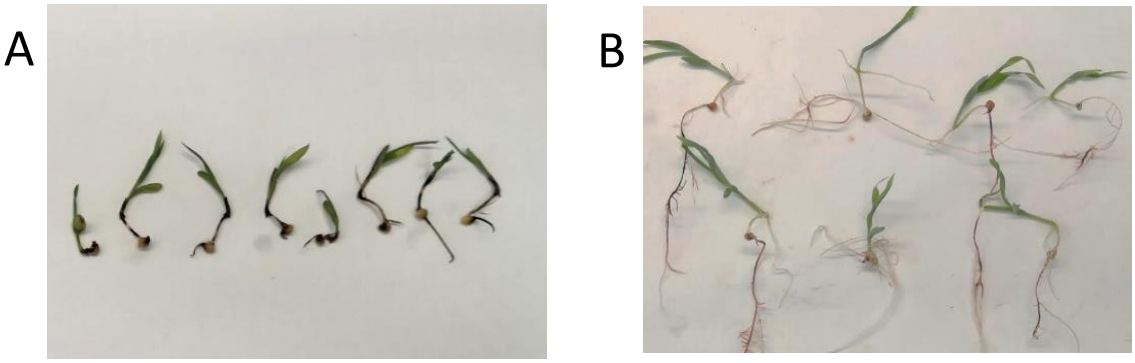


Figure 29. Antibiotic resistance of sorghum putative transgenics

Sorghum seedlings were surface sterilized and treated for 4 days in a solution of 30 mg /mL. At the left, Tx430 non-transgenic plants show black and small roots. At the right, putative transgenic sorghum plants show longer primary roots with secondary roots of white color.

Antibiotic resistant plants were screened under epifluorescence microscope and GFP positive plants were analysed with confocal microscopy (Figure 30). Despite having a good intensity of GFP, the GFP was not specifically membrane localized as expected for BR receptors.

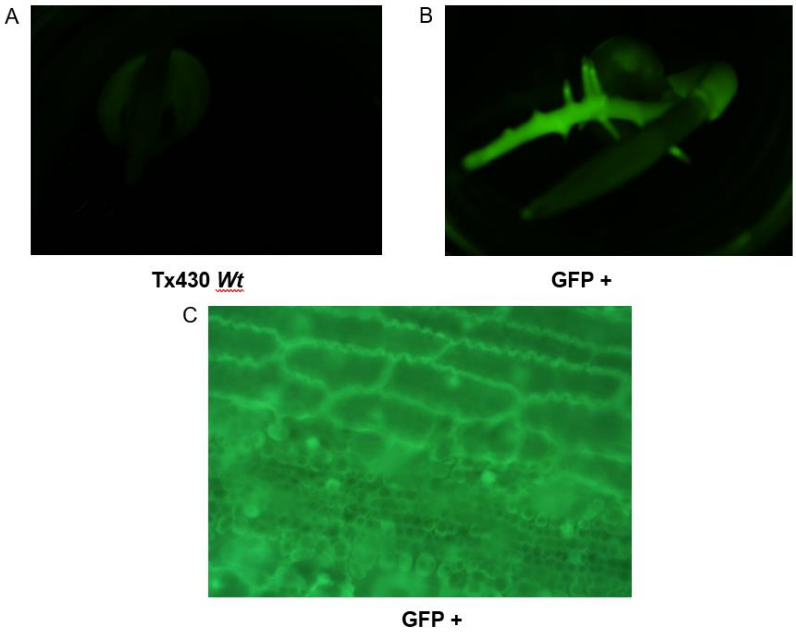


Figure 30. GFP expression of sorghum putative T1 transgenic plants,

Non-transgenic (A) and transgenic (B) Tx430 seedlings imaged under epifluorescence stereoscope. Confocal image of a transgenic sorghum leaf (C).

Different PCRs were designed to confirm the presence of the desired transgenes: nptii, *Cas9*, gRNA for the edition of the genes of interest, *BRI1* or *BRL1* in this case. Primers can be found in Table 7. In the case of the gRNA, since primers used were annealing in the promoter U6 of and the backbone of the vector, the PCR product was sequenced to confirm that the insert included the gRNA designed for *BRL1*. The primer annealing in the U6 promoter of the genomic DNA of sorghum may explain the unspecificity of the PCR in *Wt* plants. Despite these plants already showing resistance to geneticin treatment, and nptII transgene was confirmed by PCR, they did not show integration of Cas9. The vector with Cas9 was co-bombarded together with the vector containing the gRNA and nptii. In the case of the lines showing GFP fluorescence, PCRs were designed to amplify BRI1/BRL1-GFP but the results only showed amplification of PCR product in the positive control.

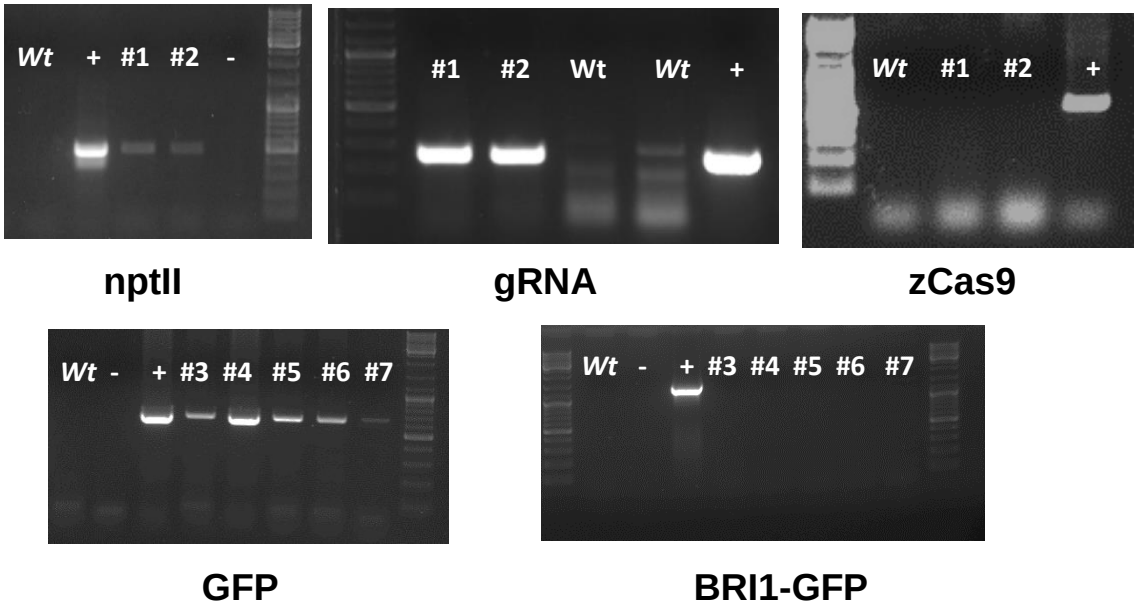


Figure 31. Identification of transgenes in putative sorghum transgenics,

PCRs for the identification of nptII, gRNA, Cas9, GFP and BRI1-GFP junctions. Antibiotic resistant plants were confirmed to be transgenic by PCR amplification of nptII and gRNA insert but not Cas9. GFP was also identified but the region of BRI1-GFP or BRL1-GFP (not shown) was not identified in these transgenic plants. The numbers correspond with different transgenic plants, negative control of the PCR is displayed as (-), the positive controls used were plasmids containing the desired sequence and are displayed as (+).

CHAPTER 5:

GENERAL DISCUSSION

Overview of the thesis

The purpose of this study was to identify and characterize the BR receptors in a crop model plant, such as sorghum and translate the knowledge obtained from Arabidopsis regarding the roles of BRs in abiotic stress responses. To be able to complete our research, we set the following approach:

1. Identify the number of BR receptors in sorghum and their similarity with other known and characterized receptors in multiple species. We took a bioinformatics approach to prove that the structure of the receptor is capable of the BL binding and could be potentially active and functional. Furthermore, we decided to investigate if the sorghum receptors could complement the most striking phenotypes of Arabidopsis mutant plants for BRI1 and BRL3. We performed multiple combinations in different background plants, and we conducted a series of characterization experiments.
2. Since sorghum BR receptors were functional in a heterologous system, we decided to study them in the crop plant. For this reason, we used a published collection of EMS mutagenized sorghum. The approach used in that part was to characterize potential phenotypes not only in M4 plants derived directly from the mutagenesis but also in plants backcrossed, to eliminate the background mutations. We tested multiple generations and different lines for more robust results. In addition, experiments that challenge the plants with abiotic stresses were performed, to be able to translate the findings that have been found for Arabidopsis.
3. Finally, since CRISPR technology allows us to generate plants with specific editing events, we tried to establish the transformation protocol for sorghum in our institute. Is it worth mentioning that very limited efforts have been tried in Europe and even less are successful. We established collaborations with well-known and experienced laboratories from different continents and we present our findings on the critical points in the sorghum transformation protocol to contribute to the efforts of scientists that are interested in this recalcitrant crop.

BR receptors are conserved in sorghum and other crops

Using publicly available databases we tried to identify the number of genes encoding for BR receptors in sorghum. The sequence similarity led to the identification of 3 members of the

BRI1-Like family in *sorghum* with high pairwise identity score: SbBRI1, SbBRL1 and SbBRL2 (Table 1). The different members of the BRI1-like family in the different tested species were grouped by phylogenetic analysis in 3 different clades as expected (Figure 4). A more exhaustive study on the origin of BR receptors found that BRL sequences are conserved in all seed plants. BRL2 was the first member of the family which appeared together with the first land plants and is conserved along the plant kingdom with the exemption of liverworts and some mosses, which suggests a selective loss of BRLs in these groups (Ferreira-Guerra et al., 2020). BR receptors appeared in the common ancestor of angiosperms and gymnosperms, although in the gymnosperm *Picea abies* no ortholog of BRI1 was found and *Pa*BRL1 appears to be the only BR receptor (Wang et al., 2021). Using 3D modelling we constructed the tertiary protein structure which showed high resemblance to the one for AtBRI1 (Figure 5). Considering the high conservation of the binding regions which are necessary for the activation of the downstream signaling, and the previous studies excluding BRL2 as a BR receptor, we considered that sorghum conserves two BR receptors, SbBRI1 and SbBRL1 (Figure 6).

The heterologous expression system of sorghum BR receptors in Arabidopsis revealed that sorghum BR receptors anchor to the plasma membrane of the cells (Figure 7). This pattern is consistent with the previous literature that showed plasma membrane localization for the BR receptors. The obvious complementation of the *bri1* dwarf phenotype in plants expressing *SbBRI1* and *SbBRL1* (Figure 8) resembled the results obtained in Caño-Delgado et al., 2004, in which both Arabidopsis *BRL1* and *BRL3* were able to complement *bri1* mutants if expressed ubiquitously. To our knowledge, the complementation of Arabidopsis *bri1* mutants with *BRI1* orthologs from other species has been assessed only with *Glycine max*, *Triticum aestivum* and *Brachypodium dystachion BRI1. GmBRI1 and TaBRI1* were able to revert the dwarf phenotype of *bri1* Arabidopsis mutants (Peng et al., 2016, Fang et al. 2020), but on the contrary, *BdBRI1* did not restore the phenotype of *bri1* mutants (Corvalán & Choe, 2017). In the last study the authors concluded that *BdBRI1* and *AtBRI1* may not be functionally equivalent, while RNAi-silenced *BdBRI1,* did show similar phenotypes than those reported for Arabidopsis know-down mutants of rice and Arabidopsis including insensitivity to BRs in root length and lamina joint bending (Feng et al., 2015). In the case of *Picea abies,* which lacks *BRI1, bri1-301* dwarf mutant was complemented with *PaBRL1* (L. Wang et al., 2021). In addition, we further proved that the insensitivity of *bri1-301* to BL was restored in both hypocotyl and root organs (Figure 9). In BR-sensitive plants, the treatment with higher concentrations of BL causes a dramatic root growth reduction and increased hypocotyl length, while Arabidopsis *bri1* mutants showed a reduced growth in both root and hypocotyl tissues

under normal conditions and was not affected by BL treatment. (Chen et al., 2013; González-García et al., 2011). The complemented mutants restored the root growth in basal conditions, but also recovered their sensitivity to BL. These results, together with the 3D modeling of sorghum BRI1 receptor and the conservation of the ID domain indicates that SbBRI1 and SbBRL1 can bind BL, supporting that both sorghum BR receptors are functional in sorghum. Finally, the last phenotype that we tested for potential complementation was the response to stress caused by heat. More specifically, we investigated if SbBRI1 and SbBRL1 could restore the inability of Arabidopsis *bri1-301* and *brl3-2* to respond to slightly elevated temperatures. Our data demonstrated that both sorghum receptors could restore the insensitivity of *bri1* mutants to elevated temperatures. Interestingly, SbBRL1 showed a stronger effect in the elongation of both roots and hypocotyl in mock treatment, while upon BL treatment the effect of *SbBRL1* was stronger in hypocotyls than in roots. However, none of sorghum receptors was able to revert the phenotype of *brl3-2* mutant. *brl3-2* mutants can elongate their hypocotyls upon BL treatment, but they show a deficient elongation upon increased temperature. The complemented *brl3-2* lines with sorghum BR receptors did show an increase in the hypocotyl length upon BL, but they were unable to restore the sensitivity to increased temperature (Figure 10).

Taking together, our findings indicate that both putative sorghum BR receptors function by binding BL and they control growth and developmental processes in Arabidopsis. According to the previous literature, the variability in the extracellular domain of the receptors is key for their specific functionality (Zheng et al., 2019). BRI1 and BRLs team up with different interactors (Jaillais et al., 2011; Lozano-Elena & Caño-Delgado, 2019) which together with the restricted localization pattern of BRLs in Arabidopsis, indicate a role for them in controlling tissue-specific responses. Some structural changes between Arabidopsis and sorghum BR receptors may lead to changes in their affinity to different BRs and other interactors. This could explain the differential response of *Sorghum* BR receptors in Arabidopsis roots and shoots.

Analysis of sorghum BR receptor mutants

The increasing popularity of the commercial and industrial uses of sorghum made necessary the research for the development of tools available to study sorghum genetics (Xin et al., 2021). We took advantage of a mutagenized population in the only available sequenced genome, at that date, BTx623 (Jiao et al., 2016). Two new alleles of SbBRI1 and four of SbBRL1 were isolated from the bulk distributed seed stocks. As presented in Table 2, all these

lines presented a high amount of background mutations. To phenotypically characterize the mutants, several M4 plants were obtained for each of the 6 different alleles. An interesting phenotype was the presence of severe dwarf plants for ARS87 and ARS72 M4 *bri1* plants. Furthermore, they showed sterility due the inability of the anther filaments to elongate as reported for some BR mutants (Kim et al., 2005; Ye et al., 2010). M4 *brl1* mutant alleles showed a reduced plant height but no other developmental phenotypes were observed (Figure 11). Due to the high number of background independent mutations that are accumulated in the plants analyzed and can have an effect on the plant growth and development, backcrossings were done to confirm these putative phenotypes.

Backcrossing mutagenized plants allowed us to reduce the number of background mutations with potential phenotypes. Due to limitations of time, in the present thesis we conducted two consecutive backcrossings which are not enough to eliminate the background mutations. For this reason, in the experiments performed we study the F2 segregation from backcrossed lines (Figure 12). In our case, M4 mutant pollen was used to pollinate BTx623 *Wt* plants previously emasculated to generate BC1F1 plants, with the exemption of M4 bri1-72 plants, which were used as acceptor of BTx623 *Wt* pollen due to the unavailability of pollen from these sterile plants. The process of backcrossing was repeated for some of the lines and helped dilute the effects of other background mutations. The analysis of the F2 progeny showed that the severe dwarf phenotypes and sterility in M4 *bri1* mutant plants were independent of *bri1*. In the three biological replicates for both *bri1-72* and *bri1-82* BC1F2 segregations, plants with normal fertility and with sterility defects were found in homozygous, heterozygous and *Wt* plants for *BRI1*. Similarly, the dwarf phenotype found in M4 *bri1-72* mutant plants, although not as severe, was also segregating with *Wt* siblings (Figure 13). Despite significant differences found in plant size for *bri1-87* and its *Wt* siblings, the average plant size from both groups was substantially different to the original *Wt* BTx623 plants, which usually exceeds 1.5m (Figure 14). These results indicate that bri*1* alleles are not responsible for the sterility observed in M4 plants and other background mutations present in these *bri1* lines might affect plant size. *brl1* mutant plants did not show any difference in plant size compared to their *Wt* siblings. BTx623 parental plants were usually taller than most of the mutagenized plants in all the lines. Regarding the grain yield of the mutant plants (Figure 15), we observed no differences between the different *brl1* alleles compared to their *Wt* siblings, and their yield was like BTx623 parental plants. For both *bri1* mutant alleles we found significant differences in plant yield compared to their *Wt* siblings,as reported also for wheat *bri1* mutants (Fang et al. 2020), *bri1-72* and *bri1-87* produced lower grain yield than their *Wt* siblings. The comparison between BTx623 *Wt* plants and the *Wt* siblings of the mutant lines revealed, as occurred with the plant size, that other background mutations play a role in yield production. *Wt-3* and *Wt-87* produced

lower yield than BTx623 plants. The background mutations from the EMS treatment in these lines are challenging for the phenotyping and are increasing the number of controls necessary for every experiment, Despite some evidence pointed that *SbBRI1* control plant size and yield in sorghum*,* the differences between F2 *Wt* siblings of the mutants with the *Wt* parental line evidence that other factors are involved in these processes. Therefore, more backcrossings should be performed to confirm these putative roles for BRI1 and get more accurate results for all the alleles.

To study the effects of the above mutants under abiotic stress conditions or BL treatment, we performed the experiments with the progeny of 3 healthy independent *Wt* and BR receptor mutant homozygous F2 plants for each line. As mentioned in chapter 3, it has been described that *DW1* is a positive regulator of BR signaling in sorghum and rice, and BTx623 plants are *dw1* mutants (Yamaguchi et al., 2016). Rice and sorghum *dw1* mutant plants show with damped BR signaling and insensitivity to BL treatment in lamina joint bending angle (Hirano et al., 2017), so this classical experiment to evaluate the sensitivity to BL was not useful in our case. In species like Arabidopsis, rice, barley, tomato and sorghum, BL treatment causes inhibition of root growth and *bri1* mutants in are insensitive to BL treatment, therefore root length does not change upon BR exogenous application (Blasco-Escámez et al., 2017; Chono et al., 2003; González-García et al., 2011; Montoya et al., 2002; Yamamuro et al., 2000). Despite the *dw1* mutation of BTx623, it reduces its root growth upon high doses of BL, indicating that BTx623 plants are sensitive to BR treatment. Once again, BTx623 roots were longer than the mutagenized lines with no BL application but all the lines showed similar levels of sensitivity to BL. As it was expected, ARS87 showed relatively less sensitivity than BTx623 and its *Wt* controls, but unexpectedly the same happened with the line *Wt-72*. To sum up, similarly to plant height and yield phenotypes, additional backcrossings are needed to elucidate the changes observed in the sensitivity to BL treatment.

Despite the limitations in phenotyping due to high variability found in many of the mutagenized lines, our results strongly indicated that *SbBRI1* is implied in important agricultural traits such as grain yield. Unexpectedly, mutations in sorghum cultivar BTx623 BRI1 did not affect plant size; this may be explained by other background mutations present in the background of each line, which may be responsible for the differences between EMS mutagenized and BTx623 parental lines. Another plausible explanation is the fact that BTx623 elite sorghum variety was selected for several dwarfing genes, including *dw1* which as previously exposed affects BR signaling negatively.

BRs receptors control adaptation to drought and heat stress in sorghum

To assess if sorghum BR receptors are involved in stress responses, we performed two different abiotic stress assays. Firstly, we exposed dark grown plants exposed to elevated temperatures (38ºC) for 7 days. We observed that plants in those conditions were elongating less their mesocotyl than grown at 28 ºC.

Mesocotyl elongation is an important agricultural trait since seedlings with the ability to elongate their mesocotyls in deep sowing conditions present an advantage to prevent drought, by uptaking the more abundant water in the deeper layers of soils. In addition, it is an advantage to avoid damage by pesticides and avoid predators. Little is known about thermomorphogenesis in monocot seedlings. Whereas other researchers found that wheat seedlings elongate their leaf sheath when increasing the temperature (14ºC and 24ºC) (Vu et al., 2021), our results showed that sorghum *Wt* and BR receptor mutant plants grown in standard temperature (28ºC) are not different in mesocotyl elongation, but when exposed to elevated temperatures sorghum *bri1* and *brl*1 mutants are deficient in mesocotyl elongation (Figure 16). These results are consistent with previous research that found that BRs application control mesocotyl elongation in *Zea mays* (Zhao et al., 2021). Our findings highlight the importance of BR receptors in adaptation to elevated temperatures and growth of sorghum plants. BR receptors trigger responses that are crucial for seedling establishment in monocot and dicot plants in stress conditions.

For the second experiment under stress conditions, we evaluated several parameters which are usually affected in plants subjected to drought. Both, *bri1-72* and *brl1-106* mutants, showed a reduced water consumption during the drought period allowing them to avoid the stress and reach critical field capacities with a significant delay than their *Wt* controls. Interestingly, the photosynthetic efficiency (PSII) and the maximum quantum yield of PSII (Fv/Fm) was increased in *bri1* at the same FC % than their *Wt* relatives, except in well-watered conditions (80% FC), in which *bri1-72* PSII efficiency and Fv/Fm was lower than for *Wt* plants. The defects of *bri1* mutants in their photosynthetic activity have also been reported in *Triticum aestivum*. The flag leaves of Ta*bri1* mutants displayed lower photosynthetic activity during grain filling, which affected the yield of the plants. A lower transpiration rate would be expected from plants with lower photosynthesis, which could explain the delay of *bri1-72* mutants in reaching critical FC percentages. We also examined whether BR receptor mutants show a differential growth under water withholding conditions. Despite the significant difference in the

growth of well-watered plantlets and plantlets subjected to drought, the growth of mutant plantlets was not different from their respective *Wt* controls. The measurements of the relative water content in the leaves of sorghum seedlings subjected to different levels of stress was also affected along the drought period. No significant differences were found in that experiment, but *bri1-72* showed a consistent trend along the drought being able to store more water in the leaves. These results are consistent with previous findings in Arabidopsis (Fàbregas et al., 2018), where *bri1-301,* consumed less water, showed improved photosynthetic efficiency and relative water content that Col-0 *Wt* plants. Also, more studies have investigated the role of BRI1 in the tolerance to drought of *Brachypodium* distachyon, (Feng et al., 2015). The authors found that down-regulation of BRI1 produced dwarf plants with enhanced tolerance to drought. The present results contribute therefore to a better understanding of the role BR signaling under drought conditions which appears to be consistent in multiple species.

Sorghum transformation

Sorghum has been characterized as a recalcitrant species for transformation due to the accumulation of phenolic compounds and low regeneration rate which null after a short period in CIM. Since the first sorghum plant was successfully transformed with an efficiency of 0.1% (Casas et al., 1997) , several publications have reported improved efficiencies and alternative protocols. One of the most efficient protocols reported up to date is based on particle bombardment using IE (Liu & Godwin, 2012). As the scheme of the timeline of a sorghum transformation event is shown in Figure 22, it is a long process in which multiple aspects should be tightly controlled. Sorghum transformation has a low reproducibility and the efficiency of transformation varies with the location, the season and the person performing the experiments. To our knowledge, sorghum transgenic plants have never been obtained in Europe, even though *Sorghum* research is active. The protocol of Ian Godwin was repeated during 3 transformation campaigns in Barcelona and Brisbane, but it only succeeded in Brisbane.

The first difference observed between the first campaign in Barcelona and Brisbane was the phenotype of the plants, sorghum plants grown in Australia were more robust than those grown in Barcelona, they had thicker shoots, bigger flowers, and green leaves, while in Barcelona older leaves senesced and they presented calcium deficiency symptoms as

splitting leaves. Efforts on improving the health of the sorghum plants in Barcelona by changing the substrate led to an improvement in better embryogenic calli formation (Figure 23). This had an effect in the growth of the calli. Calli grown for 10 days in Brisbane were compact, had a whiter color and bigger size than those growing in Barcelona, although an improvement in the color and size of the calli was noticed after ameliorating the health of the sorghum plants by using a calcium rich substrate (Substrate 3 in Table 4). The different media used and the protocols for gold preparation and precipitation of the DNA are common protocols, which are described in detail and with some tips along chapter 4. Since the particle delivery device was different in Barcelona and Brisbane, we optimized some bombardment parameters like the DNA particle quantity and the target distance (Figure 26 and figure 27). A vector containing GUS was used to assess transient expression of foreign DNA, the results using 0.72mg of gold (10 µl of gold/DNA) per bombardment (Figure 26) in an area of approximately 1.5cm at a target distance of 6cm were satisfactory (Figure 27). The regeneration efficiency of IE in the laboratory of Prof. Ian Godwin in Australia was close to 90%, during the first transformation campaign in Barcelona the regeneration of plantlets was rare. Several factors could influence calli regenerability, and therefore transformation efficiency. As happened with the embryonic calli formation, the improvement in the health of the plants was translated to a better regeneration efficiency (Figure 28). Despite a substantial improvement in the regeneration efficiency in the second campaign in Barcelona, we did not obtain any transformant plantlet. The plantlets obtained died shortly after placing them in selective rooting media and the plantlets placed in non-selective rooting media as control developed roots. To discard a problem in the antibiotic concentration the bigger plantlets were genotyped for nptii, that drives the antibiotic resistance. Adjusting the geneticin concentration could optimize the intensive labor of subculturing regenerating calli, which survived for more than ten weeks without being resistant to antibiotics.

Sorghum transformation is still a challenge for all research groups. After training in a successful laboratory, the regenerability of sorghum calli improved substantially, yet no stable transgenic plants were produced during this thesis in Barcelona. The most notable and important change was the phenotype of sorghum plants grown in control conditions in Australia and in Barcelona, more robust and healthy plants led to a better quality of explants, which in our experience impacts in the whole process of transformation.

Despite 10 putative transgenic plants were obtained in Australia by Dr. Guoquan Liu and myself, we could only confirm the presence of gRNA for BRL1 in 1 transgenic line, without the presence of the co-bombarded vector containing Cas9. Co-bombardment efficiency with a

single selective marker is reported to have an approximate efficiency of 80% (Liu & Godwin, 2012). The target gRNA site was amplified by PCR and sequenced to discard the edition while a transient expression of Cas9. In our case we had to recur to crossing of T2 gRNA positive plants with Cas9 positive plants, provided by Prof. Ian Godwin, to manage to obtain a transgenic plant with both Cas9 and gRNA and hope for the edition. The lines were observed with epifluorescence microscopy and some of them presented GFP expression. GFP was also confirmed by PCR but the junction of BRI1/BRL1-GFP was not present indicating that these plants were not the desired BRI1 or BRL1 overexpressors.

Even though the efforts made at CRAG to transform sorghum have not been fruitful yet, this work establish the first steps for the transformation of this recalcitrant specie in our laboratory. The strategy of co-bombardment proposed by Prof. Ian Godwin lab failed to us, therefore efforts in re-designing new vectors containing all the essential parts for transformation should be considered. Also, due to the high cost of gold particle bombardment, other methods using agrobacterium or electroporation could be a cheaper alternative. The quality of the starting material, in our case, immature embryos, is also of great importance. Alternative methods using leaf whorls have been also shown to be able to produce stable transgenic plants (Silva et al., 2020), and the cost and difficulty of maintaining healthy plants for months could be overcame using young seedlings.

Concluding remarks and future perspectives

BRs are important plant hormones, and research has been useful to identify targets for agricultural purposes. After nearly two decades from the discovery of BRI1-Like receptors, this study is the first to describe a phenotype for single mutants BRL1/3 receptor in crops. This research led to the identification of sorghum BR receptors, which are conserved by sequence similarity and that both, SbBRI1 and SbBRL1, are functionally binding BRs, restoring the developmental defects of bri1 mutants in Arabidopsis. Furthermore, the obtention of sorghum BR receptor mutants and their phenotypical characterization revealed conserved roles of BRs in sorghum reproduction and response to abiotic stress. *Sbbri1-72* mutant plants show less grain yield and they have enhanced tolerance to drought without being affected in the general plant growth. Contrastingly, sorghum *bri1-72* and *brl1-106* and *brl1-121* mutants showed hypersensitivity to high temperatures. A total of six BR receptor mutants are currently being curated from background mutations and transformed CRISPR and transgenic plants affecting the expression of BR receptors will be useful for future research on sorghum BR signaling.

The efforts to overcome the technical challenges of introducing a new species for research in our laboratory and setting protocols at molecular and physiological level have been fruitful. The health of the plants has substantially improved during the last years, leading to a better regeneration of potentially transformed plants, and phenotyping accuracy of adult plants. Furthermore, several methods to study drought, osmotic stress, heat-shock, cell division of embryonic roots, transcriptomics, and metabolomics have been achieved *in vitro* and in greenhouse and will be used in several publications, which will contribute to gain knowledge about BR signaling and its potential implications in adaptation of crops to climatic adverse conditions and reinforce agriculture and food security.

CONCLUSIONS

1. Brassinosteroid receptors are conserved in crops including Sorghum and Arabidopsis .

   The sequence conservation reveals the conservation of the general structure of BRI1 protein with a pocket for the binding of BRs. The similarities conserved among the different family members in their BR binding region suggest similar binding affinities to BL for the different members of the BRI1 family.

2. Sorghum BR receptors are functionally acting as BR receptors in Arabidopsis.

   Despite the role of AtBRL3 is not gained by sorghum BR receptors, both sorghum receptors complement the dwarf phenotype of bri1-301 and its insensitivity to BL and elevated temperatures. This suggests a deep conservation of BRI1 function in different species, while supports that Arabidopsis *BRL3 may have a more specific role.*

3. Sorghum BR receptors control adaptative responses to drought and elevated temperatures.

   Both sorghum bri1-72 and brl1-106 mutants consumed less water than their Wt relatives without affecting the plant growth. Furthermore, bri1-72 mutants maintained a better photosynthetic efficiency in extreme drought conditions. In elevated temperatures, bri1-72, brl1-106 and brl1-121 sorghum mutants were severely affected in the ability of sorghum plants to elongate their mesocotyls.

4. Transformation of Sorghum is not a reproducible process.

   Despite intensive labor, adaptation of bombardment and tissue culture protocols, and the training performed in Prof. Ian Godwin laboratory, stable transformation of sorghum has not been achieved at CRAG. It is notable that the starting material obtained in different times and locations led to a visually different calli, which had an impact the regeneration rate. Future efforts, will need to be done trying alternative transformation methods, and establishing sorghum in Europe.

MATERIALS AND METHODS

Plant materials and growing conditions

For experiments with Arabidopsis, seeds were surface sterilized with 35% bleach (v/v) for 5 minutes in agitation, then rinsed 5 times with sterile distilled water. The seeds were kept at 4ºC for 48h and placed in ½ Murashige & Skoog agar media in sterile conditions. Seedlings were grown vertically in INKOA chambers set at 22ºC and 16h photoperiod at 150µmol·s^{-1} of light intensity as control conditions. 10-day old seedlings were transferred to pots for adult plant imaging.

For experiments with sorghum, seeds were washed with soap and water for 30 minutes in agitation, surface sterilized with 50% bleach (v/v) for 10 minutes, rinsed with sterile distilled water 8 times and kept at 4ºC for 48h. For in vitro experiments, seeds were partially embedded in ½ Murashige & Skoog agar media in sterile conditions with the side of the embryo exposed and the radicle pointing downwards. Sorghum plants were grown in INKOA chambers set at 28ºC and 12h photoperiod at 150µmol·s^{-1} of light intensity as control conditions. Adult sorghum plants were grown in a substrate rich in calcium to avoid nutritional deficiencies.

The different plant materials used in this thesis are summarized in Table 6.

Table 6. Plant materials used in this work

Line(s) name	Gene affected	Species	Description	References
Col-0	-	*Arabidopsis thaliana*	*Wild-type* Col-0 ecotype	-
bri1-301	BRI1	*Arabidopsis thaliana*	EMS Knock-down allele	Li et al., 2010
brl3-2	BRL3	*Arabidopsis thaliana*	T-DNA Knock-out allele	Fàbregas et al., 2013
35s::SbBRI1-GFP; bri1-301	BRI1	*Arabidopsis thaliana*	Complemented *bri1* mutant	This work
35s::SbBRI1-GFP; brl3-2	BRL3	*Arabidopsis thaliana*	Complemented *brl3-2* mutant	This work
35s::SbBRL1-GFP; bri1-301	BRI1	*Arabidopsis thaliana*	Complemented *bri1* mutant	This work

Line(s) name	Gene affected	Species	Description	References
35s::SbBRL1-GFP; brl3-2	BRL3	*Arabidopsis thaliana*	Complemented *brl3-2* mutant	This work
BTx623	-	*Sorghum bicolor*	Arabidopsis parental of EMS of ARS lines	Jiao et al., 2016
ARS lines	BRI1/BRL1	*Sorghum bicolor*	Descendants of BTx623 mutagenized seeds	Jiao et al., 2016 This work †
Tx430	-	*Sorghum bicolor*	Variety used for sorghum transformation	Liu & Godwin, 2012

† See table 2 for details of the different ARS lines

3D protein representation and construction of homology model

3D model from AtBRI1 protein was retrieved from RCBS PDB, (rcbs.org - Berman et al., 2000) and the homology model for SbBRI1 sequence was built with Mol* Viewer (molstar.org - Sehnal et al., 2021).

Protein sequence alignment and phylogenetic tree construction

Protein sequences of the target species were retrieved from BLAST (https://blast.ncbi.nlm.nih.gov) and analysed with Geneious Prime®. Sequence alignment was performed with a cost matrix BLOSM62. The unrooted phylogenetic tree was built with neighbor-joining method with sequences of FLS2 from the different species as outgroup.

DNA extraction

For the DNA isolation, material from young leaf (approx. 10 mg) was collected from each plant in a 2 mL microcentrifuge tubes containing 4-8 2mm glass beads and flash-frozen in liquid nitrogen. Frozen samples were homogenized in TissueLyser II (Qiagen) at frequency of 30 Hz for 30s. Then 500 µl of extraction solution was added to each sample (0.4 M NaCl, 10 mM

Tris-HCl pH8.0, 2 mM EDTA pH8.0 and 2% (v/v) SDS) and tubes were agitated thoroughly. Samples were centrifuged for 5 min at 12000 g. The pellet was discarded, and 1 volume of isopropanol was added to the supernatant, mixed gently and incubated for 5 min at room temperature. Then centrifuged at 12000 g for 10 min. The supernatant was discarded and the pellet was washed once with 70% ethanol. After ethanol evaporation, the pellet was resuspended in 100 µl of sterile-distilled water. DNA concentration and purity was assessed with a Nanodrop® (ThermoFisher) spectrophotometer.

Cloning of sorghum BR receptors

After DNA extraction, SbBRI1 (Sb03g032990 / Sobic.003G277900; chromosome 3:61,388,358-61,393,032 reverse strand) and SbBRL1 (Sb02g019470 / Sobic.002G155400; chromosome 2:47251818-47256309 forward strand) gene fragments from sorghum BTx623 seedlings were amplified by PCR with Primestar GXL polymerase (Takara) and cloned into pDONR-221 vectors using Gateway cloning system (Thermofisher). For Arabidopsis lines, an LR reaction was performed with pGWB405 for Col-0 and bri1-301 lines, and pGWB605 for brl3-2 lines, both vectors driving the transcription with CaMV 35s promoter and eGFP tag in the C-terminal but with kanamycin or phosphinotricin resistance as selectable markers, respectively.

For sorghum, a ZmUbi promoter fused to nptII (geneticin resistance) provided by Prof. Ian Godwin was used as selectable marker (Liu & Godwin, 2012c). The ZmUbi promoter and eGFP were cloned into pDONR221 plasmids and combined with vectors containing SbBRI1 or SbBRL1 genes with pP7m34GW. The primers used are presented in TableX.

Cloning of CRISPR vectors

For generation of the CRISPR construct to edit SbBRI1 and SbBRL1, the gRNAs were selected using CRISPOR web tool (Haeussler, et al. 2016). The gRNA sequences (BRI1: GAGGCTGCCCAGCTGCAGCA, BRL1:3'-GTTCAACGGGACGCTCCCCG-5') were amplified by PCR and annealed to synthetized SbU6 promoter in 5' and to tracrRNA sequence in 3'. The resulting fragment was ligated using TypeIIS cloning to the pUKN vector carrying resistance to Geneticin. For transformation, this vector was co-bombarded with a modified

pBUN411 plasmid, provided by Prof. Ian Godwin containing zCas9 sequence under the expression of ZmUBI.

Stable transformation of Arabidopsis lines

The expression constructs generated were transformed in the *Agrobacterium tumefaciensis* strain GV3101 by electroporation and plated in YEB selective media. 4-week-old arabidopsis plants were transformed by floral dip method (X. Zhang et al., 2006) and single insertion T3 homozygous seeds were used for the experiments.

BR hormone treatment

For experiments of sensitivity to BL (Toronto Research Chemicals Inc), the desired amount of the hormone, was added from a 10 µM BL stock solution dissolved in DMSO. The same amount of DMSO was added to mock groups.

Root and hypocotyl length measurement

For root length experiments seedlings grown vertically in agar plates, for hypocotyl length seedlings were grown horizontally and hypocotyls were bent against the media for imaging with a NIKON D7000 digital camera. For experiments with sorghum, the seedlings were removed from the place and the root was stretched before imaging. For the mutations in which there is a commercially available restriction enzyme suitable for. The length was measured with ImageJ software.

Identification and isolation of sorghum BR receptor mutants

Mutagenized sorghum seed stocks were ordered from USDA ((Jiao et al., 2016). At least 30 plants from each stock were germinated and genotyped for the described mutations in BRI1 or BRL1 genes. The Cleavage Amplified Polymorphic Sequence method was chosen for the

mutations in which there is a commercially available restriction enzyme able to distinguish the mutation. For the rest of mutations PCR products were sequenced by capillary electrophoresis. The primers used are presented in Table 6. The restriction enzymes used for genotyping of the different sorghum lines are displayed in Table 7.

Table 7. Digestion enzymes used for genotyping of sorghum mutant lines

Allele	Restriction enzyme	Cuts in:
Sb_bri1-87	MspI	Wt
Sb_bri1-72	BccI	bri1-72
Sb_brl1-3	XhoI	brl1-3
Sb_brl1-121	AcuI	Wt
At_bri1-301	DpnII	Wt

Backcrossing of sorghum mutagenized lines

In order to eliminate background mutations which could affect the phenotype of the mutagenized plants, the uppermost part of the flower of BTx623 Wt plants were emasculated prior to antheresis using round thin tweezers or a mechanical pencil. Pollen from genotyped M4 individuals with the described mutations in BR receptors was collected in a petri dish and the emasculated parts of the flowers were brushed with the pollen for 2-3 days. The pollinated part of the flower was covered with a paper bag. For bri1-72 M4 mutants, which were male sterile, the pollen was collected from Wt plants and brushed directly in the entire flower. After approximately 4 weeks, the mature seeds were germinated and the seedlings were genotyped to check heterozygosity. F2 plants were also genotyped and Xi^2 test was performed. The process was repeated to get a 2^{nd} backcross and will be extended as much as possible in the next years.

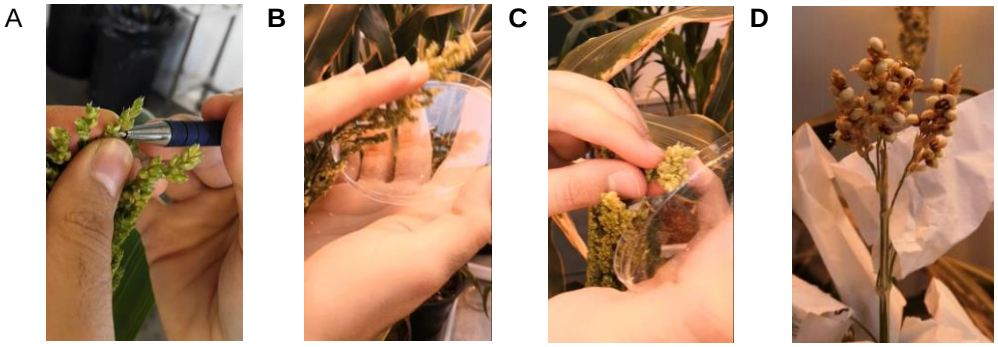


Figure 31. Crossing of sorghum plants

(A) Emasculation of sorghum flowers: the uppermost part should be emasculated to prevent self-pollination, the non-used parts of the panicle can be removed or bagged. (B) Pollen collection: fresh pollen can be collected in a petri dish. (C) Pollination: the emasculated flowers were rubbed against the collected pollen. (D) Putative F1 descendants, the mature seeds can be collected 30 days after pollination and germinated for genotyping, confirming the heterozygosity of an F1 crossing with two homozygous parentals

Phenotyping of sorghum plants under drought stress

Homozygous Wt and mutant seeds for BRI1 ARS72 line and BRL1 ARS106BC2F4 from 3 different homozygous parentals were surface sterilized and were placed in a petri dish with soak paper at 28ºC and 48h after were moved to pots with soil. 3 seedlings were planted per pot and the total soil weight of each pot after water saturation was set to 1.100g. 7 days after germination the pots were watered until saturation and the weight of the pot was measured daily to assess evapotranspiration and calculate the percentage of the FC of each pot. The treated group was not watered again while the well-watered control plants were kept in values between 60-90% of FC. This value was used to analyse the plants depending on the level of water availability instead of the days of drought. Plant height and 3rd leaf width of 16-day-old seedlings was measured (after 8 days of drought).

Photosynthetically active radiation measurements

Chlorophyll fluorescence parameters from the youngest fully expanded leaf was excised and kept in dark acclimation in a petri dish with moisturized paper for 30 minutes and then analysed

for their photosynthetically active radiation (PAR) by chlorophyll fluorescence parameters in a PAM-MAXI device (Waltz). Maximum potential quantum efficiency of Photosystem II was measured as Fv/Fm ratio. Photosystem II efficiency or YII was also measured after a light pulse excitation and acclimation to actinic light (Genty et al., 1989).

Relative water content measurement

The water content of the 2nd youngest fully expanded leave was calculated by the formula (Fresh Weight - Dry Weight) / (Turgid Weight - Dry Weight). The weight of the leave was annotated directly after collection (fresh weight), then submerged in distilled water for 24h (turgid weight) and another 24h in an oven at 60ºC (dry weight).

Primers used in this work

Table 8. Primers used for genotyping and cloning of mutant or transgenic plants

Primer pair	Left Primer Sequence	Right Primer Sequence	Gene
At_bri1-301	5' GGAAACCATTGGGAAGATCA 3'	5' GCTGTTTCACCCATCCAA 3'	AtBRI1
At_brl3-2	5' CCAGTGAACTCGTTTGAGCTC 3'	5' TTTATCGAACACTTTGTGGGC 3'	AtBRL3
Sb_bri1-72/87	5' GCTCCGGGACCTCATTC 3'	5' GCGGGATGCCACCAG 3'	SbBRI1
Sb_brl1-3/95	5' AGGTGCTTGACCTCAGTGGC 3'	5' CGCCGCCAGCACCG 3'	SbBRL
Sb-brl1-121	5' GGCACCATTCCGGACGA 3'	5' CACCAGCGTCTCCAGCG 3'	SbBRL
Sb_brl1-106	5' ATCTTGTCGGCTGGGTGA 3'	5' CTTTGGTTCGGTCTGTCGTC 3'	SbBRL
nptII	5' GATTGCACGCAGGTTCTCCG 3'	5' AGCCCCTGATGCTCTTCGTC 3'	nptII

Primer pair	Left Primer Sequence	Right Primer Sequence	Gene
zCAS9	5' CGGCCTCGATATTGGGACTAACTCT 3'	5' CTTATCTGTGGAGTCCACGAGCTTC 3'	CAS9
gRNA scaffold	5' GGCGACGTTGTTTAGTACCAC 3'	5' ACCATGATTACGCCAAGCTC 3'	plasmid
Cloning SbBRI1-NoStop	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAATCACCGGGGCTGGT 3'	5' GGGGACCACTTTGTACAAGAAAGCTGGGTAGTCCTTCTCCTCCTTGTCTT 3'	SbBRI1
Cloning SbBRL1-NoStop	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCGCCTCAACGACGGC 3'	5' GGGGACCACTTTGTACAAGAAAGCTGGGTAAATCAGCAGAGAAATCCTCG 3'	SbBRL1

Underlined sequences correspond to AttB flank sequences for cloning.

BIBLIOGRAPHY

Aldukhi, F., Deb, A., Zhao, C., Moffett, A. S., & Shukla, D. (2020). Molecular Mechanism Of Brassinosteroid Perception By The Plant Growth Receptor BRI1. *Journal of Physical Chemistry B*, 124(2):355-365.

Ali, B. (2017). Practical Applications Of Brassinosteroids In Horticulture—Some Field Perspectives. *Scientia Horticulturae*, 225:15-21.

Ali, S. S., Kumar, G. B. S., Khan, M., & Doohan, F. M. (2013). Brassinosteroid Enhances Resistance To Fusarium Diseases Of Barley. *Phytopathology*, 103(12):1260-1267.

Anjum, S. A., Wang, L. C., Farooq, M., Hussain, M., Xue, L. L., & Zou, C. M. (2011). Brassinolide Application Improves The Drought Tolerance In Maize Through Modulation Of Enzymatic Antioxidants And Leaf Gas Exchange. *Journal of Agronomy and Crop Science*, 197(3):177-185.

Aruna, C., & Visarada, K. B. R. S. (2018). Other industrial uses of sorghum. *Breeding Sorghum for Diverse End Uses*, 1:271-292.

Azpiroz A., Wu Y., LoCascio J. C. , Feldmann K. A.. (1998). An Arabidopsis Brassinosteroid-Dependent Mutant Is Blocked in Cell Elongation. *The Plant Cell,* 10(2):209-230.

Bajwa, V. S., Wang, X., Kevin Blackburn, R., Goshe, M. B., Mitra, S. K., Williams, E. L., Bishop, G. J., Krasnyanski, S., Allen, G., Huber, S. C., & Clouse, S. D. (2013). Identification and Functional Analysis of Tomato BRI1 and BAK1 Receptor Kinase Phosphorylation Sites. *Plant Physiology*, 163(1):30-42.

Baker, N. R., & Oxborough, K. (2004). Chlorophyll Fluorescence as a Probe of Photosynthetic Productivity. *Advances in Photosynthesis and Respiration*, 1:65-82.

Belide, S., Vanhercke, T., Petrie, J. R., & Singh, S. P. (2017). Robust Genetic Transformation Of Sorghum (Sorghum Bicolor L.) Using Differentiating Embryogenic Callus Induced From Immature Embryos. *Plant Methods*, 13(109).

Belkhadir, Y., Jaillais, Y., Epple, P., Balsemão-Pires, E., Dangl, J. L., & Chory, J. (2012). Brassinosteroids Modulate The Efficiency Of Plant Immune Responses To Microbe-Associated Molecular Patterns. *Proceedings of the National Academy of Sciences of the United States of America*, 109(1):297-302.

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., & Bourne, P. E. (2000). The Protein Data Bank. *Nucleic Acids Research*, 28(1):235-242 .

Bernardo-García, S., de Lucas, M., Martínez, C., Espinosa-Ruiz, A., Davière, J. M., & Prat, S. (2014). BR-Dependent Phosphorylation Modulates PIF4 Transcriptional Activity And Shapes Diurnal Hypocotyl Growth. *Genes and Development*, *28*(15):1681-1694.

Betegón-Putze, I., Mercadal, J., Bosch, N., Planas-Riverola, A., Marquès-Bueno, M., Vilarrasa-Blasi, J., Frigola, D., Burkart, R. C., Martínez, C., Conesa, A., Sozzani, R., Stahl, Y., Prat, S., Ibañes, M., & Caño-Delgado, A. I. (2021). Precise Transcriptional Control Of Cellular Quiescence By BRAVO/WOX5 Complex In Arabidopsis Roots. *Molecular Systems Biology*, *17*(6):e9864.

Bishop, G. J., Nomura, T., Yokota, T., Harrison, K., Noguchi, T., Fujioka, S., Takatsuto, S., G Jones, J. D., & Kamiya, Y. (1999). The Tomato DWARF Enzyme Catalyses C-6 Oxidation In Brassinosteroid Biosynthesis. *Plant Biology*, 96(4):1771-1776.

Blasco-Escámez, D., Lozano-Elena, F., Fàbregas, N., & Caño-Delgado, A. I. (2017). The Primary Root Of Sorghum Bicolor (L. Moench) As A Model System To Study Brassinosteroid Signaling In Crops. *Methods in Molecular Biology*, 1564:181-192.

Cai, Z., Liu, J., Wang, H., Yang, C., Chen, Y., Li, Y., Pan, S., Dong, R., Tang, G., de Dios Barajas-Lopez, J., Fujii, H., & Wang, X. (2014). GSK3-Like Kinases Positively Modulate Abscisic Acid Signaling Through Phosphorylating Subgroup III Snrk2s In Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 111(26):651-9656.

Caño-Delgado, A., Yin, Y., Yu, C., Vefeados, D., Mora-García, S., Cheng, J. C., Nam, K. H., Li, J., & Chory, J. (2004). BRL1 And BRL3 Are Novel Brassinosteroid Receptors That Function In Vascular Defferentiation In Arabidopsis. *Development*, 131(21):5341-5351.

Casas, A. M., Kononowicz, A. K., Haan, T. G., Zhang Dwight Tomes, L. T., Bressan, R. A., & Hasegawa, P. M. (1997). Transgenic Sorghum Plants Obtained After Microprojectile Bombardment Of Immature Inflorescences. *In Vitro Cellular & Developmental Biology.-Plant*, 33:97-100.

Casas, A. M., Kononowicz, A. K., Zehrt, U. B., Tomes, D. T., Axtell, J. D., Butlers, L. G., Bressan, R. A., & Hasegawa, P. M. (1993). Transgenic Sorghum Plants Via Microprojectile Bombardment (Sorghum Bicolorlgene Transfer/Particle Gun).

Proceedings of the National Academy of Sciences of the United States of America, 90:11212-11216.

Chen, I. J., Lo, W. S., Chuang, J. Y., Cheuh, C. M., Fan, Y. S., Lin, L. C., Wu, S. J., & Wang, L. C. (2013). A Chemical Genetics Approach Reveals A Role Of Brassinolide And Cellulose Synthase In Hypocotyl Elongation Of Etiolated Arabidopsis Seedlings. *Plant Science*, 209:46-57.

Chen, J., Nolan, T. M., Ye, H., Zhang, M., Tong, H., Xin, P., Chu, J., Chu, C., Li, Z., & Yina, Y. (2017). Arabidopsis WRKY46, WRKY54, And WRKY70 Transcription Factors Are Involved In Brassinosteroid-Regulated Plant Growth And Drought Responses. *Plant Cell*, 29(6):1425-1439.

Cheng, Y., Zhu, W., Chen, Y., Ito, S., Asami, T., & Wang, X. (2014). Brassinosteroids Control Root Epidermal Cell Fate Via Direct Regulation Of A MYB-Bhlh-WD40 Complex By GSK3-Like Kinases. *ELife*, 3:e02525.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D. G., Felix, G., & Boller, T. (2007). A Flagellin-Induced Complex Of The Receptor FLS2 And BAK1 Initiates Plant Defence. *Nature*, *448*(7152):497-500.

Choe S., Dilkes B.P., Fujioka S., Takatsuto S., Sakurai A., Feldmann K. A. (1998). The DWF4 Gene Of Arabidopsis Encodes A Cytochrome P450 That Mediates Multiple 22-Alpha-Hydroxylation Steps In Brassinosteroid Biosynthesis. *The Plant Cell,* 10(2):231-243.

Choe, S., Noguchi, T., Fujioka, S., Takatsuto, S., Tissier, C. P., Gregory, B. D., Ross, A. S., Tanaka, A., Yoshida, S., Tax, F. E., & Feldmann, K. A. (1999). The Arabidopsis Dwf7 / Ste1 Mutant Is Defective In The 7 Sterol C-5 Desaturation Step Leading To Brassinosteroid Biosynthesis. *The Plant Cell*, 11(2):207-221.

Chono, M., Honda, I., Zeniya, H., Yoneyama, K., Saisho, D., Takeda, K., Takatsuto, S., Hoshino, T., & Watanabe, Y. (2003). A Semidwarf Phenotype Of Barley Uzu Results From A Nucleotide Substitution In The Gene Encoding A Putative Brassinosteroid Receptor. *Plant Physiology*, 133(3):1209-1219.

Chory, J., Nagpal, P., & Peto, C. A. (1991). Phenotypic And Genetic Analysis Of det2, A New Mutant That Affects Light-Regulated Seedling Development In Arabidopsis. *The Plant Cell* 3(5):445-459.

Claeys, H., & Inzé, D. (2013). The Agony Of Choice: How Plants Balance Growth And Survival Under Water-Limiting Conditions. *Plant Physiology*, 162(4):1768-1779.

Clouse, S. D., Hall, A. F., Langford, M., Mcmorris, T. C., & Baker, M. E. (1993). Physiological And Molecular Effects Of Brassinosteroids On Arabidopsis Thaliana. *Journal of Plant Growth Regulators* 12:61-66.

Clouse, S. D., Langford, M., & Mcmorris, T. C. (1996). A Brassinosteroid-Insensitive Mutant In Arabidopsis Thaliana Exhibits Multiple Defects In Growth And Development. In *Plant Physiol* 111(3):671-678.

Clouse, S. D., & Zurek, D. (1991). Molecular Analysis Of Brassinolide Action In Plant Growth And Development. *American Chemical Society,* 474:122-140.

Coll, Y., Coll, F., Amorós, A., & Pujol, M. (2015). Brassinosteroids Roles And Applications: An Up-Date. *Biologia (Poland)*, *70*(6):726-732.

Concordet JP., Haeussler M. (2018). CRISPOR: Intuitive Guide Selection For Crispr/Cas9 Genome Editing Experiments And Screens. Nucleic Acids Research, 46(W1):242-245.

Corvalán, C., & Choe, S. (2017). Identification Of Brassinosteroid Genes In Brachypodium Distachyon. *BMC Plant Biology*, *17*(5).

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., & Abrams, S. R. (2010). Abscisic Acid: Emergence Of A Core Signaling Network. *Annual Review of Plant Biology*, *61*:651-679.

de Block', M., Herrera-Estrella', L., van Montagu', M., Schelil2, J., & Zambryski1, P. (1984). Expression Of Foreign Genes In Regenerated Plants And In Their Progeny. *The EMBO Journal* 3(8):1681-1689.

Deschamps, S., Zhang, Y., Llaca, V., Ye, L., Sanyal, A., King, M., May, G., & Lin, H. (2018). A Chromosome-Scale Assembly Of The Sorghum Genome Using Nanopore Sequencing And Optical Mapping. *Nature Communications*, *9*(1).

Dhaubhadel, S., Browning, K. S., Gallie, D. R., & Krishna, P. (2002). Brassinosteroid Functions To Protect The Translational Machinery And Heat-Shock Protein Synthesis Following Thermal Stress. *Plant Journal,* 29(6):681-91.

Divi, U. K., Rahman, T., & Krishna, P. (2016). Gene Expression And Functional Analyses In Brassinosteroid-Mediated Stress Tolerance. *Plant Biotechnology Journal*, *14*(1), 419-432.

Eremina, M., Unterholzner, S. J., Rathnayake, A. I., Castellanos, M., Khan, M., Kugler, K. G., May, S. T., Mayer, K. F. X., Rozhon, W., & Poppenberger, B. (2016). Brassinosteroids Participate In The Control Of Basal And Acquired Freezing Tolerance Of Plants. *Proceedings of the National Academy of Sciences of the United States of America*, 113(40):E5982-E5991.

Fàbregas, N., Li, N., Boeren, S., Nash, T. E., Goshe, M. B., Clouse, S. D., de Vries, S., & Caño-Delgado, A. I. (2013). The BRASSINOSTEROID INSENSITIVE1-LIKE3 Signalosome Complex Regulates Arabidopsis Root Development. *Plant Cell*, *25*(9):3377-3388.

Fàbregas, N., Lozano-Elena, F., Blasco-Escámez, D., Tohge, T., Martínez-Andújar, C., Albacete, A., Osorio, S., Bustamante, M., Riechmann, J. L., Nomura, T., Yokota, T., Conesa, A., Alfocea, F. P., Fernie, A. R., & Caño-Delgado, A. I. (2018). Overexpression Of The Vascular Brassinosteroid Receptor BRL3 Confers Drought Resistance Without Penalizing Plant Growth. *Nature Communications*, *9*(1).

Feng, Y., Yin, Y., & Fei, S. (2015). Down-Regulation Of Bdbri1, A Putative Brassinosteroid Receptor Gene Produces A Dwarf Phenotype With Enhanced Drought Tolerance In Brachypodium Distachyon. *Plant Science*, 234:163-173.

Feng, Z., Wu, C., Wang, C., Roh, J., Zhang, L., Chen, J., Zhang, S., Zhang, H., Yang, C., Hu, J., You, X., Liu, X., Yang, X., Guo, X., Zhang, X., Wu, F., Terzaghi, W., Kim, S. K., Jiang, L., & Wan, J. (2016). SLG Controls Grain Size And Leaf Angle By Modulating Brassinosteroid Homeostasis In Rice. *Journal of Experimental Botany*, *67*(14):4241-4253.

Ferreira-Guerra, M., Marquès-Bueno, M., Mora-García, S., & Caño-Delgado, A. I. (2020). Delving Into The Evolutionary Origin Of Steroid Sensing In Plants. *Current Opinion In Plant Biology*, 57:87-95.

Friedrichsen, D., & Chory, J. (2001). Steroid Signaling In Plants: From The Cell Surface To The Nucleus. *BioEssays,* 23:1028-1036.

Fujioka, S., Li, J., Choi, Y.-H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., & Sakurai, A. (1997). The Arabidopsis deetiolated2 Mutant is Blocked Early In Brassínosteroid Biosynthesis. *The Plant Cell* 9(11):1959-1962.

Fujioka, S., Noguchi, T., Takatsuto, S., & Yoshida, S. (1998). Activity Of Brassinosteroids In The Dwarf Rice Lamina Inclination Bioassay. *Phytochemistry,* 49(7):1841-1848.

Gampala, S. S., Kim, T. W., He, J. X., Tang, W., Deng, Z., Bai, M. Y., Guan, S., Lalonde, S., Sun, Y., Gendron, J. M., Chen, H., Shibagaki, N., Ferl, R. J., Ehrhardt, D., Chong, K., Burlingame, A. L., & Wang, Z. Y. (2007). An Essential Role for 14-3-3 Proteins In Brassinosteroid Signal Transduction in Arabidopsis. *Developmental Cell*, 13(2):177-189.

Gao, X., Zhang, J. Q., Zhang, X., Zhou, J., Jiang, Z., Huang, P., Tang, Z., Bao, Y., Cheng, J., Tang, H., Zhang, W., Zhang, H., & Huang, J. (2019). Rice QGL3/OSPPKL1 Functions With The GSK3/Shaggy-Like Kinase OsGSK3 To Modulate Brassinosteroid Signaling. *Plant Cell*, 31(5):1077-1093.

Genty, B., Briantais, J. M., & Baker, N. R. (1989). The Relationship Between The Quantum Yield Of Photosynthetic Electron Transport And Quenching Of Chlorophyll Fluorescence. *Biochimica et Biophysica Acta - General Subjects*, 990(1):87-92.

Gomes, M. M. A. (2011). Physiological Effects Related To Brassinosteroid Application In Plants. *Brassinosteroids: A Class of Plant Hormone*. 193-242.

González-García, M. P., Vilarrasa-Blasi, J., Zhiponova, M., Divol, F., Mora-García, S., Russinova, E., & Caño-Delgado, A. I. (2011). Brassinosteroids Control Meristem Size By Promoting Cell Cycle Progression In Arabidopsis Roots. *Development*, *138*(5):849-859.

Gou, X., He, K., Yang, H., Yuan, T., Lin, H., Clouse, S. D., & Li, J. (2010). Genome-wide cloning and sequence analysis of leucine-rich repeat receptor-like protein kinase genes in Arabidopsis thaliana. *BMC Genomics*, 11(1).

Grove, M., Spencer GF, Rohwedder WK, Mandava NB, & Worley JF. (1979). Brassinolide, a plant growth-promotingsteroid isolated fromBrassica napus pollen. *Nature*. 281: 216-217

Gudesblat, G. E., Schneider-Pizoń, J., Betti, C., Mayerhofer, J., Vanhoutte, I., van Dongen, W., Boeren, S., Zhiponova, M., de Vries, S., Jonak, C., & Russinova, E. (2012). SPEECHLESS Integrates Brassinosteroid And Stomata Signaling Pathways. *Nature Cell Biology*, *14*(5), 548-554.

Guimarães, Z. T. M., dos Santos, V. A. H. F., & Ferreira, M. J. (2022). Chlorophyll A Fluorescence Parameters Are Related To The Leaf Economics Spectrum Of Tropical Tree Species In A Mixed Plantation. *Trees*. 36(2):763-775.

Gurel, S., Gurel, E., Miller, T. I., & Lemaux, P. G. (2012). Agrobacterium-mediated transformation of sorghum bicolor using immature embryos. *Methods in Molecular Biology*, 847:109-122.

Hacham, Y., Holland, N., Butterfield, C., Ubeda-Tomas, S., Bennett, M. J., Chory, J., & Savaldi-Goldstein, S. (2011). Brassinosteroid perception in the epidermis controls root meristem size. *Development*, 138(5):839-848.

Hartwig, T., Chuck, G. S., Fujioka, S., Klempien, A., Weizbauer, R., Potluri, D. P. v., Choe, S., Johal, G. S., & Schulz, B. (2011). Brassinosteroid control of sex determination in maize. *Proceedings of the National Academy of Sciences of the United States of America*, 108(49):19814-19819.

He, G., Liu, J., Dong, H., & Sun, J. (2019). The Blue-Light Receptor CRY1 Interacts with BZR1 and BIN2 to Modulate the Phosphorylation and Nuclear Function of BZR1 in Repressing BR Signaling in Arabidopsis. *Molecular Plant*, 12(5):689-703.

He, J. X., Gendron, J. M., Sun, Y., Gampala, S. S. L., Gendron, N., Sun, C. Q., & Wang, Z. Y. (2005). BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. *Science*, *307*(5715):1634-1638.

Hee Nam, K., & Li, J. (2002). BRI1/BAK1, a Receptor Kinase Pair Mediating Brassinosteroid Signaling. *Cell,* 110(2):203-212.

Hiei, Y., & Komari, T. (2008). Agrobacterium-Mediated Transformation Of Rice Using Immature Embryos Or Calli Induced From Mature Seed. *Nature Protocols*, 3(5):824-834.

Hirano, K., Kawamura, M., Araki-Nakamura, S., Fujimoto, H., Ohmae-Shinohara, K., Yamaguchi, M., Fujii, A., Sasaki, H., Kasuga, S., & Sazuka, T. (2017). Sorghum DW1 Positively Regulates Brassinosteroid Signaling By Inhibiting The Nuclear Localization Of BRASSINOSTEROID INSENSITIVE 2. *Scientific Reports*, 7(126).

Hohmann, U., Lau, K., & Hothorn, M. (2017). The Structural Basis of Ligand Perception and Signal Activation by Receptor Kinases. *Annual Review Plant Biology*, 68:109-137.

Hohmann, U., Nicolet, J., Moretti, A., Hothorn, L. A., & Hothorn, M. (2018). The SERK3 Elongated Allele Defines A Role For BIR Ectodomains In Brassinosteroid Signalling. *Nature Plants*, *4*(6):345-351.

Holzwart, E., Huerta, A. I., Glöckner, N., Gómez, B. G., Wanke, F., Augustin, S., Askani, J. C., Schürholz, A. K., Harter, K., & Wolf, S. (2018). BRI1 Controls Vascular Cell Fate In

The Arabidopsis Root Through RLP44 And Phytosulfokine Signaling. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 115(46):11838-11843.

Hong, Z., Ueguchi-Tanaka, M., Shimizu-Sato, S., Inukai, Y., Fujioka, S., Shimada, Y., Takatsuto, S., Agetsuma, M., Yoshida, S., Watanabe, Y., Uozu, S., Kitano, H., Ashikari, M., & Matsuoka, M. (2002). Loss-Of-Function Of A Rice Brassinosteroid Biosynthetic Enzyme, C-6 Oxidase, Prevents The Organized Arrangement And Polar Elongation Of Cells In The Leaves And Stem. *The Plant Journal,* 32(4):495-508.

Hothorn, M., Belkhadir, Y., Dreux, M., Dabi, T., Noel, J. P., Wilson, I. A., & Chory, J. (2011). Structural Basis Of Steroid Hormone Perception By The Receptor Kinase BRI1. *Nature*, 474(7352):467-472.

Hou, L., Zhang, A., Wang, R., Zhao, P., Zhang, D., Jiang, Y., Diddugodage, C. J., Wang, X., Ni, Z., & Xu, S. (2019). Brassinosteroid Regulates Root Development With Highly Redundant Genes In Hexaploid Wheat. *Plant & Cell Physiology*, 60(8):1761-1777.

Huang S., Zheng C., Zhao Y., Li Q., Rui L., Tiantian D., Shufen L.,Xiaofeng W. (2021). RNA Interference Knockdown Of The Brassinosteroid Receptor BRI1 In Potato (Solanum Tuberosum L.) Reveals Novel Functions For Brassinosteroid Signaling In Controlling Tuberization. *Scientia Horticulturae,* 290(110516).

Hussain, M. A., Fahad, S., Sharif, R., Jan, M. F., Mujtaba, M., Ali, Q., Ahmad, A., Ahmad, H., Amin, N., Ajayo, B. S., Sun, C., Gu, L., Ahmad, I., Jiang, Z., & Hou, J. (2020). Multifunctional Role Of Brassinosteroid And Its Analogues In Plants. *Plant Growth Regulation*, 92(2):141-156.

Ibañes, M., Fàbregas, N., Chory, J., & Caño-Delgado, A. I. (2009). Brassinosteroid Signaling And Auxin Transport Are Required To Establish The Periodic Pattern of Arabidopsis Shoot Vascular Bundles. *Proceedings Of The National Academy Of Sciences Of The United States Of America*,106(32):13630-13635.

Ibañez, C., Delker, C., Martinez, C., Bürstenbinder, K., Janitza, P., Lippmann, R., Ludwig, W., Sun, H., James, G. V., Klecker, M., Grossjohann, A., Schneeberger, K., Prat, S., & Quint, M. (2018). Brassinosteroids Dominate Hormonal Regulation Of Plant Thermomorphogenesis Via BZR1. *Current Biology*, *28*(2):303-310.

Ishida, Y., Hiei, Y., & Komari, T. (2007). Agrobacterium-Mediated Transformation Of Maize. *Nature Protocols*, *2*(7):1614-1621.

Ishida, Y., Tsunashima, M., Hiei, Y., & Komari, T. (2015). Wheat (Triticum Aestivum L.) Transformation Using Immature Embryos. *Methods In Molecular Biology*, *1223*:189-198.

Ismagul, A., Yang, N., Maltseva, E., Iskakova, G., Mazonka, I., Skiba, Y., Bi, H., Eliby, S., Jatayev, S., Shavrukov, Y., Borisjuk, N., & Langridge, P. (2018). A Biolistic Method For High-Throughput Production Of Transgenic Wheat Plants With Single Gene Insertions. *Bmc Plant Biology*, *18*(1).

Iwasaki, T., & Shibaoka, H. (1991). Brassinosteroids Act As Regulators Of Tracheary-Element Differentiation In Isolated Zinnia Mesophyll Cells. *Plant & Cell Physiology* 32(7):1007-1014.

Jaillais, Y., Hothorn, M., Belkhadir, Y., Dabi, T., Nimchuk, Z. L., Meyerowitz, E. M., & Chory, J. (2011). Tyrosine Phosphorylation Controls Brassinosteroid Receptor Activation By Triggering Membrane Release Of Its Kinase Inhibitor. *Genes And Development*, *25*(3):232-237.

Jia, C., Zhao, S., Bao, T., Zhao, P., Peng, K., Guo, Q., Gao, X., & Qin, J. (2021). Tomato Bzr/Bes Transcription Factor SlBZR1 Positively Regulates Br Signaling And Salt Stress Tolerance In Tomato And Arabidopsis. *Plant Science*, *302:1107-1119*.

Jiang, C., Li, B., Song, Z., Zhang, Y., Yu, C., Wang, H., Wang, L., & Zhang, H. (2021). PtBRI1.2 Promotes Shoot Growth And Wood Formation Through A Brassinosteroid-Mediated PtBZR1-PtWNDS Module In Poplar. *Journal Of Experimental Botany*, *72*(18):6350-6364.

Jiang, J., Zhang, C., & Wang, X. (2015). A Recently Evolved Isoform Of The Transcription Factor BES1 Promotes Brassinosteroid Signaling And Development In Arabidopsis Thaliana. *The Plant Cell*, *27*(2):361-374.

Jiang, W. B., Huang, H. Y., Hu, Y. W., Zhu, S. W., Wang, Z. Y., & Lin, W. H. (2013). Brassinosteroid Regulates Seed Size And Shape In Arabidopsis. *Plant Physiology*, *162*(4):1965-1977.

Jiang, Y. P., Huang, L. F., Cheng, F., Zhou, Y. H., Xia, X. J., Mao, W. H., Shi, K., & Yu, J. Q. (2013). Brassinosteroids Accelerate Recovery Of Photosynthetic Apparatus From Cold Stress By Balancing The Electron Partitioning, Carboxylation And Redox Homeostasis In Cucumber. *Physiologia Plantarum*, *148*(1):133-145.

Jiao, Y., Burke, J., Chopra, R., Burow, G., Chen, J., Wang, B., Hayes, C., Emendack, Y., Ware, D., & Xin, Z. (2016). A Sorghum Mutant Resource As An Efficient Platform For Gene Discovery In Grasses. *The Plant Cell*, *28*(7), 1551-1562.

Jin, Y. L., Tang, R. J., Wang, H. H., Jiang, C. M., Bao, Y., Yang, Y., Liang, M. X., Sun, Z. C., Kong, F. J., Li, B., & Zhang, H. X. (2017). Overexpression Of Populus Trichocarpa Cyp85a3 Promotes Growth And Biomass Production In Transgenic Trees. *Plant Biotechnology Journal*, 15(10):1309-1321.

Jung C., Till B. (2021). Mutagenesis And Genome Editing In Crop Improvement: Perspectives For The Global Regulatory Landscape. *Trends In Plant Science*, 16(12):1258-1269.

Köhler, F., Cardon, G., Pöhlman, M., Gilp, R., & Schieder, O. (1989). Enhancement Of Transformation Rates In Higher Plants By Low-Dose Irradiation: Are Dna Repair Systems Involved In The Incorporation Of Exogenous Dna Into The Plant Genome? *Plant Molecular Biology,* 12(2):189-199.

Kagale, S., Divi, U. K., Krochko, J. E., Keller, W. A., & Krishna, P. (2007). Brassinosteroid Confers Tolerance In Arabidopsis Thaliana And Brassica Napus To A Range Of Abiotic Stresses. *Planta*, *225*(2):353-364.

Kajava, A. V. (1998). Structural Diversity Of Leucine-Rich Repeat Proteins11edited By F. Cohen. *Journal Of Molecular Biology*, *277*(3):519-527.

Kang, Y. H., Breda, A., & Hardtke, C. S. (2017). Brassinosteroid Signaling Directs Formative Cell Divisions And Protophloem Differentiation In Arabidopsis Root Meristems. *Development,* 144(2):272-280.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L., & Altmann, T. T. (1996). Genetic Evidence For An Essential Role Of Brassinosteroids In Plant Development. *The Plant Journal* 9(5):701-713.

Keyi, Y., Hui, L., Yanglin, D., Yiting, S., Chunpeng Song, Zhzhong, G., & Shuhua, Y. (2019). Brassinosteroid-Insensitive2 Negatively Regulates Thestability Of Transcription Factor Ice1 In Response To Coldstress In Arabidopsis. The *Plant Cell*, 31(9):2682-2696.

Kim, T. W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J. X., Sun, Y., Burlingame, A. L., & Wang, Z. Y. (2009). Brassinosteroid Signal Transduction From Cell-Surface Receptor

Kinases To Nuclear Transcription Factors. *Nature Cell Biology*, 11(10), 1254-1260. Https://Doi.Org/10.1038/Ncb1970

Kim, T. W., Hwang, J. Y., Kim, Y. S., Joo, S. H., Soo, C. C., June, S. L., Takatsuto, S., & Kim, S. K. (2005). Arabidopsis Cyp85a2, A Cytochrome P450, Mediates The Baeyer-Villiger Oxidation Of Castasterone To Brassinolide In Brassinosteroid Biosynthesis. *Plant Cell*, 17(8):2397-2412.

Kim, T. W., Michniewicz, M., Bergmann, D. C., & Wang, Z. Y. (2012). Brassinosteroid Regulates Stomatal Development By Gsk3-Mediated Inhibition Of A Mapk Pathway. *Nature*, 482(7385):419-422.

Kinoshita, T., Caño-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S., & Chory, J. (2005). Binding Of Brassinosteroids To The Extracellular Domain Of Plant Receptor Kinase Bri1. *Nature*, 433(7022):167-171.

Kir, G., Ye, H., Nelissen, H., Neelakandan, A. K., Kusnandar, A. S., Luo, A., Inzé, D., Sylvester, A. W., Yin, Y., & Kir, G. (2015). Rna Interference Knockdown Of Brassinosteroid Insensitive1 In Maize Reveals Novel Functions For Brassinosteroid Signaling In Controlling Plant Architecture. *Plant Physiology*, *169*(1):826-839.

Klümper, W., & Qaim, M. (2014). A Meta-Analysis Of The Impacts Of Genetically Modified Crops. *Plos One*, 9(11): e111629.

Koka, C. V, Cerny, R. E., Gardner, R. G., Noguchi, T., Fujioka, S., Takatsuto, S., Yoshida, S., & Clouse, S. D. (2000). A Putative Role For The Tomato Genes Dumpy And Curl-3 In Brassinosteroid Biosynthesis And Response. *Plant Physiology*, 122(1):85-98.

Krysiak, C., Mazu, B., & Buchowicz, J. (1999). Generation Of Dna Double-Strand Breaks And Inhibition Of Somatic Embryogenesis By Tungsten Microparticles In Wheat. *Plant Cell, Tissue And Organ Culture*, 58:163-170.

Kushiro, T., Nambara, E., & Mccourt, P. (2003). The Key To Signalling: Hormone Evolution. *Nature,* 422:122.

Lee, H. S., Kim, Y., Pham, G., Kim, J. W., Song, J. H., Lee, Y., Hwang, Y. S., Roux, S. J., & Kim, S. H. (2015). Brassinazole Resistant 1 (BZR1)-Dependent Brassinosteroid Signalling Pathway Leads To Ectopic Activation Of Quiescent Cell Division And Suppresses Columella Stem Cell Differentiation. *Journal Of Experimental Botany*, 66(15):4835-4849.

Li, H., Ye, K., Shi, Y., Cheng, J., Zhang, X., & Yang, S. (2017). BZR1 Positively Regulates Freezing Tolerance Via Cbf-Dependent And Cbf-Independent Pathways In Arabidopsis. *Molecular Plant*, 10(4), 545-559.

Li, J., & Chory, J. (1997). A Putative Leucine-Rich Repeat Receptor Kinase Involved In Brassinosteroid Signal Transduction. *Cell* 90(5):929-938.

Li, J., Kang, B., Wang, H., Hee Nam, K., & Li, J. (2010). Activation-Tagged Suppressors Of A Weak Brassinosteroid Receptor Mutant. *Molecular Plant*, *3*(1):260-268.

Li, J., Zhou, H., Zhang, Y., Li, Z., Yang, Y., & Guo, Y. (2020). The Gsk3-Like Kinase Bin2 Is A Molecular Switch Between The Salt Stress Response And Growth Recovery In Arabidopsis Thaliana. *Developmental Cell*, 55(3):367-380.

Li, Q. F., & He, J. X. (2016). BZR1 Interacts With Hy5 To Mediate Brassinosteroid- And Light-Regulated Cotyledon Opening In Arabidopsis In Darkness. *Molecular Plant*, 9(1):113-125.

Li, Q. F., Lu, J., Yu, J. W., Zhang, C. Q., He, J. X., & Liu, Q. Q. (2018). The Brassinosteroid-Regulated Transcription Factors BZR1/BES1 Function As A Coordinator In Multisignal-Regulated Plant Growth. *Biochimica Et Biophysica Acta - Gene Regulatory Mechanisms* 1861(6):561-571.

Li, Z., Ou, Y., Zhang, Z., Li, J., & He, Y. (2018). Brassinosteroid Signaling Recruits Histone 3 Lysine-27 Demethylation Activity To Flowering Locus C Chromatin To Inhibit The Floral Transition In Arabidopsis. *Molecular Plant*, 11(9):1135-1146.

Liang, T., Mei, S., Shi, C., Yang, Y., Peng, Y., Ma, L., Wang, F., Li, X., Huang, X., Yin, Y., & Liu, H. (2018). Uvr8 Interacts With BES1 And Bim1 To Regulate Transcription And Photomorphogenesis In Arabidopsis. *Developmental Cell*, 44(4):512-523.

Liu, G., Campbell, B. C., & Godwin, I. D. (2014). Sorghum Genetic Transformation By Particle Bombardment. *Methods In Molecular Biology*, 1099:219-234.

Liu, G., Gilding, E. K., & Godwin, I. D. (2015). A Robust Tissue Culture System For Sorghum [Sorghum Bicolor (L.) Moench]. *South African Journal Of Botany*, 98:157-160.

Liu, G., & Godwin, I. D. (2012). Highly Efficient Sorghum Transformation. *Plant Cell Reports*, 31(6):999-1007.

Liu, G., Li, J., & Godwin, I. D. (2019). Genome Editing By Crispr/Cas9 In Sorghum Through Biolistic Bombardment. *Methods In Molecular Biology*,1931:169-183.

Liu, J., Chen, J., Zheng, X., Wu, F., Lin, Q., Heng, Y., Tian, P., Cheng, Z. J., Yu, X., Zhou, K., Zhang, X., Guo, X., Wang, J., Wang, H., & Wan, J. (2017). Gw5 Acts In The Brassinosteroid Signalling Pathway To Regulate Grain Width And Weight In Rice. *Nature Plants*, 3(17043).

Liu, T., Zhang, J., Wang, M., Wang, Z., Li, G., Qu, L., & Wang, G. (2007). Expression And Functional Analysis Of Zmdwf4, An Ortholog Of Arabidopsis Dwf4 From Maize (Zea Mays L.). *Plant Cell Reports*, 26(12):2091-2099.

Lozano-Durán, R., Macho, A. P., Boutrot, F., Segonzac, C., Somssich, I. E., & Zipfel, C. (2013). The Transcriptional Regulator BZR1 Mediates Trade-Off Between Plant Innate Immunity And Growth. *Elife*, *2013*(2):e00983.

Lozano-Elena, F., & Caño-Delgado, A. I. (2019). Emerging Roles Of Vascular Brassinosteroid Receptors Of The Bri1-Like Family. *Current Opinion In Plant Biology* 51:105-113.

Lozano-Elena, F., Fàbregas, N., Coleto-Alcudia, V., & Caño-Delgado, A. I. (2022). Analysis Of Metabolic Dynamics During Drought Stress In Arabidopsis Plants. *Scientific Data*, 9(1):90.

Lozano-Elena, F., Planas-Riverola, A., Vilarrasa-Blasi, J., Schwab, R., & Caño-Delgado, A. I. (2018). Paracrine Brassinosteroid Signaling At The Stem Cell Niche Controls Cellular Regeneration. *Journal Of Cell Science*, 131(2).

Lv, M., Li, M., Chen, W., Wang, Y., Sun, C., Yin, H., He, K., & Li, J. (2018). Thermal-Enhanced Bri1-301 Instability Reveals A Plasma Membrane Protein Quality Control System In Plants. *Frontiers In Plant Science*, 9(1620).

Maeda, E. (1965). Rate Of Lamina Inclination In Excised Rice Leaves. *Physiologia Plantarum*, 18(3):813-827

Makarevitch, I., Thompson, A., Muehlbauer, G. J., & Springer, N. M. (2012). Brd1 Gene In Maize Encodes A Brassinosteroid C-6 Oxidase. *Plos One*, *7*(1):e30798.

Mandava, N. B. (1988). Plant Growth-Promoting Brassinosteroids. *Annual Review Of Plant Physiology And Plant Molecular Biology,* 39:23-52.

Mann, D. G. J., Lafayette, P. R., Abercrombie, L. L., King, Z. R., Mazarei, M., Halter, M. C., Poovaiah, C. R., Baxter, H., Shen, H., Dixon, R. A., Parrott, W. A., & Neal Stewart, J. (2012). Gateway-Compatible Vectors For High-Throughput Gene Functional Analysis In

Switchgrass (Panicum Virgatum L.) And Other Monocot Species. *Plant Biotechnology Journal*, 10(2):226-236.

Mantilla Perez, M. B., Zhao, J., Yin, Y., Hu, J., & Salas Fernandez, M. G. (2014). Association Mapping Of Brassinosteroid Candidate Genes And Plant Architecture In A Diverse Panel Of Sorghum Bicolor. *Theoretical And Applied Genetics*, 127(12):2645-2662.

Martínez, C., Espinosa-Ruíz, A., Lucas, M., Bernardo-García, S., Franco-Zorrilla, J. M., & Prat, S. (2018). PIF4-Induced Br Synthesis Is Critical To Diurnal And Thermomorphogenic Growth . *The Embo Journal, 37(2*3):e99552.

Martins, S., Dohmann, E. M. N., Cayrel, A., Johnson, A., Fischer, W., Pojer, F., Satiat-Jeunemaître, B., Jaillais, Y., Chory, J., Geldner, N., & Vert, G. (2015). Internalization And Vacuolar Targeting Of The Brassinosteroid Hormone Receptor Bri1 Are Regulated By Ubiquitination. *Nature Communications*, 6(6251).

Martins, S., Montiel-Jorda, A., Cayrel, A., Huguet, S., Roux, C. P. Le, Ljung, K., & Vert, G. (2017). Brassinosteroid Signaling-Dependent Root Responses To Prolonged Elevated Ambient Temperature. *Nature Communications*, 8(309).

Matsushima, N., Tanaka, T., Enkhbayar, P., Mikami, T., Taga, M., Yamada, K., & Kuroki, Y. (2007). Comparative Sequence Analysis Of Leucine-Rich Repeats (LRRs) Within Vertebrate Toll-Like Receptors. *BMC Genomics*, 8(124).

Mitchell, J., Mandawa N, Worley Jf, & Plimmer, J. (1970). Brassins. A New Family Of Plant Hormones From Rape Pollen. *Nature*, 225(5237):1065-1066.

Montoya, T., Nomura, T., Farrar, K., Kaneta, T., Yokota, T., & Bishop, G. J. (2002). Cloning The Tomato Curl3 Gene Highlights The Putative Dual Role Of The Leucine-Rich Repeat Receptor Kinase Tbri1/Sr160 In Plant Steroid Hormone And Peptide Hormone Signaling. *Plant Cell*, 14(12):3163-3176.

Mü, P., Li, X.-P., & Niyogi, K. K. (2001). Update On Photosynthesis Non-Photochemical Quenching. A Response To Excess Light Energy. *Plant physiology,* 125(4):1558–1566.

Mullet, J., Morishige, D., Mccormick, R., Truong, S., Hilley, J., Mckinley, B., Anderson, R., Olson, S. N., & Rooney, W. (2014). Energy Sorghum-A Genetic Model For The Design Of C4 Grass Bioenergy Crops. In *Journal Of Experimental Botany*, 65 (13):3479-3489.

Murtadha, H. M., Maran Ille, J. W., Clark, R. B., & Murtadha, H. (1988). Calcium Deficiency In Sorghum Grown In Controlled Environments In Relation To Nitrate/Ammonium Ratio And Nitrogen Source. *Agronomy Journal* 80(1):125-130.

Nakamura, A., Fujioka, S., Sunohara, H., Kamiya, N., Hong, Z., Inukai, Y., Miura, K., Takatsuto, S., Yoshida, S., Ueguchi-Tanaka, M., Hasegawa, Y., Kitano, H., & Matsuoka, M. (2006). The Role Of Osbri1 And Its Homologous Genes, Osbrl1 And OsBRL3, In Rice. *Plant Physiology*, 140(2):580-590.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., & Yoshida, S. (2002). Brassinosteroid Functions In A Broad Range Of Disease Resistance In Tobacco And Rice. *The Plant Journal,* 33(5):887-898.

Nakayama, C., Adachi, N., & Koyama, H. (1998). Bleomycin Enhances Random Integration Of Transfected Dna Into A Human Genome. *Mutation Research,* 409(1):1-10.

Nam, K. H., & Li, J. (2002). Regulation Of Brassinosteroid Signaling By A Gsk3/Shaggy-Like Kinase. Science, 295(5558):1299-1301.

Navarro, C., Moore, J., Ott, A., Baumert, E., Mohan, A., Gill, K. S., & Sandhu, D. (2015). Evolutionary, Comparative And Functional Analyses Of The Brassinosteroid Receptor Gene, Bri1, In Wheat And Its Relation To Other Plant Genomes. *Plos One*, 10(5).

Nguyen, T. Van, Thanh Thu, T., Claeys, M., & Angenon, G. (2007). Agrobacterium-Mediated Transformation Of Sorghum (Sorghum Bicolor (L.) Moench) Using An Improved In Vitro Regeneration System. *Plant Cell, Tissue And Organ Culture*, 91(2):155-164.

Nie, S., Huang, S., Wang, S., Cheng, D., Liu, J., Lv, S., Li, Q., & Wang, X. (2017). Enhancing Brassinosteroid Signaling Via Overexpression Of Tomato (Solanum Lycopersicum) SlBRI1 Improves Major Agronomic Traits. *Frontiers In Plant Science*, 8(1386).

Nie, S., Huang, S., Wang, S., Mao, Y., Liu, J., Ma, R., & Wang, X. (2019). Enhanced Brassinosteroid Signaling Intensity Via Slbri1 Overexpression Negatively Regulates Drought Resistance In A Manner Opposite Of That Via Exogenous Br Application In Tomato. *Plant Physiology And Biochemistry*, 138:36-47.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., Feldmann, K. A., & Tax, F. E. (1999). Brassinosteroid-Insensitive Dwarf Mutants Of Arabidopsis Accumulate Brassinosteroids. *Plant physiology*, 121(3):743-752.

Nolan, T. M., Brennan, B., Yang, M., Chen, J., Zhang, M., Li, Z., Wang, X., Bassham, D. C., Walley, J., & Yin, Y. (2017). Selective Autophagy Of BES1 Mediated By Dsk2 Balances Plant Growth And Survival. *Developmental Cell*, 41(1):33-46.

Nolan, T. M., Vukasinović, N., Liu, D., Russinova, E., & Yin, Y. (2020). Brassinosteroids: Multidimensional Regulators Of Plant Growth, Development, And Stress Responses. *Plant Cell*, 32(2):298-318.

Nomura, T., Bishop, G. J., Kaneta, T., Reid, J. B., Chory, J., & Yokota, T. (2003). The Lka Gene Is A Brassinosteroid Insensitive 1 Homolog Of Pea*. The Plant Journal*, *36*(3):291-300.

Oh, E., Zhu, J. Y., Ryu, H., Hwang, I., & Wang, Z. Y. (2014). Topless Mediates Brassinosteroid-Induced Transcriptional Repression Through Interaction With BZR1. *Nature Communications*, 5:4140.

Oh, E., Zhu, J. Y., & Wang, Z. Y. (2012). Interaction Between BZR1 And Pif4 Integrates Brassinosteroid And Environmental Responses. *Nature Cell Biology*, 14(8):802-809.

O'kennedy, M. M., Grootboom, A., & Shewry, P. R. (2006). Harnessing Sorghum And Millet Biotechnology For Food And Health. In *Journal Of Cereal Science*, 44(3):224-235.

O'kennedy, M. M., Stark, H. C., & Dube, N. (2011). Biolistic-Mediated Transformation Protocols For Maize And Pearl Millet Using Pre-Cultured Immature Zygotic Embryos And Embryogenic Tissue. Methods In Molecular Biology 710:343-354.

Ortiz-Morea, F. A., He, P., Shan, L., & Russinova, E. (2020). It Takes Two To Tango - Molecular Links Between Plant Immunity And Brassinosteroid Signalling. *Journal Of Cell Science*, 133:22.

Paterson, A. H., Bowers, J. E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., Haberer, G., Hellsten, U., Mitros, T., Poliakov, A., Schmutz, J., Spannagl, M., Tang, H., Wang, X., Wicker, T., Bharti, A. K., Chapman, J., Feltus, F. A., Gowik, U., … Rokhsar, D. S. (2009). The Sorghum Bicolor Genome And The Diversification Of Grasses. *Nature*, *457*(7229):551-556.

Peng, P., Yan, Z., Zhu, Y., & Li, J. (2008). Regulation Of The Arabidopsis Gsk3-Like Kinase Brassinosteroid-Insensitive 2 Through Proteasome-Mediated Protein Degradation. *Molecular plant,* 1(2):338-346.

Peng, S., Tao, P., Xu, F., Wu, A., Huo, W., & Wang, J. (2016). Functional Characterization Of Soybean Glyma04g39610 As A Brassinosteroid Receptor Gene And Evolutionary

Analysis Of Soybean Brassinosteroid Receptors. *International Journal Of Molecular Sciences*, 17(6):897-920

Planas-Riverola, A., Gupta, A., Betegón-Putze, I., Bosch, N., Ibañes, M., & Caño-Delgado, A. I. (2019). Brassinosteroid Signaling In Plant Development And Adaptation To Stress. *Development*, *146*(5).

Poppenberger, B., Rozhon, W., Khan, M., Husar, S., Adam, G., Luschnig, C., Fujioka, S., & Sieberer, T. (2011). Cesta, A Positive Regulator Of Brassinosteroid Biosynthesis. *Embo Journal*, *30*(6), 1149-1161.

Praat, M., De Smet, I., & Van Zanten, M. (2021). Protein Kinase And Phosphatase Control Of Plant Temperature Responses. *Journal Of Experimental Botany*, 72(21):7459-7473.

Ruban, A. V., & Wilson, S. (2021). The Mechanism Of Non-Photochemical Quenching In Plants: Localization And Driving Forces. In *Plant And Cell Physiology*, 62(7):1063-1072).

Russinova, E., Borst, J. W., Kwaaitaal, M., Caño-Delgado, A., Yin, Y., Chory, J., & De Vries, S. C. (2004). Heterodimerization And Endocytosis Of Arabidopsis Brassinosteroid Receptors BRI1 And AtSERK3 (BAK1). *The Plant Cell*, 16(12):3216-3229.

Ryu, H., Cho, H., Bae, W., & Hwang, I. (2014). Control Of Early Seedling Development By BES1/TPL/HDA19-Mediated Epigenetic Regulation Of ABI3. *Nature Communications*, *5(4138)*.

Sakamoto, T., Morinaka, Y., Ohnishi, T., Sunohara, H., Fujioka, S., Ueguchi-Tanaka, M., Mizutani, M., Sakata, K., Takatsuto, S., Yoshida, S., Tanaka, H., Kitano, H., & Matsuoka, M. (2006). Erect Leaves Caused By Brassinosteroid Deficiency Increase Biomass Production And Grain Yield In Rice. *Nature Biotechnology*, 24(1):105-109.

Salazar-Henao, J. E., Lehner, R., Betegón-Putze, I., Vilarrasa-Blasi, J., & Caño-Delgado, A. I. (2016). BES1 Regulates The Localization Of The Brassinosteroid Receptor BRL3 Within The Provascular Tissue Of The Arabidopsis Primary Root. *Journal Of Experimental Botany*, 67(17):4951-4961.

Savaldi-Goldstein, S., Peto, C., & Chory, J. (2007). The Epidermis Both Drives And Restricts Plant Shoot Growth. *Nature*, 446(7132):199-202.

Sehnal, D., Bittrich, S., Deshpande, M., Svobodová, R., Berka, K., Bazgier, V., Velankar, S., Burley, S. K., Koča, J., & Rose, A. S. (2021). Mol∗Viewer: Modern Web App For 3d

Visualization And Analysis Of Large Biomolecular Structures. *Nucleic Acids Research*, *49*(1):431-437.

She, J., Han, Z., Zhou, B., & Chai, J. (2013). Structural Basis For Differential Recognition Of Brassinolide By Its Receptors. *Protein And Cell*, 4(6):475-482.

Shiu, S.-H., & Bleecker, A. B. (2001). Receptor-Like Kinases From Arabidopsis Form A Monophyletic Gene Family Related To Animal Receptor Kinases. *Proceedings Of The National Academy Of Sciences Of The United States Of America,* 98(19):10763-10768

Shiu, S.-H., & Bleecker, A. B. (2001). Plant Receptor-Like Kinase Gene Family: Diversity, Function, And Signaling. Science's STKE, 113.

Silva, T. N., Kelly, M. E., & Vermerris, W. (2020). Use Of Sorghum Bicolor Leaf Whorl Explants To Expedite Regeneration And Increase Transformation Throughput. *Plant Cell, Tissue And Organ Culture*, *141*(2):243-255

Somssich, M. (2019). A Short History Of Plant Transformation. *PeerJ Preprints*.

Stirk, W. A., Bálint, P., Tarkowská, D., Novák, O., Strnad, M., Ördög, V., & Van Staden, J. (2013). Hormone Profiles In Microalgae: Gibberellins And Brassinosteroids. *Plant Physiology And Biochemistry*, *70*:348-353.

Sun, H., Xu, H., Li, B., Shang, Y., Wei, M., Zhang, S., Zhao, C., Qin, R., Cui, F., & Wu, Y. (2021). The Brassinosteroid Biosynthesis Gene, Zmd11, Increases Seed Size And Quality In Rice And Maize. *Plant Physiology And Biochemistry*, *160*:281-293.

Sun, S., Chen, D., Li, X., Qiao, S., Shi, C., Li, C., Shen, H., & Wang, X. (2015). Brassinosteroid Signaling Regulates Leaf Erectness In Oryza Sativa Via The Control Of A Specific U-Type Cyclin And Cell Proliferation. *Developmental Cell*, *34*(2):220-228.

Sun, Y., Fan, X. Y., Cao, D. M., Tang, W., He, K., Zhu, J. Y., He, J. X., Bai, M. Y., Zhu, S., Oh, E., Patil, S., Kim, T. W., Ji, H., Wong, W. H., Rhee, S. Y., & Wang, Z. Y. (2010). Integration Of Brassinosteroid Signal Transduction With The Transcription Network For Plant Growth Regulation In Arabidopsis. *Developmental Cell*, *19*(5):765-777.

Swigoňová, Z., Lai, J., Ma, J., Ramakrishna, W., Llaca, V., Bennetzen, J. L., & Messing, J. (2004). Close Split Of Sorghum And Maize Genome Progenitors. *Genome Research*, 14(10):1916-1923.

Szekeres, M. S., & Né, K. (1996). Brassinosteroids Rescue The Deficiency Of Cyp90, A Cytochrome P450, Controlling Cell Elongation And De-Etiolation In Arabidopsis. *Cell* 85(2):171-182.

Takeno, K., & Pharis, R. P. (1982). Brassinosteroid-Induced Bending Of The Leaf Lamina Of Dwarf Rice Seedlings: An Auxin-Mediated Phenomenon. *Plant & Cell Physiology*, 23(7):1275-1281.

Tang, W., Kim, T. W., Oses-Prieto, J. A., Sun, Y., Deng, Z., Zhu, S., Wang, R., Burlingame, A. L., & Wang, Z. Y. (2008). Bsks Mediate Signal Transduction From The Receptor Kinase Bri1 In Arabidopsis. *Science*, 321(5888):557-560.

Tang, W., Yuan, M., Wang, R., Yang, Y., Wang, C., Oses-Prieto, J. A., Kim, T. W., Zhou, H. W., Deng, Z., Gampala, S. S., Gendron, J. M., Jonassen, E. M., Lillo, C., Delong, A., Burlingame, A. L., Sun, Y., & Wang, Z. Y. (2011). Pp2a Activates Brassinosteroid-Responsive Gene Expression And Plant Growth By Dephosphorylating BZR1. *Nature Cell Biology*, 13(2):124-131.

Taylor, J. R. N. (2018). Sorghum And Millets: Taxonomy, History, Distribution, And Production. *Sorghum And Millets: Chemistry, Technology, And Nutritional Attributes,* 1-21.

Tong, H., & Chu, C. (2018). Functional Specificities Of Brassinosteroid And Potential Utilization For Crop Improvement. *Trends In Plant Science,* 23(11):1016-1028.

Tong, H., Xiao, Y., Liu, D., Gao, S., Liu, L., Yin, Y., Jin, Y., Qian, Q., & Chu, C. (2014). Brassinosteroid Regulates Cell Elongation By Modulating Gibberellin Metabolism In Ricec W Open. *Plant Cell*, *26*(11):4376-4393

Tůmová, L., Tarkowská, D., Řřová, K., Marková, H., Kočová, M., Rothová, O., Čečetka, P., & Holá, D. (2018). Drought-Tolerant And Drought-Sensitive Genotypes Of Maize (Zea Mays L.) Differ In Contents Of Endogenous Brassinosteroids And Their Drought-Induced Changes. *Plos One*, 13(5).

Tunc-Ozdemir, M., & Jones, A. M. (2017). BRL3 And Atrgs1 Cooperate To Fine Tune Growth Inhibition And Ros Activation. *Plos One*, 12(5).

Tunc-Ozdemir, M., Li, B., Jaiswal, D. K., Urano, D., Jones, A. M., & Torres, M. P. (2017). Predicted Functional Implications Of Phosphorylation Of Regulator Of G Protein Signaling Protein In Plants. *Frontiers In Plant Science*, 8(1456).

Vain, P., Memullen, M. D., & Finer, J. J. (1993). Plant Cell Reports Osmotic Treatment Enhances Particle Bombardment-Mediated Transient And Stable Transformation Of Maize. *Plant Cell Reports,* 12(2):84-88.

Vilarrasa-Blasi, J., González-García, M. P., Frigola, D., Fàbregas, N., Alexiou, K. G., López-Bigas, N., Rivas, S., Jauneau, A., Lohmann, J. U., Benfey, P. N., Ibañes, M., & Caño-Delgado, A. I. (2014). Regulation Of Plant Stem Cell Quiescence By A Brassinosteroid Signaling Module. *Developmental Cell*, 30(1):36-47.

Vriet, C., Russinova, E., & Reuzeaua, C. (2012). Boosting Crop Yields With Plant Steroids. *The Plant Cell*, 24(3):842-857.

Vukašinović, N., & Russinova, E. (2018). Brexit: Possible Brassinosteroid Export And Transport Routes. *Trends In Plant Science*, 23(4):285-292.

Vukašinović, N., Wang, Y., Vanhoutte, I., Fendrych, M., Guo, B., Kvasnica, M., Jiroutová, P., Oklestkova, J., Strnad, M., & Russinova, E. (2021). Local Brassinosteroid Biosynthesis Enables Optimal Root Growth. *Nature Plants*, 7(5):619-632.

Wada, K., Marumo, S., Ikekawa, N., Morisaki, M., & Mori, K. (1981). Brassinolide And Homobrassinolide Promotion Of Lamina Inclination Of Rice Seedlings. *Plant & Cell Physiology*, 22(2):232-325.

Wang, F., Gao, Y., Liu, Y., Zhang, X., Gu, X., Ma, D., Zhao, Z., Yuan, Z., Xue, H., & Liu, H. (2019). BES1-Regulated Bee1 Controls Photoperiodic Flowering Downstream Of Blue Light Signaling Pathway In Arabidopsis. *New Phytologist*, 223(3), 1407-1419.

Wang, H., & Mao, H. (2014). On The Origin And Evolution Of Plant Brassinosteroid Receptor Kinases. *Journal Of Molecular Evolution*, 78(2), 118-129.

Wang, H., Tang, J., Liu, J., Hu, J., Liu, J., Chen, Y., Cai, Z., & Wang, X. (2018). Abscisic Acid Signaling Inhibits Brassinosteroid Signaling Through Dampening The Dephosphorylation Of Bin2 By Abi1 And Abi2. *Molecular Plant*, 11(2), 315-325.

Wang, J., Shi, H., Zhou, L., Peng, C., Liu, D., Zhou, X., Wu, W., Yin, J., Qin, H., Ma, W., He, M., Li, W., Wang, J., Li, S., & Chen, X. (2017). OsBSK1-2, An Orthologous Of AtBSK1, Is Involved In Rice Immunity. *Frontiers In Plant Science*, 8(908).

Wang, L., Liu, J., Shen, Y., Pu, R., Hou, M., Wei, Q., Zhang, X., Li, G., Ren, H., & Wu, G. (2021). Brassinosteroids Synthesised By Cyp85a/A1 But Not Cyp85a2 Function Via A Bri1-Like Receptor But Not Via Bri1 In Picea Abies. *Journal Of Experimental Botany*, *72*(5):1748-1763.

Wang, Q., Yang, S., Wan, S., & Li, X. (2019). The Significance Of Calcium In Photosynthesis. *International Journal Of Molecular Sciences*, 20(1353).

Wang, W., Lu, X., Li, L., Lian, H., Mao, Z., Xu, P., Guo, T., Xu, F., Du, S., Cao, X., Wang, S., Shen, H., & Yang, H. Q. (2018). Photoexcited Cryptochrome1 Interacts With Dephosphorylated BES1 To Regulate Brassinosteroid Signaling And Photomorphogenesis In Arabidopsis. *Plant Cell*, 30(9):1989-2005.

Wang, X., & Chory, J. (2006). Brassinoteroids Regulate Dissociation Of Bki1, A Negative Regulator Of Bri1 Signaling, From The Plasma Membrane. *Science*, 313(5790), 1118-1122.

Wang, Z., Setp, H., Fujioka, S., Yoshida, S., & Chory J. (2000). BRI1 Is A Critical Component Of A Plasma-Membrane Receptor For Plant Steroids. *Nature,* 410(6826):380-383.

Wang, Z.-Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., & Chory, J. (2002). Nuclear-Localized BZR1 Mediates Brassinosteroid-Induced Growth And Feedback Suppression Of Brassinosteroid Biosynthesis Dwarfism, Sterility, And Photomorphogenesis In The Dark. *Developmental Cell,* 2(4):505-513.

Wu, J., Wang, W., Xu, P., Pan, J., Zhang, T., Li, Y., Li, G., Yang, H., & Lian, H. (2019). Phyb Interacts With BES1 To Regulate Brassinosteroid Signaling In Arabidopsis. *Plant And Cell Physiology*, *60*(2), 353-366.

Xia, X., Dong, H., Yin, Y., Song, X., Gu, X., Sang, K., Zhou, J., Shi, K., Zhou, Y., Foyer, C. H., & Yu, J. (2021). Brassinosteroid Signaling Integrates Multiple Pathways To Release Apical Dominance In Tomato. *Proceedings Of The National Academy Of Sciences Of The United States Of America* 118(11):e2004384118.

Xiang, L., Nolan, T. M., Bao, Y., Elmore, M., Tuel, T., Gai, J., Shah, D., Wang, P., Huser, N. M., Hurd, A. M., Mclaughlin, S. A., Howell, S. H., Walley, J. W., Yin, Y., & Tang, L. (2021). Robotic Assay For Drought (Road): An Automated Phenotyping System For Brassinosteroid And Drought Responses. *Plant Journal*, 107(6):1837-1853.

Xie, Z., Nolan, T., Jiang, H., Tang, B., Zhang, M., Li, Z., & Yin, Y. (2019). The Ap2/Erf Transcription Factor Tiny Modulates Brassinosteroid-Regulated Plant Growth And Drought Responses In Arabidopsis. *Plant Cell*, 31(8):1788-1806.

Xin, Z., Wang, M., Cuevas, H. E., Chen, J., Harrison, M., Pugh, N. A., & Morris, G. (2021). Sorghum Genetic, Genomic, And Breeding Resources. *Planta,* 254(114).

Xu, W., Huang, J., Li, B., Li, J., & Wang, Y. (2008). Is Kinase Activity Essential For Biological Functions Of Bri1? *Cell Research*, 18(4):472-478.

Yamaguchi, M., Fujimoto, H., Hirano, K., Araki-Nakamura, S., Ohmae-Shinohara, K., Fujii, A., Tsunashima, M., Song, X. J., Ito, Y., Nagae, R., Wu, J., Mizuno, H., Yonemaru, J., Matsumoto, T., Kitano, H., Matsuoka, M., Kasuga, S., & Sazuka, T. (2016). Sorghum Dw1, An Agronomically Important Gene For Lodging Resistance, Encodes A Novel Protein Involved In Cell Proliferation. *Scientific Reports*, *6*(1):28366.

Yamamoto, R., Fujioka, S., Demura, T., Takatsuto, S., Yoshida, S., & Fukuda, H. (2001). Brassinosteroid Levels Increase Drastically Prior To Morphogenesis Of Tracheary Elements. *Plant Physiology*, Volume 125(2):556-563.

Yamamuro, C., Ihara, Y., Wu, X., Noguchi, T., Fujioka, S., Takatsuto, S., Ashikari, M., Kitano, H., & Matsuoka, M. (2000). Loss Of Function Of A Rice Brassinosteroid Insensitive1 Homolog Prevents Internode Elongation And Bending Of The Lamina Joint. *The Plant Cell*, 12(9):1596-1606.

Yang, X., Bai, Y., Shang, J., Xin, R., & Tang, W. (2016). The Antagonistic Regulation Of Abscisic Acid-Inhibited Root Growth By Brassinosteroids Is Partially Mediated Via Direct Suppression Of Abscisic Acid Insensitive 5 Expression By Brassinazole Resistant 1. *Plant Cell And Environment*, 39(9):1994-2003.

Yang, Z., Zhang, C., Yang, X., Liu, K., Wu, Z., Zhang, X., Zheng, W., Xun, Q., Liu, C., Lu, L., Yang, Z., Qian, Y., Xu, Z., Li, C., Li, J., & Li, F. (2014). Pag1, A Cotton Brassinosteroid Catabolism Gene, Modulates Fiber Elongation. *New Phytologist, 203(2):*437-448.

Ye, H., Liu, S., Tang, B., Chen, J., Xie, Z., Nolan, T. M., Jiang, H., Guo, H., Lin, H. Y., Li, L., Wang, Y., Tong, H., Zhang, M., Chu, C., Li, Z., Aluru, M., Aluru, S., Schnable, P. S., & Yin, Y. (2017). RD26 Mediates Crosstalk Between Drought And Brassinosteroid Signalling Pathways. *Nature Communications*, 8(14573).

Ye, Q., Zhu, W., Li, L., Zhang, S., Yin, Y., Ma, H., & Wang, X. (2010). Brassinosteroids Control Male Fertility By Regulating The Expression Of Key Genes Involved In Arabidopsis Anther And Pollen Development. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 107(13):6100-6105.

Yin, Y., Vafeados, D., Tao, Y., Yoshida, S., Asami, T., & Chory, J. (2005). A New Class Of Transcription Factors Mediates Brassinosteroid-Regulated Gene Expression In Arabidopsis. *Cell*, *120*(2):249-259.

Yin, Y., Wang, Z.-Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., & Chory, J. (2002). BES1 Accumulates In The Nucleus In Response To Brassinosteroids To Regulate Gene Expression And Promote Stem Elongation Extensive Loss-Of-Function Genetic Screens For Br-Insensitive Dwarf Mutants Have Identified Mutations. *Cell*, 109(2):181-191.

Yokota, T., Ohnishi, T., Shibata, K., Asahina, M., Nomura, T., Fujita, T., Ishizaki, K., & Kohchi, T. (2017). Occurrence Of Brassinosteroids In Non-Flowering Land Plants, Liverwort, Moss, Lycophyte And Fern. *Phytochemistry*, 136:46-55.

Yu, X., Li, L., Zola, J., Aluru, M., Ye, H., Foudree, A., Guo, H., Anderson, S., Aluru, S., Liu, P., Rodermel, S., & Yin, Y. (2011). A Brassinosteroid Transcriptional Network Revealed By Genome-Wide Identification Of Besi Target Genes In Arabidopsis Thaliana. *Plant Journal*, 65(4):634-646.

Yuan, G. F., Jia, C. G., Li, Z., Sun, B., Zhang, L. P., Liu, N., & Wang, Q. M. (2010). Effect Of Brassinosteroids On Drought Resistance And Abscisic Acid Concentration In Tomato Under Water Stress. *Scientia Horticulturae*, 126(2):103-108.

Zhang, C., Bai, M. Yi, & Chong, K. (2014). Brassinosteroid-Mediated Regulation Of Agronomic Traits In Rice. In *Plant Cell Reports* (33, Issue 5, Pp. 683-696). Springer Verlag.

Zhang, C., Xu, Y., Guo, S., Zhu, J., Huan, Q., Liu, H., Wang, L., Luo, G., Wang, X., & Chong, K. (2012). Dynamics Of Brassinosteroid Response Modulated By Negative Regulator Lic In Rice. *Plos Genetics*, *8*(4).

Zhang, H., Zhu, J., Gong, Z., & Zhu, J. K. (2022). Abiotic Stress Responses In Plants. In *Nature Reviews Genetics* (23, Issue 2, Pp. 104-119). Nature Research.

Zhang, L. Y., Bai, M. Y., Wu, J., Zhu, J. Y., Wang, H., Zhang, Z., Wang, W., Sun, Y., Zhao, J., Sun, X., Yang, H., Xu, Y., Kim, S. H., Fujioka, S., Lin, W. H., Chong, K., Lu, T., & Wanga, Z. Y. (2009). Antagonistic Hlh/Bhlh Transcription Factors Mediate Brassinosteroid Regulation Of Cell Elongation And Plant Development In Rice And Arabidopsis. *Plant Cell*, 21(12):3767-3780.

Zhang, S., Cai, Z., & Wang, X. (2009). *The Primary Signaling Outputs Of Brassinosteroids Are Regulated By Abscisic Acid* Signaling. *Proceedings of the National Academy of Sciences of the United States of America* 106(11):4543-4548.

Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W., & Chua, N. H. (2006). Agrobacterium-Mediated Transformation Of Arabidopsis Thaliana Using The Floral Dip Method. *Nature Protocols*, *1*(2):641-646.

Zhang, X., Zhou, L., Qin, Y., Chen, Y., Liu, X., Wang, M., Mao, J., Zhang, J., He, Z., Liu, L., & Li, J. (2018). A Temperature-Sensitive Misfolded Bri1-301 Receptor Requires Its Kinase Activity To Promote Growth 1[Open]. *Plant Physiology*, *178*(4):1704-1719.

Zhang, Y., Li, B., Xu, Y., Li, H., Li, S., Zhang, D., Mao, Z., Guo, S., Yang, C., Weng, Y., & Chong, K. (2013). The Cyclophilin Cyp20-2 Modulates The Conformation Of Brassinazole-Resistant1, Which Binds The Promoter Of Flowering Locus D To Regulate Flowering In Arabidopsis. *Plant Cell*, *25*(7):2504-2521.

Zhao, H., & Bao, Y. (2021). PIF4: Integrator Of Light And Temperature Cues In Plant Growth. *Plant Science* 313(111086).

Zhao, Z.-Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, M., Schroeder, S., Hondred, D., Seltzer, J., & Pierce, D. (2000). Agrobacterium-Mediated Sorghum Transformation. *Plant Molecular Biology*,44(6):789-798

Zheng, B., Bai, Q., Wu, L., Liu, H., Liu, Y., Xu, W., Li, G., Ren, H., She, X., & Wu, G. (2019). Ems1 And Bri1 Control Separate Biological Processes Via Extracellular Domain Diversity And Intracellular Domain Conservation. *Nature Communications*, 10(1).

Zheng, L., Ma, S., Shen, D., Fu, H., Wang, Y., Liu, Y., Shah, K., Yue, C., & Huang, J. (2021). Genome-Wide Identification Of Gramineae Histone Modification Genes And Their Potential Roles In Regulating Wheat And Maize Growth And Stress Responses. *BMC Plant Biology*, *21*(1).

Zhi, X., Tao, Y., Jordan, D., Borrell, A., Hunt, C., Cruickshank, A., Potgieter, A., Wu, A., Hammer, G., George-Jaeggli, B., & Mace, E. (2022). Genetic Control Of Leaf Angle In Sorghum And Its Effect On Light Interception. *Journal Of Experimental Botany*, 73(3):801-816.

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