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ANA LAURA GARCIA LEME PERES

O PAPEL DA VIA DE SINALIZAÇÃO DE BRASSINOSTERÓIDE NO DESENVOLVIMENTO DA CANA-DE-AÇÚCAR

THE ROLE OF BRASSINOSTEROID SIGNALING PATHWAY ON SUGARCANE DEVELOPMENT

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de doutora em genética e biologia molecular.

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Orientador: PROF. DR. MARCELO MENOSSI TEIXEIRA

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This thesis is dedicated to my

beloved son Arthur

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RESUMO

A cana-de-açúcar é uma cultura de grande importância, sendo principal matéria prima para a produção de etanol e açúcar. Porém ainda apresenta grandes lacunas de conhecimento sobre a regulação hormonal de seu desenvolvimento. Os brassinosteróides (BR) são hormônios esteroides que modulam diversos processos fisiológicos e de desenvolvimento ao longo do ciclo de vida das plantas, como a produção de etileno, síntese da parede celular, componentes da fotomorfogênese, resposta a estresse abiótico e metabolismo de carboidratos. Diversos estudos em outras espécies mostram que o fator de transcrição BZR1 é um dos principais componentes da via de sinalização de BR. Um segundo fator de transcrição, LIC, exclusivo de monocots, possui função antagônica, a BZR1, atenuando a via de sinalização de BR através do mecanismo de *feedback* negativo. Em cana-de-açúcar não são conhecidos os efeitos de BR na fisiologia e desenvolvimento, bem como dos componentes moleculares que regulam a ação desse hormônio. Frente a isso, os principais objetivos desse projeto são desenvolver um estudo molecular que busque elucidar quais são os BR endógenos presentes em cana de açúcar, avaliar como a modulação da expressão dos genes *ScBZR1* e *ScLIC* podem interferir no desenvolvimento da cana-de-açúcar e em respostas ao déficit hídrico e determinar como os principais genes de signalização e biossíntese são modulados em linhagens transgênicas superexpressando e silenciando os dois fatores de transcrição. O presente estudo mostrou a castasterona como o principal BR endógeno de cana, sendo compatível com estudos prévios de espécies próximas a cana, como o arroz. Observamos que os principais genes que ativam a via de sinalização estão presentes em linhagens com maior expressão de *ScBZR1*, enquanto os componentes que agem inibindo a via estão presentes em linhagens com maior expressão de *ScLIC*. E por fim foi observado que altos níveis de *ScBRZ1* conferem maior tolerância ao déficit hídrico, sendo o oposto verificado em linhagens com altos níveis de *ScLIC*. No entanto, não foram observadas diferenças no crescimento e desenvolvimento das canas transgênicas após modulação da expressão de *SBZR1* e *ScLIC*. O estudo aprofundado desses genes será fundamental para auxiliar no entendimento dessa via de sinalização em cana e em monocotiledôneas de maneira geral, abrindo perspectivas para modulação de componentes com objetivo de regular diversos *traits* agronômicos de interesse econômico.

ABSTRACT

Sugarcane is an important crop and the main raw material to sugar and ethanol production. However, there are still big gaps on the knowledge about hormonal regulation of its development. Brassinosteroids (BR) are steroidal hormones which modulate a range of physiological and developmental processes along plants life cycle such as ethylene production, cell wall biosynthesis, components of photomorphogenesis, responses to abiotic stresses and carbohydrates metabolism. Many studies in other species showed that transcriptional factor BZR1 is one of the major components of BR signaling pathway. A second transcriptional factor, LIC, exclusive for monocots, presents antagonistic function to BZR1, acting on BR signaling pathway through negative feedback mechanism. The role of BR pathway on physiology and development of sugarcane is still unknown, as well as the molecular components that regulate the mode of action of this hormone. Therefore, the main objectives of this research project are providing a molecular study to elucidate what are the endogenous BR in sugarcane, how the modulation of *ScBZR1* and *ScLIC* may interfere in sugarcane development and stress response in greenhouse conditions and how the main signaling and biosynthetic genes express themselves in transgenic lines of sugarcane, which overexpress and silence the two transcriptional factors. The present work showed that castasterone is the main endogenous BR in sugarcane, confirming the results of previous studies with close related species as rice. In addition, the genes that activate BR signaling pathway are observed in the lines with high levels of *ScBZR1* and genes that inhibit BR signaling pathway are observed in the lines with high levels of *ScLIC*. Moreover, it was observed that high levels of *ScBRZ1* confers more tolerance to abiotic stresses, in opposite to observed in the lines with high levels of *ScLIC*. However, the transgenic sugarcane lines did not presented any changes in growth rate and development in relation to WT. The deep study of BR signaling pathway in sugarcane will be a fundamental tool to help to understand how this pathway occurs in sugarcane and monocots in general, opening new perspectives to modulate components of BR pathway, objectifying the regulation of many agronomic traits with economic importance.

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1. GENERAL INTRODUCTION

1.1 Sugarcane around the world

Sugarcane is a monocot of the Poaceae family (Gramineae) and belongs to the *Saccharum* genus. The crossing species produce vigorous hybrids which are the mainstay of the modern sugarcane industry (Irvine, 1999).

The global interest in sugarcane increased in the past few years due to the high demand for renewable energy production. In addition, sugarcane is the main renewable energy source in Brazil according with Brazilian Energy Balance 2021, where 19.1% of sugarcane biomass represents 39.5% of all renewable energy source used in the country. Currently, sugarcane is responsible for approximately 80% of the world sugar production, corresponding to almost 182 mi of tons in season 2021/22 (Statista, 2022).

Brazil stands out in this scenario as the biggest producer of sugarcane due to the great development of sugar and alcohol department and favorable weather conditions. It has been estimated that the cultivated area will reach 8,345 thousand hectares, with a sugar production of 33.89 mi tons and 26.4 bi litters of ethanol in season 2021/2022 in Brazil (4^{th} data entry - Conab, april 2022).

Sugarcane can accumulate high levels of sugar in the culms. In physiologic terms, sucrose accumulation and internodes growth are dynamic and complex processes that involve a range of changes in plant metabolism, including various regulatory genes and BR.

1.2 Agricultural use of BR

Over the past few decades, the discovery of brassinolide and the production of synthetic BR compounds caught the attention of the scientific community due to their versatile use in agriculture. There are several reports on the applications of BR and their effects on plants: a foliar spray of 24-epibrassinolide (EBL) in strawberry improved tolerance against *Botrytis cinerea* (Furio et al., 2019); tomato seedlings treated with 28-homobrassinolide (HBR) showed improved fruits production, with higher contents of lycopene and β-carotene(Ali et al., 2006); BRs application in grape increased the length, width and weight of clusters (Champa et al., 2015); BR also helped to keep postharvest quality, controlling grey mould of postharvest grapes

(Liu et al., 2016) and accelerating the ripening process by increasing the soluble solids and ethylene production in tomato (Zhu et al., 2015[1]), mango fruits (Zaharah et al., 2012) and potato tubers (Korableva et al., 2002).

However, due to the high cost of the production of synthetic BR compounds and the variability of the results, this compound is still poorly used in the agriculture. On the other side, the modulation of the endogenous activity of BR through the direct manipulation of genes involved in the biosynthesis and signaling pathways may allow the increase of productivity and performance of the plants in predictable and homogeneous ways (Divi and Krishna, 2009).

Two of the bigest potential genes to be modulated are the BR signaling pathway genes, *BZR1* and *LIC*, the two major transcriptional factors of this pathway, which control several genes in different physiological processes. As we will describe in the next chapter, *BZR1* plays a positive role in growth and stress responses, whereas *LIC* has the opposite effect. Therefore, the hypothesis of this work is that *ScBZR1* would increase sugarcane height and stress tolerance, while *ScLIC* would increase tillering and repress stress tolerance.

Molecular studies of sugarcane were leveraged with the development of the SUCEST project, identifying more than 238 thousand sequences of cDNAs and genes involved in signaling, response to biotic and abiotic stresses, pest resistance, carbohydrate metabolism, sugar storage, flowering development, nutrients absorption, cell cycle regulation, nitrogen assimilation and tolerance to aluminum and other metals (Vettore et al., 2001). In 2004 the "Sugarcane Transcriptome" Project, financed by São Paulo Research Foundation (FAPESP, SP, Brazil), Cane Technology Center (CTC, Piracicaba, SP, Brazil) and Lucélia Alcohol Central (Lucélia, SP, Brazil), performed the first experiments of wide-genome expression analysis in sugarcane (Papini-Terzi, 2005). Plant breeding programs contributed to increase sugarcane production of the past 30 years and with biotechnology techniques, there is a great expectation that this will leverage this progress (Cheavegatti-Gianotto et al., 2011).

1.3 Objectives

The main objective of this thesis is to promote a deep molecular study of the BR signaling pathway in sugarcane, attempting to better understand the role of this pathways component on sugarcane growth and development.

Specific objectives:

I. Produce transgenic plants overexpressing and silencing *ScBZR1* and *ScLIC* genes separately to evaluate how the balance of these genes may affect growth and other developmental processes of sugarcane plants.

II. Elucidate how the feedback mechanism of BZR1 (positive regulator) and LIC (negative regulator) occurs on the BR pathway in overexpressing and silencing transgenic lines.

III. Perform a molecular analysis of the BR signaling pathway in sugarcane, elucidating how the expression of important genes of BR signaling and biosynthesis pathways occur in transgenic and non-transgenic lines.

IV. Evaluate how transgenic lines respond to drought stress under greenhouse conditions.

V. Identify the main endogenous BR of sugarcane.

2. CHAPTER 1

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Review

Brassinosteroids, the Sixth Class of Phytohormones: A Molecular View from the Discovery to Hormonal Interactions in Plant Development and Stress Adaptation

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Abstract: Phytohormones are natural chemical messengers that play critical roles in the regulation of plant growth and development as well as responses to biotic and abiotic stress factors, maintaining plant homeostasis, and allowing adaptation to environmental changes. The discovery of a new class of phytohormones, the brassinosteroids (BRs), almost 40 years ago opened a new era for the studies of plant growth and development and introduced new perspectives in the regulation of agronomic traits through their use in agriculture. BRs are a group of hormones with significant growth regulatory activity that act independently and in conjunction with other phytohormones to control different BR-regulated

activities. Genetic and molecular research has increased our understanding of how BRs and their cross-talk with other phytohormones control several physiological and developmental processes. The present article provides an overview of BRs' discovery as well as recent findings on their interactions with other phytohormones at the transcriptional and post-transcriptional levels, in addition to clarifying how their network works to modulate plant growth, development, and responses to biotic and abiotic stresses.

Keywords: brassinosteroids; plant hormones; hormonal cross-talk

1. Introduction

In the first years of the 20th Century, the only known plant hormones with recognized roles in development were indole-acetic acid and gibberellic acid. Some early experiments demonstrated that the application of the spores or pollen of some plants to the stigmas of other species promotes the development of parthenocarpic fruits therein. Even pollen extracts [1], and some growth-promoting chemicals [2], were shown to promote parthenocarpy. When applied to the first internode of intact bean plants, ethereal extracts from corn pollen caused pronounced elongation compared to control and even plants treated with natural or synthetic auxins [3]. The same effect was obtained with extracts prepared from immature bean seeds [4]. It was further shown that *Brassica napus* and *Alnus glutinosa* pollens contain some plant growth regulators, termed brassins, considered plant hormones as they were supposed to be "specific translocatable organic compounds isolated from a plant and have induced measurable growth control when applied in minute amounts to another plant" [5]. The pollen extracts of many other plant species showed the same effects [6], but it was not possible at that time to attribute the physiological effects observed by the application of brassins to any known compound. After a time-consuming and expensive multidisciplinary effort [7]—that involved the processing of at least 400 pounds of rape pollen by a newly-developed method for obtaining brassins [8] as well as physiological and agronomical assays with the active fractions—brassinolide (BL) (Figure 1) [9] was identified as the compound responsible for the different physiological effects produced by brassins. The first syntheses of BL [10,11] and similar compounds [12–15] were soon reported, and the development of a micromethod for their detection [16] (from which many others derived [17,18]) revealed compounds resembling BL in many plant species. In the coming years the isolation of many other compounds with structures similar to BL gave rise to the family of brassinosteroids (BRs) [19–24], defined as the "3-

oxygenated (20β)-5α-cholestane-22α,23α-diols or their derived compounds isolated from plants, bearing additional alkyl or oxy substituents" [25], now recognized as the sixth class of plant hormones. This class of phytohormones is represented by more than 60 compounds (Figure 1) that have been isolated or detected from more than 100 plant species, from algae to angiosperms, revealing their ubiquitous distribution in the plant kingdom [25,26].

Simultaneous to efforts being made to isolate the active principle(s) of the brassins, experiments were being conducted to verify their possible beneficial effects on crops [27,28] as well as to determine their hormonal functions [29–31]. The first syntheses of BL [10,11], 28-homobrassinolide [12], 24-epibrassinolide [13], and other BRs allowed pure compounds to be assayed by the methods used for testing other established plant hormones, such as auxins [32,33], cytokinin, and gibberellin [34,35]. It also allowed their interactions with other plant hormones to be tested [36,37], providing a solid basis for understanding their actions in plant growth and development [38–41], including the role of BL in the germination and growth of pollen tubes [42]. Molecular analyses of BRs' action soon appeared [43], and the discovery of BR-deficient mutants [44], BR-signaling mutants [45], and of BR biosynthesis inhibitors [46,47] made it possible to further determine their mechanisms of action at the molecular level. The elucidation of the BL structure and its receptor kinase BRASSINOSTEROID INSENSITIVE 1 (BRI1) provided insight into the recognition of BRs by their receptor and the activation of the BL-BRI1 complex [48,49]. The evolution of the research into the physiological and biochemical aspects of brassinosteroids is reviewed elsewhere [50].

Previous and recent studies have indicated how the cross-talk between BRs and other phytohormones might contribute to the regulation of an extensive spectrum of biological processes. The present review provides an overview of the current knowledge on the cross-talk between brassinosteroids and other phytohormones, such as auxin (AUX), gibberellins (GAs), cytokinins (CKs), ethylene (ET), abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA) at the transcriptional and post-transcriptional levels, as well as how their networks may contribute to the modulation of plant growth, development, and other biological processes. Our major objective is to provide a clear understanding of how BR in conjunction with other phytohormones controls different activities in plant metabolism.

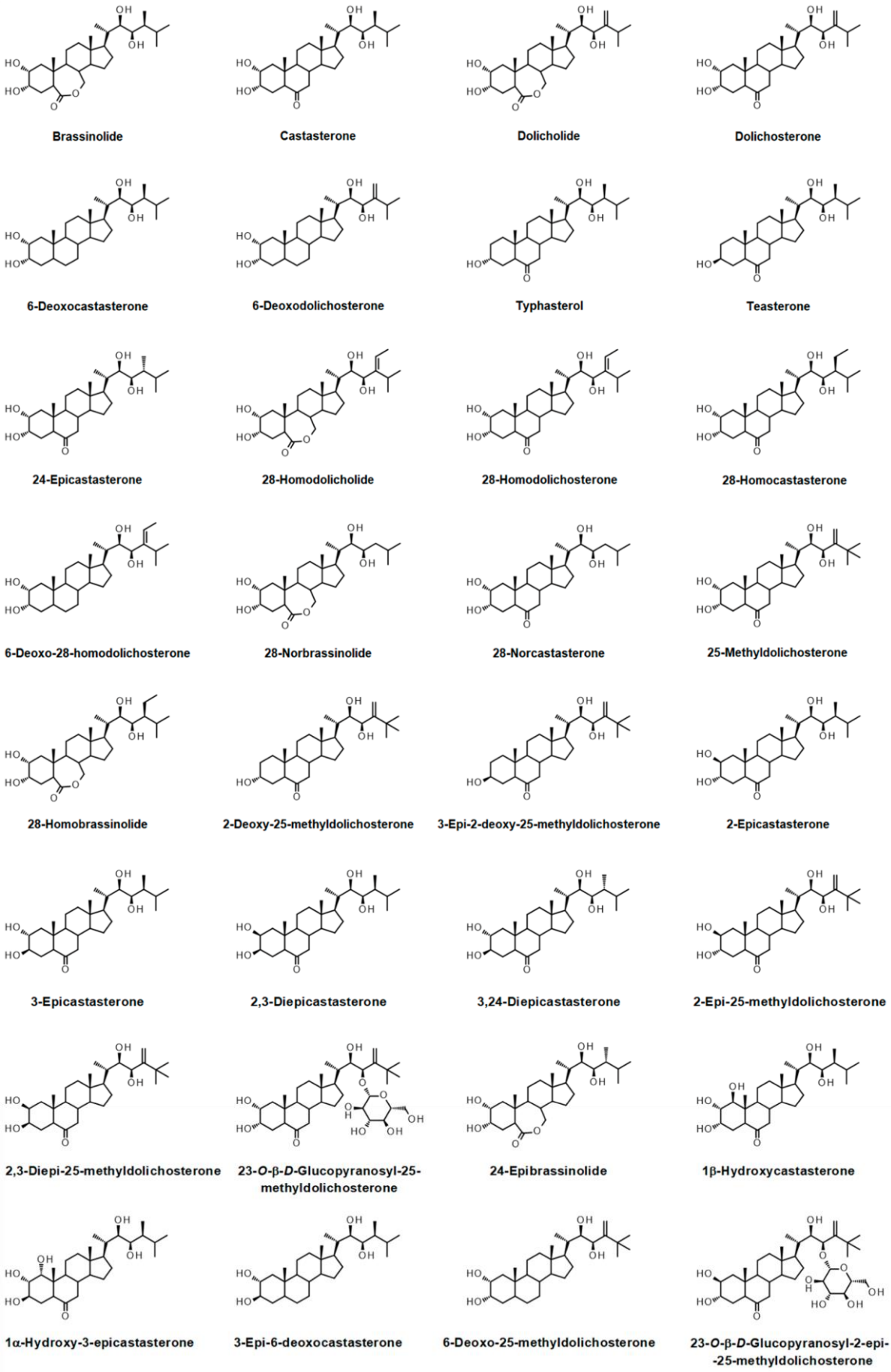

Figure 1. *Cont.*

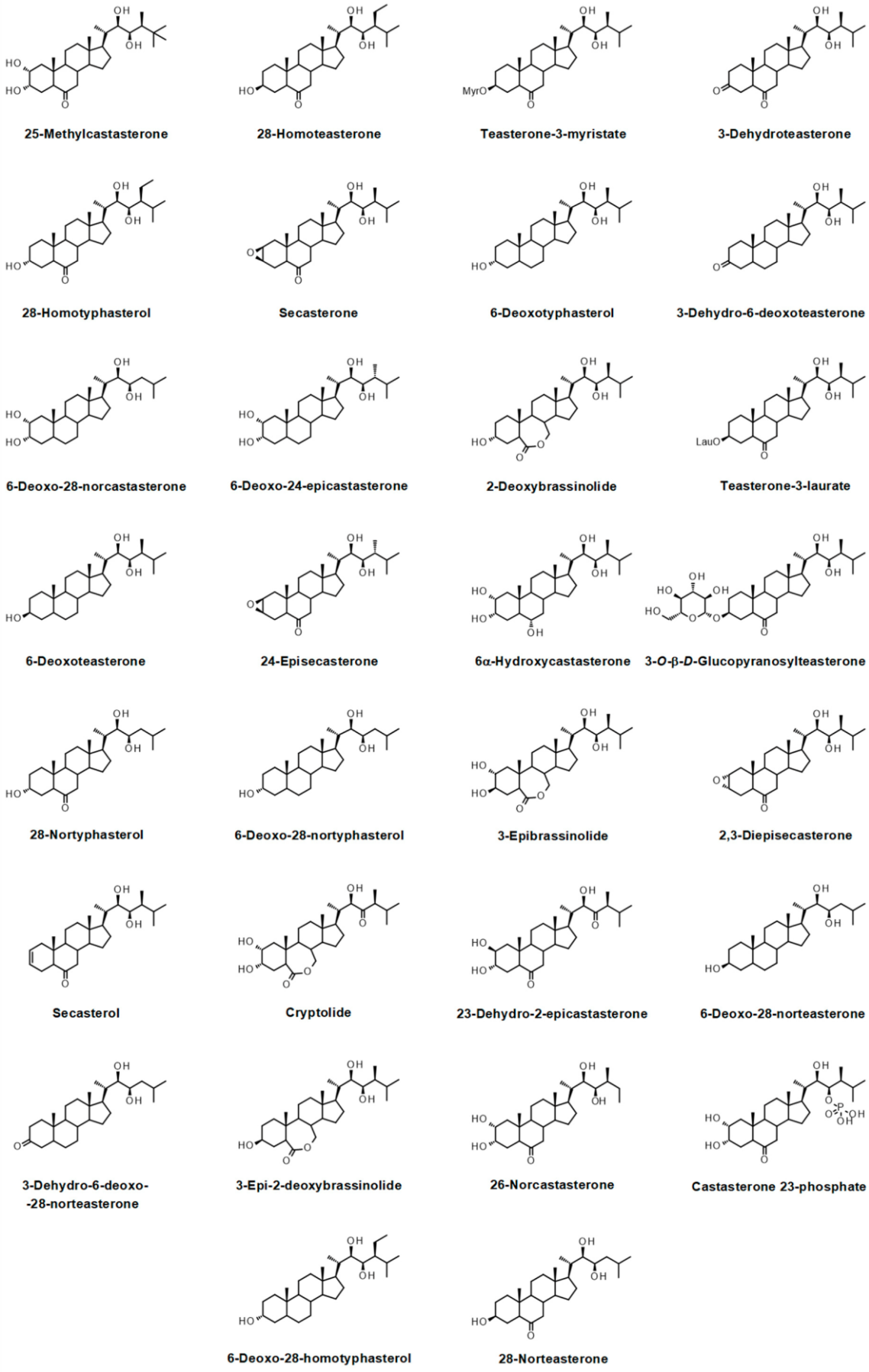

Figure 1. Natural brassinosteroids isolated from or detected in plant sources.

2. Brassinosteroids: Functions and Signaling Pathway

Due to BRs' growth regulator activity, this class of phytohormones is involved in a range of developmental processes, including cell division and elongation, vascular differentiation, reproductive development and the modulation of gene expression [51]. BR-deficient and -insensitive mutants in *Arabidopsis thaliana* (hereafter called *Arabidopsis*) present dwarfism, short petioles, delayed flowering, and reduction in fertility phenotypes. Equivalent mutants in other eudicot species such as tomato (*Solanum lycopersicum*), pea (*Pisum sativum*), and petunia (*Petunia hybrida*), as well as in monocots, like rice (*Oryza sativa*), barley (*Hordeum vulgare*), and maize (*Zea mays*) showed comparable phenotypes [52–54].

The main responsible for BR-mediated responses are BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (BRI1-EMS SUPPRESSOR 1), also named BZR2, the two major BR signaling pathway transcription factors, which regulate a range of genes involved in different physiological processes, such as developmental responses, protein metabolism, cellular transport and signaling, cell wall biosynthesis, chromatin and cytoskeleton components, environmental responses, and hormone responses [55].

Signaling Pathway

In previous years, a combination of genetic, biochemical and proteomic approaches have accelerated the understanding of the BR signaling pathway in *Arabidopsis* [52,56–58]. Upon BR binding, BRI1 (BRASSINOSTEROID INSENSITIVE 1), a plasmatic membrane leucine-rich repeat (LRR) receptor-like kinase (RLK) [59,60], which functions with its coreceptor BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) [61–63], generates a phosphorylation cascade [64,65]. Activation of the receptor and coreceptor stimulates the phosphorylation of BKI1, the inhibitor of BRI1 [66,67], leading to its dissociation from the plasma membrane and further association with 14-3-3 proteins. The 14-3-3 proteins are involved in the interaction and cytoplasmic retention of BZR1 and BES1 [68–72]. Concomitantly, activated BRI1 is also involved in the phosphorylation of the BSKs (BR-SIGNALING KINASE 1) and CDG1 (CONSTITUTIVE DIFFERENTIAL GROWTH 1), which both subsequently activate BSU1 phosphatase (BRI1 SUPPRESSOR 1) [57,73–75]. BSU1 is responsible for dephosphorylating BIN2 (BRASSINOSTEROID-INSENSITIVE 2), a GSK3-like kinase and the major repressor of the BR signaling pathway [72], which is posteriorly repressed by KIB1 (KINK SUPPRESSED IN BZR1-1D), an F-box ubiquitin ligase that does not allow the association of BIN2 with

BZR1/BES1, culminating in its ubiquitination and degradation [76]. Upon BIN2 inactivation, BZR1 and BES1 are rapidly dephosphorylated by PP2A (PHOSPHATASE 2A) and subsequently dissociated from 14-3-3 proteins, causing them to accumulate into the nucleus, resulting in the regulation of many BR-responsive genes [77].

In the absence of BR, BKI1 binds to the intracellular domain of BRI1, preventing its association with its coreceptor BAK1 [66]. In turn, BIN2 is activated, and 14-3-3 proteins are associated with BZR1 and BES1, maintaining their dephosphorylated form and blocking their capability of shuttling to the nucleus for the regulation of thousands of BR responsive genes [67]. It is worth mentioning that previous studies have indicated that BR increases the expression of SBI1 (SUPPRESSOR OF BRI1), a positive regulator of BR1 degradation that methylates PP2A and controls its membrane-associated subcellular localization. As such, the relocation of methylated PP2A at membranes facilitates its association with the BR-activated BRI1, leading to BRI1 dephosphorylation and degradation, and, in turn, the termination of BR signaling. These data indicate that PP2A and SBI1 provide a negative feedback mechanism that triggers BRI1 turnover after activation of the BR signaling pathway [78]. The current model of the BR signaling pathway can be observed in Figure 2.

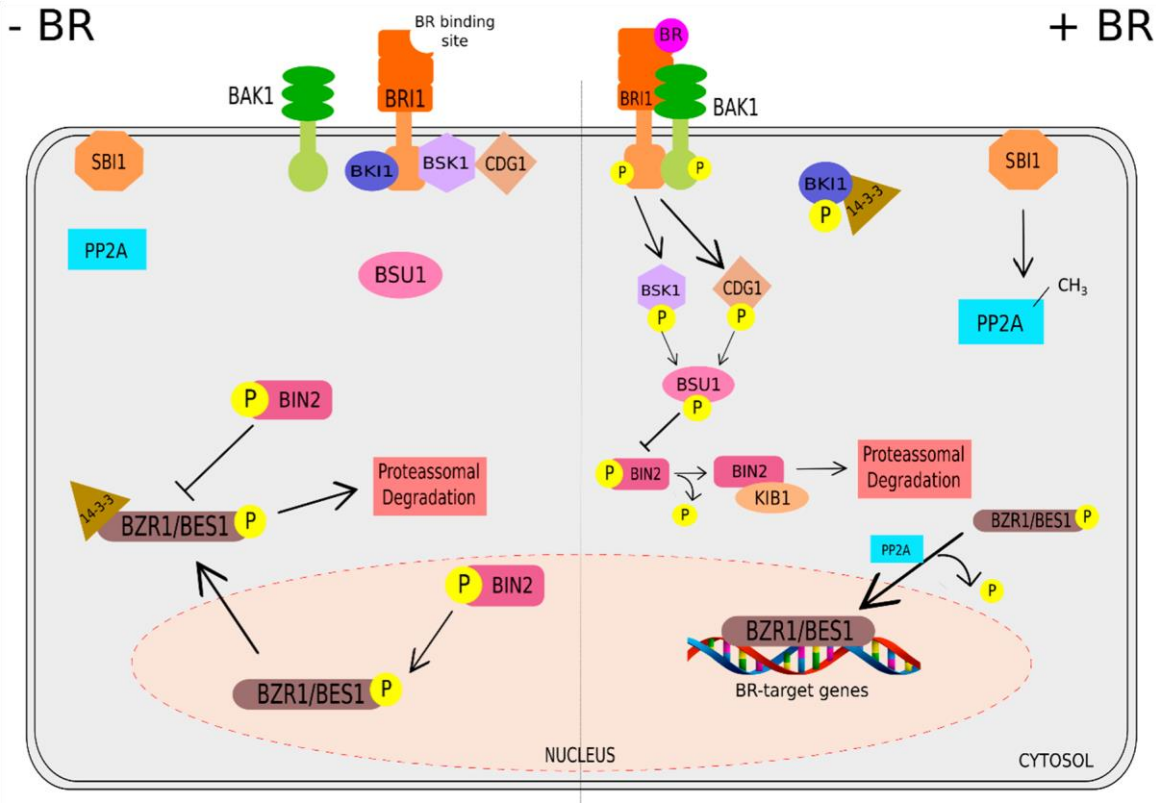

Figure 2. Current model of the signaling pathway in the presence or absence of brassinosteroids (BRs) in *Arabidopsis*. In the absence of BR, the receptor kinase BRI1 (BRASSINOSTEROID INSENSITIVE 1) does not heterodimerize with its coreceptor BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1), maintaining their inactive forms. Consequently, BIN2 (BRASSINOSTEROID-INSENSITIVE 2), a negative regulator of BR signaling pathway, is free to constitutively phosphorylate BZR1 (BRASSINAZOLE RESISTANT 1) and BES1 (BRI1-EMS SUPPRESSOR 1), the two master transcription factors of BR-induced responses, inducing their interactions with 14-3-3 proteins that, in turn, promotes the cytoplasmic retention of BZR1/BES1, suppressing their DNA-binding activity. On the other hand, in the presence of BR, the activation of BRI1 triggers its autophosphorylation and partial kinase activity and dissociation from its inhibitor BKI1, which is attached at the BRI1 kinase domain. This leads to its heterodimerization with BAK1, and transphosphorylation to complete BRI1 kinase activity. Activated BRI1 then phosphorylates BSKs (BR-SIGNALING KINASES) and CDG1 (CONSTITUTIVE DIFFERENTIAL GROWTH 1) which both phosphorylate BSU1 (BRI1 SUPPRESSOR 1), leading to BIN2 dephosphorylation. BIN2 is subsequently restrained by KIB1 (KINK SUPPRESSED IN BZR1-1D), which prevents the association of BIN2 with BZR1/BES1 and facilitates its ubiquitination and degradation. The inactivated form of BIN2 allows BZR1 and BES1 to enter into the nucleus and regulate the expression of BR target genes. Additionally, PP2A (PHOSPHATASE 2A) also positive regulates BR signaling by dephosphorylating BZR1 and BES1, whereas SBI1 (SUPPRESSOR OF BRI1) deactivates BRI1 through the methylation of PP2A.

3. Cross-talk between BRs and Other Phytohormones in Plant Growth, Development, and Stress Responses

3.1. Brassinosteroids and Auxins

The events along the plant life cycle rely on coordinated changes at the molecular level in plant growth in a complex network, requiring a synchronism involving different hormone signals. Over the years, BR and auxin have been considered as two important phytohormones that function as master regulators in different plant development processes such as root development and stem elongation [79,80].

The interaction between BR and auxin has been observed in different processes. Hypocotyl elongation assays showed that auxin-responsive mutants display reduced BR sensitivity [81]. Similarly,

BR treatment significantly enhanced auxin response in hypocotyl elongation, indicating that the auxin response depends on the presence of a functional BR signal transduction pathway [82].

Similar to BR, auxin is a growth-promoting hormone that is synthesized mostly in the shoot apical meristem (SAM), young leaves and in the root along the meristem [83,84] that binds to the TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) receptor protein, which triggers the degradation of the AUXIN/INDOLE ACETIC ACID (AUX/IAA) transcriptional repressor protein. Upon ubiquitylation and subsequent degradation of the Aux/IAA proteins, AUXIN RESPONSE FACTOR (ARF), a family of transcription factors, including 25 and 23 members in rice and *Arabidopsis*, respectively, are released to activate gene expression through the recognition of auxin-responsive DNA Elements (AuxREs) [85–87]. The balance between AUX/IAA and ARF is a key control point in auxin signaling and orchestrates the molecular mechanisms by which auxin-BR impacts plant growth and development [88]. Besides, dual roles have been reported for ARFs: transcriptional activation and repression of gene expression.

The first molecular evidence of transcriptional regulation of ARF genes by BR came from the downregulation of *ARF4* and *ARF8* in BL-treated hypocotyls of *Arabidopsis* wild-type (WT) seedlings, contrasting the high level of expression observed in BR-deficient mutants [89]. In another study, the overexpression of *ARF8* in *Arabidopsis* inhibited the hypocotyl growth and resulted in a weaker apical dominance [90]. These results indicate that *ARF8* negatively regulates the auxin response in shoot elongation. The transcriptional activation activity of *ARF* was observed by chromatin immunoprecipitation-sequencing (ChiP-seq) and transgenic analyses where the interaction between BZR1 and ARF6 enhanced their DNA-binding activity capacity and promoted the activation of shared-target genes involved in hypocotyl elongation [91,92]. In addition, the ChIP assay confirmed that BZR1 binds to *IAA19* and *ARF7* promoters to potentiate the auxin response [93]. Interestingly, the application of high concentrations of BL or the hypersensitive *bzr1-1D* mutant resulted in curved and shorter hypocotyls [94]. All of these results indicate that BZR1 and an appropriate BR concentration are required for the auxin promotion of hypocotyl elongation in *Arabidopsis* seedlings grown in the dark. On the other hand, at low BR levels, another component of the BR signaling pathway, BIN2, phosphorylates ARF7 and ARF19, enhancing their DNA-binding capacity during lateral root development [95]. This corroborates with the inhibition of root growth by high levels of BR [96]. Nevertheless, the BIN2-mediated phosphorylation of ARF2 in the gain-of-function *bin2* mutant was shown to reduce ARF2 DNA-binding

and its repressing activity on shoot and root growth [82]. These results are a clear indication that the auxin-BR response involves a dynamic coordination of both transcriptional and post-transcriptional regulation of ARFs via BZR1 and BIN2 to control plant growth and development in a spatiotemporal context.

Root development is determined by the balance between cell division and differentiation in the root meristem. Despite the well-known synergistic interaction in various developmental processes, in the case of root tips, BR and auxin interact antagonistically in controlling gene expression, stem cell maintenance and cell elongation. Additionally, a finely balanced concentration between these hormones is required for optimal root growth [97]. BR affects root growth in a concentration-dependent manner to control the root meristem size. The short root phenotype of the BR-insensitive *bri1-116* mutant is suppressed by low concentrations of BL [98]. Additionally, specific cell types of the root meristem are affected by different levels of BR. Chaiwanon et al. (2015) [97] observed that the expression of *bzr1-1D* in the *bri1-116* mutant epidermis cells increased the elongation zone of the root meristem. On the other hand, high levels of BR/BZR1 in the endodermis or in the quiescence center (QC) had no effect on the *bri1-116* phenotype, indicating the requirement of different concentrations of BR/BZR1 for the normal function of root cells [97]. Collectively, these observations support a model whereby, under different levels of BR, BZR1 contributes to the gene expression pattern by targeting different genes in distinct cells, as is the case in the induction of genes expressed in the transition-elongation zone, but repressing genes in the QC and surrounding stem cells [97].

BES1 is another transcription factor of the BR signaling pathway and shares 88% sequence identity with its closest paralog, BZR1. BES1 also tightly connects the BR pathway to other hormone responses in *Arabidopsis*. In the gain-of-function *bes1-1D*, a dominant mutation that leads to overaccumulation of BES1, some auxin-responsive genes are induced [99]. The auxin-responsive gene *SAUR15* is upregulated in the *bes1-1D* mutant and induced by BR without increasing the endogenous auxin levels [100]. Interestingly, the auxin efflux carriers PIN4 and PIN7, which maintain the distribution and endogenous auxin gradient, are controlled by BES1 [101]. When grown in the dark, the phenotype of the *bes1-1D* mutant was shown to be similar to *bzr1-1D* [77]. However, both mutants have distinct light-grown phenotypes that are consistent with their effects on the feedback regulation of BR biosynthetic genes [99]. While the *bzr1-1D* mutant has reduced BR levels and lower expression of the BR biosynthetic pathway gene *CONSTITUTIVE PHOTOMORPHISM AND DWARFISM* (*CPD*), *bes1-*

1D has only a small effect on *CPD* gene expression [99]. This suggests that BZR1 plays a major role in the activation of the BR negative feedback pathway that inhibits BR biosynthetic genes [77]. Interestingly, another BR biosynthetic gene, *BREVIS RADIX* (*BRX*), is under a feedback loop during *Arabidopsis* root development and mediates feedback between auxin and BR signaling [102]. In the future, it would be interesting to evaluate the effects of BZR1 on *BRX* gene expression in different root tissues at different BR levels.

From the molecular point of view, the question that needs to be addressed is: what is the conversion point of different hormone signals at different stages of development, at different organs and under different hormone levels? Unfortunately, there is no clear answer yet. Studies on the relationship between BR and auxin might clarify the complex biological significance of the question above.

In summary, Figure 3 shows a schematic working model for the cross-talk between BR and auxin. The concept behind this model is a mechanism involving the control of BR–auxin interaction by a tissue-specific transcriptional/post-transcriptional regulation circuit in a hormone dose-dependent manner. A detailed molecular link between the interaction of BR and auxin in plant growth remains elusive, and further investigations will be essential to understand the spatiotemporal pattern of BR–auxin cross-talk.

3.2. Brassinosteroids and Gibberellins

3.2.1. BR–GA Cross-talk: The Signaling Model

A long-standing theme in plant development is how, when and where hormonal cross-talk orchestrates a myriad of developmental cues while simultaneously transmitting environmental inputs. Over the years, this multidynamic mapping of hormonal signaling has elegantly been deciphered by transcriptional and post-transcriptional regulatory mechanism models. Therefore, it is not surprising that there has been a strong effort over the last two decades, particularly in the last six years, to develop an improved integrated model of BR–GA coordination. To date, three out of eight classes of hormones in plants have been identified as major classes of growth-promoting hormones which include auxins, gibberellins, and brassinosteroids. Despite their interdependences in playing a wide range of growth

and developmental processes in different contexts throughout the life cycle of plants, they also act through a woven network, regulating themselves and several downstream effects [103].

Gibberellins are a group of tetracyclic diterpenoids, synthesized by a multistep process, which act as mobile signals [104] with diverse intermediates being processed into different cellular compartments [105]. Several studies have shown the complex spatiotemporal regulation of their biosynthesis in different tissues, cell types and developmental phases [106]. GAs' distribution and mobility, recently clarified through the report of two GA transporters (i.e.; the nitrate transporter 1/peptide transporter family (NPF) [107] and SWEET13/14 proteins [108]) have long been described to long-distance movement, but their combinatorial effects on GA activity at a cellular resolution have only recently been clarified through novel approaches using the GA biosensor (termed GPS1) [109] and a fluorescently labeled version of active GA_3 and GA_4 (termed GA–FI) [110]. In contrast to this multifaceted regulation, their signal transduction mechanism seems to be relatively straightforward, whereas GA-induced DELLA degradation acts as a central regulatory switch for GA signaling (Figure 4). Briefly, active GAs are recognized and bound to their receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1), which, in turn, binds to the N-terminal of DELLA proteins, relieving their repression by promoting their degradation via the ubiquitin–proteasome pathway [111]. Of note, the existence of a DELLA-independent signaling pathway has also been reported through the increase of $[Ca^{2+}]_{cyt}$ within a few minutes after GA treatment [112].

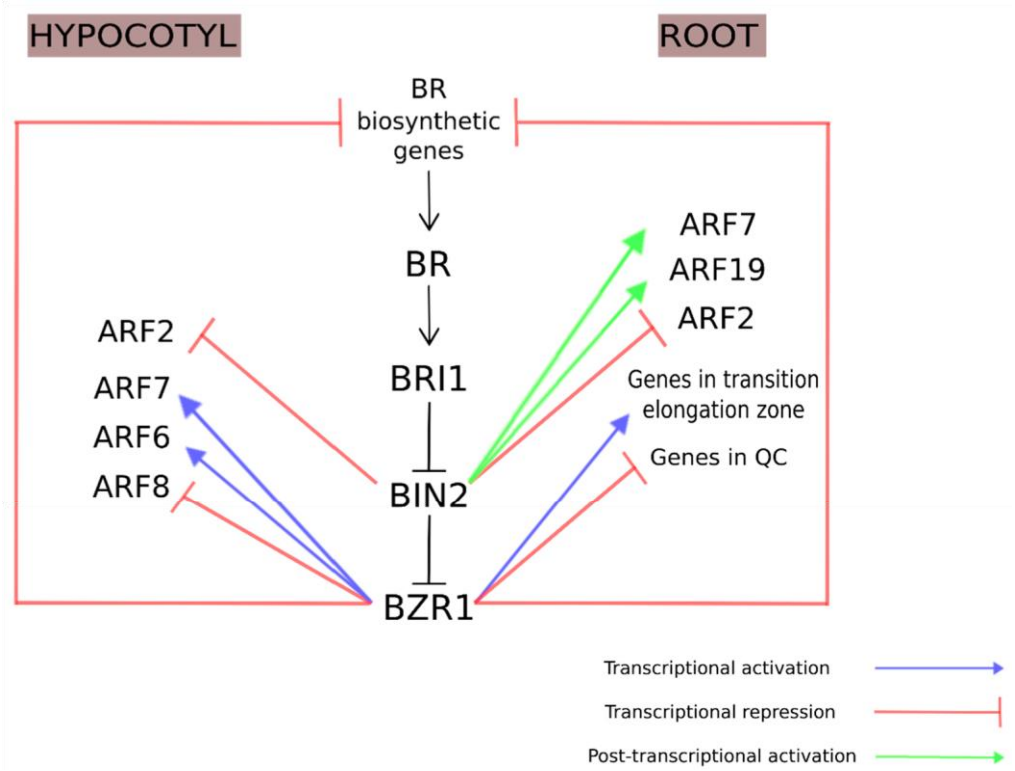

Figure 3. Schematic working model of regulatory interactions between BR and auxin in root and hypocotyl growth. The green arrows represent the post-transcriptional activation of AUXIN RESPONSE FACTOR (ARF) by BRASSINOSTEROID-INSENSITIVE 2 (BIN2). The blue arrows represent the transcriptional activation of ARF and auxin-responsive genes in the root transition elongation zone by BRASSINAZOLE RESISTANT 1 (BZR1). The red arrows represent the transcriptional repression of ARF and auxin-responsive genes in the root quiescence center (QC) by BZR1. The negative feedback of biosynthetic genes coordinated by BZR1 in both root and hypocotyl elongation is also represented by red arrows.

The most convincing evidence of this tangled interaction between BR and GA came in the late 1990s, with the discovery of the remarkably resembled phenotypes (being de-etiolated in the dark and dwarf stature in the light) between GA- and BR-deficient *Arabidopsis* mutants [96,113–117]. Subsequently, several detailed physiological, metabolic and genetic studies in pea (*Pisum sativum*) [118], mung bean (*Vigna radiate*) [35], cucumber (*Cucumis sativus*) [119], rice (*Oryza sativa* L.) [120], and, in particular, in *Arabidopsis* [121], started to reveal evidence of a cooperative and interdependent relationship between BRs and GAs, but with multiple layers of this complex interaction acting in a species-, tissue-, and dose-dependent manner. The elusive nature of such responses in this complex interplay was clarified further when, in 2012, a direct physical cross-talk between their signaling

pathways was revealed, and the signaling model was proposed (Figure 4). In fact, DELLA not only interacts with BZR1/BES1, but also exerts an inhibitory effect on BZR1 transcriptional activity [122–124].

This mechanistic molecular framework became the stepping stone towards expand the understanding of the integration between BR–GA activities, whereas if DELLAs inhibit BZR1 activity and GA-induce DELLA degradation, GA and BR should affect the expression of BZR1 target genes similarly in the control of plant growth and development.

Consequently, this strengthened notion was further examined and validated through the coregulation of common target genes mediated by the BZR1–DELLA interaction. Bai and coworkers elegantly firstly demonstrated that 419 (35%) out of 1,194 genes differentially expressed in *ga1-3* (GA-biosynthesis deficient) compared to WT plants, were also affected in the *bri1-116* (BR-insensitive) mutant, of which 387 (92.3%) of the coregulated genes were affected in the same way by these mutants. Secondly, they analyzed RNA-sequencing data from GA-treated WT and GA-treated WT grown on PPZ (a specific inhibitor of BR biosynthesis) medium, identifying 3,570 and 1,629 differentially regulated genes, respectively. Again, this striking data suggested that around 66.7% of GA-regulated genes require BR, emphasizing the important role of BR in the GA regulation of genome expression [122]. Consistent with these data, other groups showed that hypocotyl elongation promoted by GA was eliminated in *Arabidopsis* seedlings with reduced BR biosynthesis (i.e.; *de-etiolated-2* (*det2*) mutants or brassinazole (BRZ) treatment), indicating that cell elongation largely relies on the appropriate action of both hormones [123,125]. Later experiments, discussed in more detail below, showed that the capacity of GA to rescue the growth defects of BR mutants is dependent on the developmental stage, on the physiological conditions and also on the fact that the GA pathway is only one of the branched pathways of BR-regulated growth [126].

Even in the absence of BR, GAs might also regulate BZR1-dependent gene expression, at least in part, since GA treatment slightly increases the dephosphorylation state of BZR1, its active form, likely through phosphatase PP2A proteins [124]. This action might explain the increased BZR1–DNA binding in vivo and GA-induced the modulation of BR transcriptional outputs [122]. Interestingly, this slight rise in the dephosphorylated BZR1 concentration was abolished in the presence of the protein phosphatase inhibitor okadaic acid (OA), and, in the same manner, in paclobutrazol-treated plants, which also showed a reduced level of two PP2AB' subunits (PP2AB'α and PP2AB'β) [124]. In future studies, it will be exciting to elucidate how GA and DELLA act on PP2A regulation to promote the phosphorylation state

of BZR1. The fact that DELLA proteins interact exclusively with the dephosphorylated BZR1 indicates that BR signaling enhances GA signaling by promoting the BZR1–DELLA interaction and, therefore, the alleviation of DELLA's restraint imposed on GA-mediated growth [124]. This BZR1 titration might explain why, surprisingly, BR was shown to strongly increase the abundance of the DELLA protein at the early elongation stages postgermination in *Arabidopsis* [125]. However, on the other hand, another group showed that neither BR treatment, nor BR biosynthesis or signaling mutants affected the accumulation of DELLA proteins in seedlings of 12-day-old *Arabidopsis* plants [124]. One explanation for these seemingly contradictory findings might be related to the developmental stage and tissue studied, evidencing the complexity of this hormonal interaction.

3.2.2. The Expanded and Integrated Model

Although this attractive signaling model could shed some light on the BR–GA interaction, recent detailed results on the potential interaction between BR and GA biosynthesis brought an informative readout at the level of hormonal biosynthesis, providing a novel expanded and integrated model of BR–GA cross-talk. Nonetheless, it is worth mentioning that a previous study had already demonstrated that BR promotes the expression of GA biosynthetic genes, and that DELLA can also modulate negative feedback in the BR biosynthetic genes by preventing the DNA-binding ability of BES1 and BZR1 proteins [125]. This overlooked biosynthetic cross-talk gained some attention following the recent demonstration by independent groups that the active GA contents (and various GA intermediates therein for *Arabidopsis*) were reduced in *Arabidopsis* (*ASK$_{\theta}$-oe*) and rice (*d11*, *GSK2oe*, and *dlt*) BR deficient mutants in comparison to those in WT plants. Similarly, an increase in the GA_1 level in BR-accumulating rice (*Do* and *m107*) lines was observed [126,127]. Strengthening these findings, and also in line with previous results, the expression levels of two genes (*GA20ox* and *GA3ox*) encoding key enzymes in the rate-limiting step of GA production were shown to be impaired in BR mutants, but were also strongly increased after BR treatment in *Arabidopsis* and rice plants, clearly indicating that BR influences GA biosynthesis in dicot and monocot plants. Such findings became more evident through the use of bioinformatics, ChIP, and in vitro DNA binding studies, which demonstrated that BZR1/BES1 can directly bind to the target promoters of *GA20ox*, *GA3ox*, and *GA2ox* from *Arabidopsis* and rice plants. These analyses revealed that BZR1/BES1 binding *cis*-elements are highly enriched on these promoters, including the BR-response element (BRRE, $CGTG^{T}/_{C}G$), G-box (CACGTG) and a type of E-box (CATGTG) in rice, and a non-E-box ($AA^{T}/_{A}CAAnnnC^{C}/_{T}T$) motif in *Arabidopsis* [126,127]. Importantly,

there was a higher enrichment of BES1 on these promoters followed by BR treatment, evidencing that the dephosphorylation of BZR1/BES1 increases GA production.

Extending the analysis to the effects of *GA20ox* expression on BR mutant phenotypes, complementation of the *bri1-301* mutant with *GA20ox1* under the control of the *BRI1* promoter restored various growth defects of the BR-deficient seedlings, demonstrating that some defects are related to GA deficiency [126]. Additionally, in contrast with the previous observations that BR- deficient and -insensitive mutants conferred insensitivity to GA, two independent groups demonstrated that externally applied GA could restore growth defects of *Arabidopsis* and rice BR mutants [126,127]. However, the developmental stage, environmental context, tissue specificities, hormone concentration, and species must be considered during the study of this positive loop between GA and BR.

At this stage, the proposed model postulates that BR activates BZR1/BES1 post-translationally to induce GA biosynthesis, and the increased GA induces DELLA degradation to further release BZR1/BES1 activity (Figure 4). Although this expanded model has incited a debate around the relative importance of the biosynthesis and signaling pathways [128–130], it is essential to highlight the applicability of this model to different contexts, as described above. Nevertheless, recent mathematical modeling and analysis of BR–GA cross-talk revealed that the signaling model (BZR1/BES1–DELLA interaction) exerts a stronger influence on the dynamics of the BR and GA signaling pathways than the BZR1/BES1-mediated biosynthesis of GA. Besides, the stability of this feed-forward model is mainly dependent on the mechanisms involved in the phosphorylation state of BZR1/BES1 proteins and the cellular localization of these processes [131].

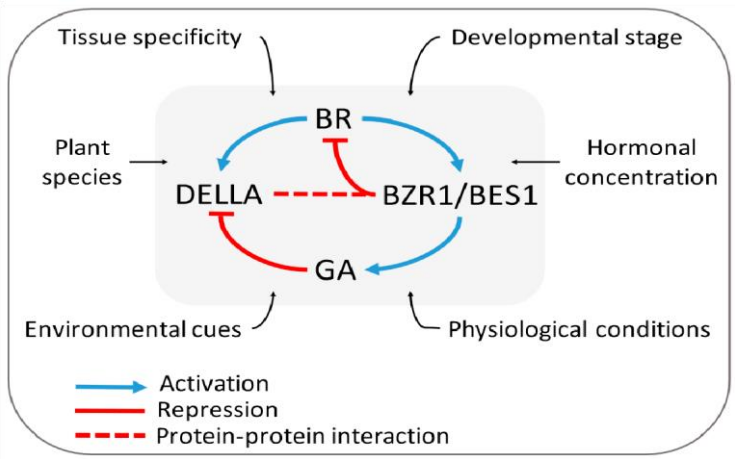

Figure 4. The integrated model for BR–Gibberellin (GA) cross-talk. BR activates BRASSINAZOLE RESISTANT 1/BRI1-EMS SUPPRESSOR 1 (BZR1/BES1) to promote GA biosynthesis and production. As a consequence, GAs degrade DELLA proteins, releasing their repressive action on BZR1/BES1 activity. Some critical factors, emphasized above, may influence and alter this interaction over time and should be considered when discussing BR–GA coordination in plants (e.g.; BR-induced accumulation of DELLA at dawn in the early stages of *Arabidopsis* development).

3.2.3. Is the BR–GA Antagonism an Alternate Strategy to Tackle Biotic and Abiotic Stresses?

As pointed out above (Figure 4), the high degree of complexity in BR–GA cross-talk can be attributed to several factors that profoundly influence BR and GA homeostasis. One interesting example is the fact that plant pathogens may exploit endogenous hormones or produce deceptive BR or GA (or mimics thereof) signals for their own advantages in order to manipulate and subdue their host's immunity. In rice, the root pathogen *Pythium graminicola* uses endogenous BR as a virulence factor and manipulates the host BR signaling to alleviate effective GA-mediated defenses. In this case, BR suppresses GA biosynthesis and induced GA repressor genes (*GA2ox3*) that indirectly stabilize the rice DELLA protein, SLENDER RICE 1 (SLR1) [132]. Thus, as a virulence strategy, BR promotes susceptibility to this particular pathogen, disarming the plant's defense signaling circuitry, which is in contrast to the protective effects of BRs that have been unveiled so far against myriad fungal, viral, and bacterial pathogens.

Intriguingly, the same BR–GA antagonism mechanism was reported in the submergence response in rice [133]. The tolerant *M202-Sub1* line adopts a quiescent strategy that limits shoot elongation during transient flooding, conserving energy until floodwaters retreat. The increased BR level in these plants during submergence induces a GA catabolic gene (*GA2ox7*) and the DELLA protein SLR1, restricting growth through the repression of GA signaling. In keeping with this data, BR pretreatment of the intolerant *M202* line before inundation was shown to restrict shoot elongation, conferring submergence tolerance [133].

In contrast to the antagonistic control of BR on GA metabolism, the positive effect of BR on DELLA protein stability may offer a mechanistic explanation for the abiotic stress tolerance conferred by BR. The positive correlation between DELLA protein levels and tolerance to abiotic stresses has been attributed to elevated expression of reactive oxygen species (ROS)-scavenging enzymes [134].

However, the dynamics and stability of DELLA and BZR1 protein complexes in response to pathogen and abiotic stresses remain elusive.

In summary, the intricate interconnection of BR with GA illustrates the functional versatility of these hormones whereby the integration of their outputs and signals of adverse conditions stimulates a balance between plant defense and growth responses. Nonetheless, the understanding of how the BR–GA interplay acts in biotic and abiotic stresses is still far behind that of the classic defensive hormones JA, ET, and SA.

3.3. Brassinosteroids and Cytokinins

Cytokinins are a group of phytohormones that play important roles in several biological processes, such as the development of aerial and subterranean organs, light responses, mineral enrichment, and responses to abiotic stresses [135–137]. The key enzymes involved in CK metabolism are isopentenyltransferases (IPTs), which are responsible for the biosynthesis of bioactive CKs, and CK oxidases/dehydrogenases (CKXs), which are responsible for the inactivation of bioactive CKs [135], both targets of BR-mediated responses.

The main interplay between CKs and BRs seems to be related to plant growth regulation [138]. The *CKX3* gene from *Arabidopsis* directs the breakdown of CKs, and when overexpressed under the control of a root-specific promoter *PYK10*, lower CKs levels in roots were observed, causing a reduction of root growth and also a weak reduction of leaf growth in *Arabidopsis* [136]. On the other hand, plants ectopically expressing both *CKX3* and *BRI1* present a synergistic increase in leaf and root growth. In agreement, *PYK10::CXK3* transgenic plants treated with exogenous BR showed an accentuated growth of lateral roots compared to WT plants, strongly suggesting a cross-talk between BRs and CKs that controls growth and developmental processes [138].

Moreover, the interplay between BR and CK can be observed in CK-induced anthocyanin production [139]. *Arabidopsis* mutant seedlings defective in BR biosynthesis (*dwf4*, *dwf4-102*, and *psc1*) and BR signaling (*bri1-4*), were submitted to different trials to evaluate the effects of BR on CK-induced anthocyanin accumulation. The *dwf4* and *bri1-4* plants presented reduced CK-induced accumulation of anthocyanin, but when WT plants were treated with exogenous BR, an increase in anthocyanin levels was observed. Similarly, CK-induced expression of anthocyanin biosynthetic genes, such as *dihydroflavonol reductase*, *leucoanthocyanidin dioxygenase*, and *UDP-glucose:flavonoid-3-O-glucosyl*

transferase, presented an accentuated reduction in the *dwf4-102* and *bri1-4* lines compared to WT. In addition, WT plants treated with CK presented higher expression of transcription factors related to anthocyanin production, including anthocyanin *pigment 1* (*PAP1*), *glabra 3* (*GL3*), and *enhancer of glabra 3* (*EGL3*), but the same was not observed in the *bri1-4* and *dwf4-102* lines. These data provide evidence that BR may boost CK-induced anthocyanin biosynthesis by positively mediating the expression of biosynthesis and signaling genes as well as transcription factors involved in both cases [139].

As with various phytohormones, later evidence suggested that CKs play important roles in several abiotic stress responses [140–142]. Studies of the gain- and loss-of-function of selected genes suggested that CKs negatively regulate several stress responses. Constitutive overexpression of *CKX* genes was implicated in CK deficiency and an increase in drought and salt tolerance, while the loss-of-function of *IPT* genes also led to increased stress tolerance due to decreasing bioactive CK levels [137]. Parallel experiments showed that the negative relation between the CK content and stress tolerance might be associated with a mutual interplay between CKs and ABA [143]. The treatment of *CKX* overexpressing lines and *IPT* silencing lines with exogenous ABA similarly resulted in the decrease of biologically active CK contents. Nevertheless, CK-deficient mutants were shown to be more sensitive to ABA compared to WT plants, leading to a higher induction of ABA-signaling marker genes under stress conditions (e.g.; *AIL1*, *COR47*, *RAB18*, *RD29B*, and *SAG29*) and subsequently, enhancing stress tolerance. These data suggest that the elevated stress tolerance in CK-deficient plants compared to WT plants may be related to the ability of these mutant plants to react more quickly to ABA and stressful conditions by further repression of the CK signaling pathway.

Besides the interplay of ABA and CK in stress tolerance regulation, other studies in rice (*Oryza sativa*) showed that BR might be associated with CK-mediated responses to drought stress in a different way. Rice transgenic lines expressing the *IPT* gene driven by a stress- and maturation-induced promoter (P_{SARK}) presented an increase in CK content before the beginning of senescence as well as the upregulation of several genes involved in the activation of BR signaling (*BRL3*, *BRI1*, *BH1*, *BIM1*, and *SERK1*) and biosynthesis (*DWF5* and *HYD1*), in water-stressed and well-watered plants. Under stress conditions, this resulted in a delay in stress symptoms such as leaf rolling, senescence, and decreased photosynthesis activity, which contributed to an increased grain yield [144].

It is well documented that CKs have an important role in the source/sink relationship [145]. During the vegetative and premature reproductive stages of cereal plants, the assimilated carbon is temporarily stocked in the stem and leaf sheaths in carbohydrate form. In the later stages of plant development, these stored compounds are subsequently remobilized to reproductive sink tissue as flowers and grain filling [146]. However, the maintenance of source/sink homeostasis is a major challenge during stress conditions, causing yield losses. In *P_{SAPK}::IPT* lines, the increase of CK content enabled the maintenance of source strength during drought stress, keeping higher yields compared to WT plants. It is also known that the application of BR is a powerful biotechnological tool to enhance crop yield [147–152]. According to the presented scenario, the changes in hormonal profile, including the upregulation of BR-related genes, can modify the source/sink relationship, providing a strong sink capacity to *P_{SAPK}::IPT* line plants during water stress. Together, these data suggest that BR–CK cross-talk may contribute to the modification of source/sink relations, improving crop yield and stress responses.

It has been observed that BR and ABA present antagonistic actions [153]. BR-mediated signaling is regulated by ABA through the upregulation of *BIN2* and downregulation of genes from the PP2C family, causing decreased activity of the BR signaling pathway [153]. The relative expression of three members of the *PP2C* family (*PP2C7*, *PP2C6*, and *PP2C53*) was increased in WT plants under water stress. However, the expression of *BIN2* was upregulated in plants of *P_{SAPK}::IPT* lines [153]. ABA is responsible for inhibiting BR effects during stress conditions. Therefore, the observed hormonal profile in the mentioned study and its consequences may be due to the interplay not only between CK and BR, but also between the three hormones—CK, ABA, and BR—in a complex manner that remains unclear [144]. The role of ABA in abiotic stress and its cross-talk with BR are discussed in more detail in Section 3.5. A suggested interplay between BR, CK, and ABA is represented in Figure 5.

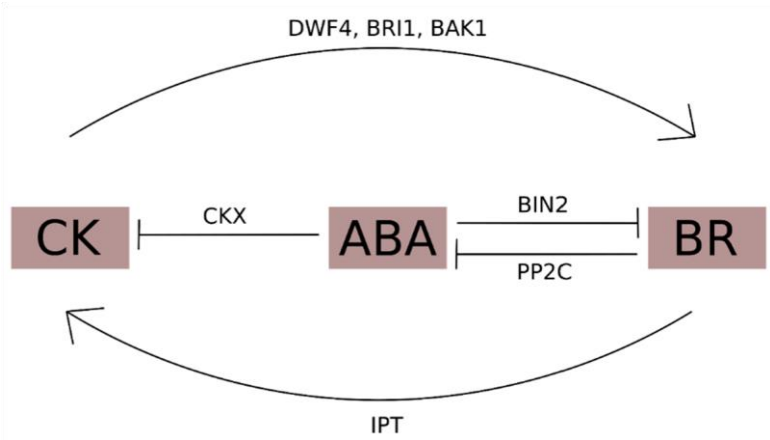

Figure 5. A putative interplay between brassinosteroid (BR), cytokinin (CK), and abscisic acid (ABA). ABA is responsible for inhibiting BR effects during stress conditions by upregulating *BIN2* (BRASSINOSTEROID-INSENSITIVE 2), a major negative regulator of BR signaling, whereas BR is responsible for inhibiting ABA effects during growth processes through *PP2C* (PROTEIN PHOSPHATASE 2C), a major negative regulator of Snark proteins (positive regulators of ABA signaling). ABA is also responsible for inhibiting CK signaling by upregulating *CKX* (CK oxidases/dehydrogenases), which play a major role in inactivating bioactive CKs. Despite ABA's role, BR and CK present positive interactions. While CK upregulates BR biosynthetic (*DFW4*) and signaling (*BRI1*, *BAK1*) genes, BR upregulates IPT (isopentenyltransferases), which are major enzymes responsible for the biosynthesis of bioactive CKs.

3.4. Brassinosteroids and Ethylene

Ethylene is a gaseous phytohormone with a simple structure. Because volatile substances move rapidly, they can act as regulators and coordinators of several growth and development processes, both in the tissue and in the whole organism, as well as facilitating plant-to-plant communication. Although the main function attributed to ethylene is fruit ripening promotion, other physiological processes, such as seed germination, senescence, and responses to abiotic and biotic stress factors, are also regulated by this hormone [154]. Ethylene biosynthesis requires the participation of five major components: the amino acid methionine which is converted into S-adenosyl methionine (SAM^2) and subsequently modified by the ACC-synthase enzyme (ACS) to form 1-aminocyclopropane-1-carboxylic acid (ACC), the direct precursor of ethylene. In turn, ACC is converted by the enzyme ACC-oxidase (ACO) into ethylene, a stable compound that can be transported throughout the plant [155].

Brassinosteroids influence ethylene biosynthesis mainly by regulating *ACS* and *ACO* activities [156]. The cross-talk between these two phytohormones presents two scenarios, with BR regulating ethylene production at the transcriptional and post-transcriptional levels. Regarding protein regulation, previous studies in *Arabidopsis* indicated that seedlings treated with exogenous BR show elevated levels of ethylene biosynthesis, at least partly through an increase in ACS5 protein stability by elevating its half-life [156]. Additionally, other studies have already found that BR may also regulate ethylene biosynthesis through the induction of *ACS5* gene expression in *Arabidopsis* [157].

The regulation of ethylene biosynthesis by BR happens in a dose-dependent manner, where BRs can be positive as well as negative regulators, depending on the exogenous application dose

(Figure 6) [158]. High levels of BRs stimulate ethylene biosynthesis by enhancing the stability of the ACS protein by preventing its degradation by the 26S proteasome. On the other hand, low levels of BRs repress ethylene biosynthesis by increasing the activity of *BZR1/BES1*, the two major BR signaling pathway transcription factors that inhibit the transcription of *ACS* genes [158]. Experiments with banana fruit (*Musa acuminata* L.) showed that BZR proteins bind specifically to BRRE elements (CGTGT/CG) of at least one *ACS* gene (*MaACS1*) and two *ACO* genes (*MaACO13* and *MaACO14*) in this species. An expression analysis showed that the expression of *MaBZR1*, *MaBZR2*, and *MaBZR3* decreases continuously during fruit ripening. Moreover, *MaBZR1* and *MaBZR2* are capable of suppressing the transcription of these three ethylene biosynthetic genes, which is increased during the fruit ripening process. Additionally, the exogenous application of BR promotes banana fruit ripening due to the acceleration of *MaACS1*, *MaACO13*, and *MaACO14* expression, and consequently, ethylene production occurs, confirming the action of BZR proteins as transcriptional repressors of ethylene biosynthesis [159].

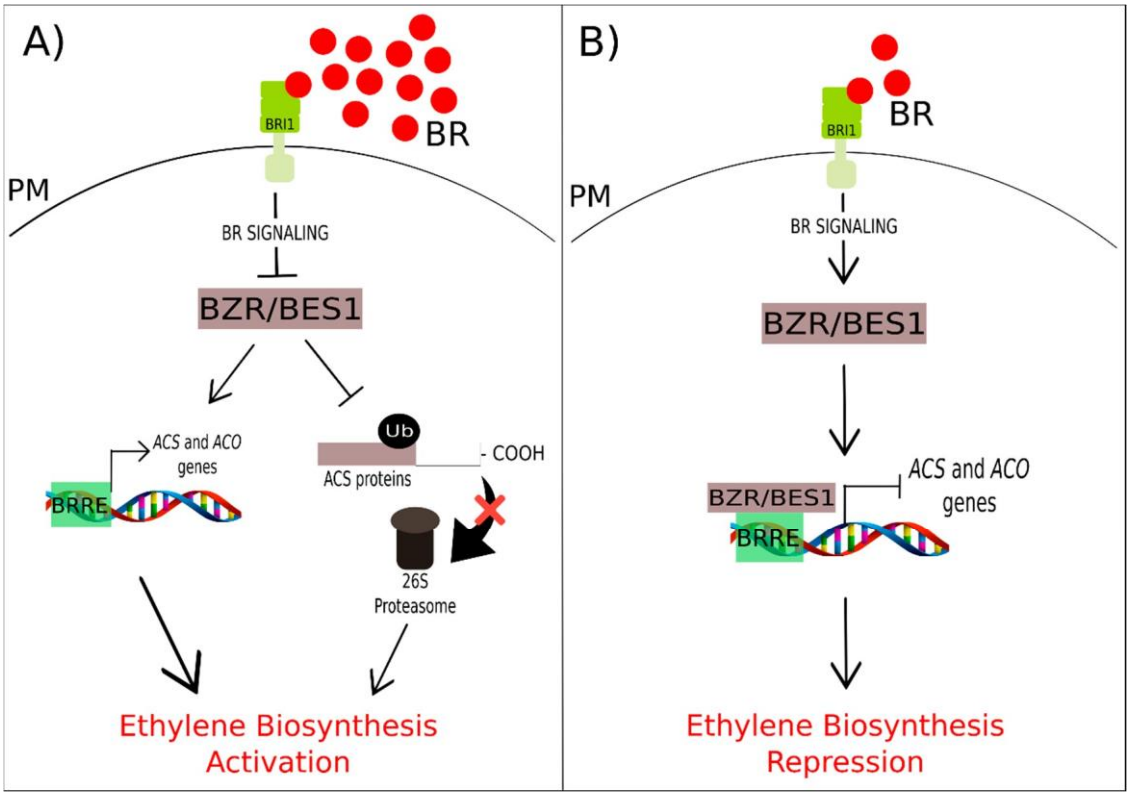

Figure 6. A general simplified model of BR and ethylene cross-talk. The perception of BR begins in its receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) which activates BR signaling, which, in turn, controls ethylene biosynthesis in a dose-dependent manner. (**A**) High levels of BR decrease the activity of BRASSINAZOLE RESISTANT 1/BRI1-EMS SUPPRESSOR 1 (BZR1/BES1), the major transcription factors of the BR signaling

pathway while enhancing the stability of the 1-aminocyclopropane-1-carboxylic acid (ACC)-synthase enzyme (ACS) proteins by preventing its degradation by the 26S proteasome and consequently, activating ethylene biosynthesis. (**B**) Low levels of BR increase the activity of BZR1/BES1, which, in turn, bind to the promoter of the *ACC-synthase* (*ACS*) and *ACC-oxidase* (*ACO*) genes, inhibiting their transcription and consequently, repressing ethylene biosynthesis. PM represents the plasma membrane.

The application of exogenous BR can also accelerate postharvest ripening, enhancing the development of quality attributes and consequently, promoting ethylene production in *Solanum lycopersicum* by increasing transcriptional levels of *ACS2* and *ACS4* genes [160]. Tomato fruits with enhanced BR levels or BR signaling due to overexpression of the BR biosynthetic gene *DWARF* and the signaling gene *BRI1* showed elevated ethylene production and quick-ripening, respectively [161,162]. On the other hand, tomato plants silenced for the *BRI1* gene and insensitive to BR presented no changes in ethylene accumulation, ACC content, and ACS and ACO activities during BR treatment, reinforcing that BRI1 downstream components may be involved in ethylene accumulation [163], as also suggested by Lv et al. 2018 [158].

Ethylene is also known to be involved in a range of stress responses, such as heat stress [164] and pathogen and pest attacks [165]. Studies using mutants that are deficient and insensitive to ET showed higher thermal and salt tolerance when 24-epibrassinolide (EBR) was applied. EBR was capable of increasing the survival rates of the ET-insensitive mutant *ein2* under heat stress in *Arabidopsis* plants. Moreover, the treatment of *Brassica napus* seeds with EBR reduced the inhibition *ein2* mutant germination under salt stress, reverting this line's hypersensitivity to salt to a level similar to those of WT plants [166].

Lettuce plants present high emission of ET and an increase in ACC content during salt stress [167]. However, the increase in ethylene production under salt stress leads to the inhibition of plant growth and induction of senescence and consequently, premature death [168]. The treatment of lettuce plants under salt stress with DI-31, a brassinosteroid analog, was shown to be capable of reducing the ACC content and consequently, ET production, avoiding premature death, alleviating weight loss, and showing a good protective effect of BR against salinity.

3.5. Brassinosteroids and Abscisic Acid

Abscisic acid (ABA) is a phytohormone that is involved in a wide range of plant responses and is essential for plant development and survival. The hormone acts as a major abiotic stress sensor, leading to protective responses such as stomatal closure, seed dormancy, and inhibition of growth and germination [169–173]. Even in the early stages of plant development, ABA drives stress tolerance and/or avoidance mechanisms, helping plants to survive in adverse conditions [174].

The serine-threonine kinases SnRK2.2/2.3/2.6 (SNF1-related protein kinases) play a central role in the ABA pathway response as positive regulators of ABA signaling [173,175–177]. The kinases regulate the expression of stress-responsive genes and transcription factors, leading to ABA-related responses. The kinases' activity is modulated by their interactions with PHOSPHATASE 2C (PP2C), which inactivates SnRK2s by dephosphorylation [178]. In the presence of the hormone, the complex formed by ABA and PYL/PYR/RCAR receptors inactivates the phosphatase by blocking the substrate's entry [179–183].

Despite the essential roles of PP2C and SnRK2s in activating ABA responses, their effects in plant cells are influenced by cross-talk with other phytohormones. For example, seed dormancy is affected by the interplay of abscisic acid with gibberellins and ethylene [184]. Also, stomatal movement is regulated under stress by jasmonic acid, cytokinins, ethylene, auxin, and also, brassinosteroids [185,186]. In general, under favorable conditions, the cross-talk between growth-related hormones and ABA results in the attenuation of ABA-related responses by diverse molecular mechanisms, allowing plant growth and development.

The antagonism between ABA and the growth-related hormone brassinosteroid has been known for several years. The negative cross-talk between these hormones has been observed during seed germination, early seedling development, root growth, and stomatal closure [153,187]. Moreover, mutants with defective BR signaling (i.e.; *bin2-1*, *bri1*, *constitutive photomorphogenesis and dwarfism* (*cpd*), and *de-etiolated-2 mutant* (*det2*)) have enhanced ABA sensibility during seed germination, early seedling development, and/or primary root formation [96,187–189]. Despite all these observations, the molecular mechanism behind the negative cross-talk remained poorly understood until recently.

Essentially, ABA and BR antagonism includes two types of regulation: post-translational modification at the protein level and transcriptional repression at the gene level. Regarding protein–

protein regulation, phosphorylation and dephosphorylation events play a key role in ABA–BR cross-talk. Similar to the ABA signaling pathway, the activity of kinases and phosphatases is crucial to brassinosteroid sensing and responses. The presence of brassinosteroid triggers the activation of the BRI1 kinase-like receptor, the kinases BAK1 and BRI1 and the phosphatase BSU1. This phosphatase is responsible for the dephosphorylation of the kinase BIN2, a major repressor of BR signaling [190].

A considerable body of evidence indicates that BIN2 is one of the key players in ABA–BR cross-talk. This kinase can interact and phosphorylate *Arabidopsis* SnRK2.2 and SnRK2.3 in vitro [191]. BIN2-mediated phosphorylation was shown to increase SnRK2.3 activity in vitro. While the in vivo overexpression of SnRK2.3 caused ABA hypersensitivity, plants overexpressing SnRK2.3^{T180A} presented sensibility to ABA at levels similar to WT plants. These data suggest a role of T180 phosphorylation in ABA signaling in vivo.

BIN2 activity also affects another ABA pathway element downstream of SnRK2s, the basic leucine-zipper (bZIP) transcription factor ABA Insensitive 5 (ABI5). In the presence of ABA, ABI5 regulates seed germination, and seedling growth, leading to seed dormancy and growth arrest responses [192–194]. Moreover, ABI5 activates *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) genes in vegetative tissues [194]. A recent study showed that ABI5 interacts with BIN2, which then phosphorylates ABI5 in vitro [195]. In vivo, seeds from the gain-of-function *bin2* mutant (*bin2-1*) presented higher expression of ABI5 target genes during ABA-treatment compared to the triple knockout mutant (*bin2-3 bil1 bil2)*. The effect of BIN2 on ABI5 phosphorylation and target regulons expression indicates that BIN2 might modulate ABA signaling during seed germination and early seed development.

Despite all evidence showing some ABA pathway key elements are targets of BIN2, a recent study suggests that ABI1 and ABI2 [166] might regulate kinase activity [196]. Overexpression of the PP2C family phosphatases ABI1 and ABI2 in *Arabidopsis* resulted in decreased expression of the gene markers of BR suppression: CPD and DWF4. Moreover, phosphatase overexpression led to the accumulation of BES1 in its dephosphorylated form. Similar results were previously observed in *abi1* and *abi2* mutants after ABA treatment [153]. The direct interactions between ABI1, ABI2, and BIN2 could be the mechanism behind these effects: the BR-repressor BIN2 is dephosphorylated by the phosphatases, leading to the accumulation of active BES1 [196]. This mechanism may also explain why only BIN2 extracted from ABA-treated seedlings can phosphorylate ABI5 in vitro [195].

Aside from BIN2, the kinase BAK1 also seems to be involved in ABA–BR cross-talk. A recent study showed that BAK1 can interact with and phosphorylate SnRK2.6 in vitro [197]. As the kinase SnRK2.6 is the primary regulator of stomatal closure [169–171], the lack of BAK1-mediated activation of SnRK2.6 could explain the increased water loss by transpiration observed in *bak1-3* mutants, even during ABA treatment [197].

In addition to protein interactions and post-translational modification, ABA–BR cross-talk also comprises mechanisms of regulation at the transcriptional level. Real-time quantitative reverse transcription-PCR (qRT-PCR) analysis revealed low expression of the ABA-related transcription factors—ABFs, *ABI3*, and *ABI5*—in the gain-of-function mutant *bes1-D* seedlings [198]. On the other hand, *bes1* knockout mutant (*bes1*^{ko}) seedlings displayed high expression of the same transcription factors. Additionally, this mutant presented an enhanced ABA response during root growth and seed germination compared to WT plants. The negative role of BES1 in the ABA signaling pathway relies on the interaction of BES1 with TOPLESS (TPL)/HISTONE DEACETYLASE 19 (HDAC19). Once bound to the *ABI3* promoter, BES1 represses *ABI3* expression through histone deacetylation by assembling the TPL–HDAC19 complex. As BES1 cannot interact with the *ABI5* promoter, the decreased expression of this ABA transcription factor observed in *bes1-D* is a consequence of the repression of the upstream element *ABI3* [192,198].

The direct inhibition of *ABI5* expression seems to be controlled by the BZR1 transcription factor. The BR-induced transcription factor binds to G-box sequences present in the *ABI5* promoter, reducing their expression [199]. The regulation of *ABI5* by BZR1 could be the cause of the *ABI5* downregulation in the gain-of-function *brz1-1D* mutants after ABA treatment. Therefore, the ABA insensibility of *bzr1-1D* mutants in root growth assays might be a consequence of ABI5 repression by BZR1, and this could be suppressed by ABI5 overexpression.

Recent findings suggest that ABA–BR cross-talk involves multiple players acting on two fronts: modulation of protein activity and regulation of gene expression. In summary, under optimal conditions, brassinosteroids trigger BR cascade activation and antagonize ABA responses by decreasing *ABI3* and *ABI5* expression during seed germination and seed growth (Figure 7A) [198,199]. The absence of ABA triggers BR responses by repressing BIN2 through PP2C ABI1 and ABI2 phosphatases [196]. However, during BR signaling repression, BIN2 stimulates and enhances ABA responses through direct

phosphorylation of SnRK2.3 and ABI5, leading to ABA-modulated seed dormancy and seedling growth arrest responses [191,195]. Under the same conditions, BAK1 increases stomata responses by SnRK2.6 phosphorylation (Figure 7B) [197].

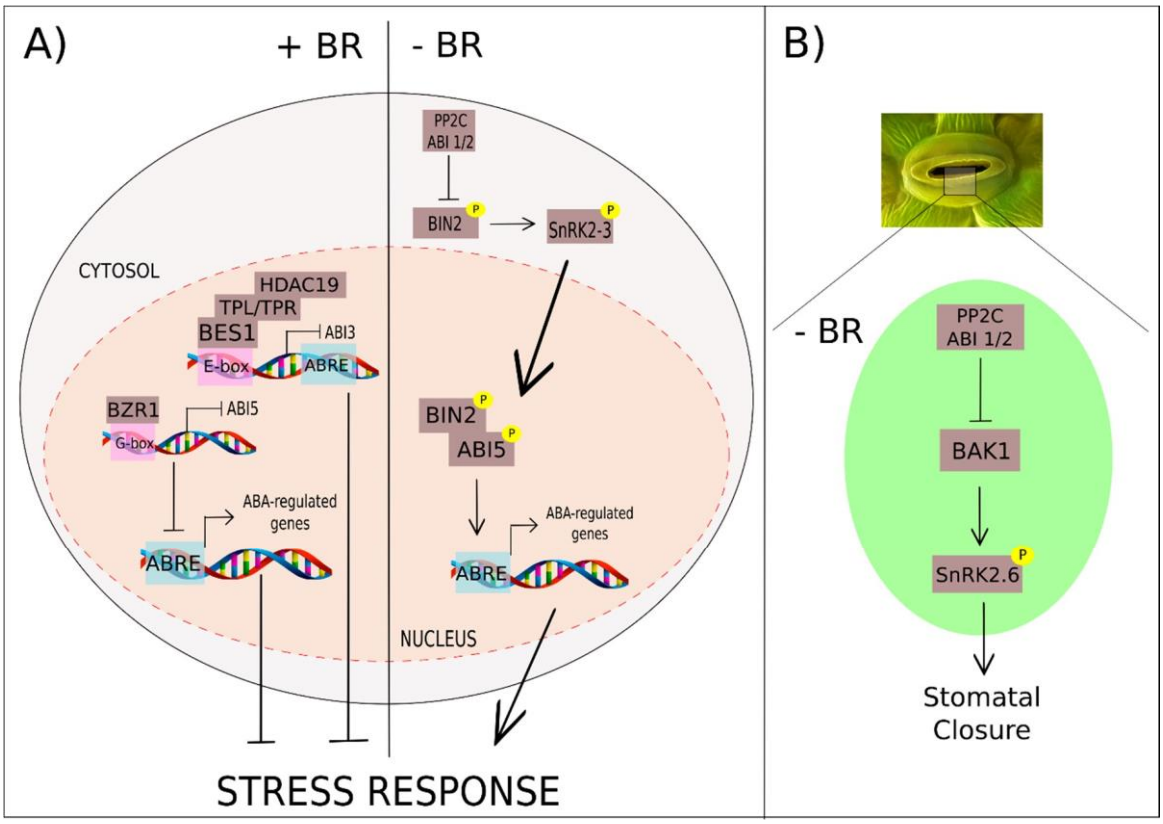

Figure 7. BR and ABA cross-talk relies on protein activity modulation and gene expression regulation. (**A**) In the presence of BR, the complex formed by BRI1-EMS SUPPRESSOR 1 (BES1), TOPLESS/(TPL/TPR) and HISTONE DEACETYLASE 19 (HDAC19) inhibits *ABA Insensitive 3* (*ABI3*) expression by interacting with E-box promoter sequences. The transcription factor BRASSINAZOLE RESISTANT 1 (BZR1) interacts with the G-box sequences of the *ABI5* promoter, leading to gene repression. Repression of the *ABI3* and *ABI5* genes results in lower expression of ABA-regulated genes and decreased stress responses. At low levels of BR, stress responses are stimulated by SnRK2.3 activation by BRASSINOSTEROID-INSENSITIVE 2 (BIN2). Additionally, the BR-repressor BIN2 phosphorylates the transcription factor *ABI5*, resulting in the expression of ABA-related genes. (**B**) In guard cells, BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) phosphorylates the kinase SnRK2.6 at low levels of BR, driving stomatal closure responses. At low levels of ABA, the PHOSPHATASE 2C (PP2C) phosphatase ABI1 and ABI2 repress SnRK2.6 phosphorylation by BAK1 and also the phosphorylation of SnRK2.3 by BIN2, decreasing stress responses related to ABA–BR cross-talk.

Despite the substantial evidence supporting the molecular mechanism behind ABA–BR cross-talk, key points remain to be clarified. The previously reported lower auto-activation of SnRK2.2 and SnRK2.3 compared to SnRK2.6 suggests the need for activation by an upstream kinase [200]. However, ABA-related SnRK2s have been under investigation for several years, and different studies have shown that kinase auto-activation is sufficient for kinase activity and activation of downstream ABA-related targets [175,200–202]. In this sense, further studies are required to elucidate the importance of the brassinosteroid kinases BIN2 and BAK1 in SnRK2s activation and their roles in the ABA response in vivo. Additionally, the understanding of the interplay between brassinosteroid and ABA network elements in particular tissues and plant developmental stages, considering protein spatial distribution and expression, represents a challenge for future studies [192].

3.6. Brassinosteroids and Jasmonic and Salicylic Acids

Plants present a range of defense mechanisms whose costs represent a tradeoff between growth and immunity [203–206], in which phytohormones fulfill central roles in protection against biotic stressors agents. Studies have already proven that BR can induce disease resistance in tobacco (*Nicotiana tabacum*) and rice (*Oryza sativa*) [207] in a complex network which involves crucial functions of the receptor BRI1 and its coreceptor BAK1 [203,208–210].

Flagellin 22 (flg22) and chitin are both pathogen- and microbe-associated molecules patterns, also named PAMPs and MAMPs, respectively, which are recognized by the cells of innate immune system as alert signals of invaders. Flg22 binds to its receptor flg-sensing 2 (FLS2), which initiates signals to prevent pathogen proliferation [209–211]. Curiously, the binding of flg22 to FLS2 generates an association and transphosphorylation with BAK1 as happens in BR-induced BRI1 signaling, activating FLS2. The activated FLS2 then phosphorylates BIK1 (BOTRYTIS-INDUCED KINASE 1), a receptor-like cytoplasmic kinase responsible for associating with a flagellin receptor complex, triggering plant innate immunity and transducing the target response [209,212,213]. The association of BAK1 as a coreceptor of both BR-induced BRI1 signaling and flg22-induced FLS2 signaling suggests a possible tradeoff between BR and FLS2 signaling responses mediated by BAKI1.

However, another study suggested the potential existence of BAK-independent immune signaling [214]. *Arabidopsis* plants treated with both exogenous BR and flg22 showed decrease of flg22-induced MAMP-triggered immunity responses (MTI) by BR. However, on the other hand, flg22 did not

affect the BR-induced responses. Additionally, when BR and flg22 were applied separately, they induced distinct gene profiles and biological responses (i.e.; the treatment with flg22 induced the stress markers ROS and MAPKs (mitogen activated protein kinases), which were not observed in plants treated only with BR). These data suggest the inhibition of FLS2-mediated immune signaling by BR, independently of a complex formation with its coreceptor BAK1 and associated downstream phosphorylation when different pools of BAK1 exist and are not interchangeable: the BAK1 recruited by FL2S complex is different from BAK1 recruited by BRI1 signaling [214]. Another independent study corroborated these ideas by providing evidence that the association between BRs and MTI responses depends on the endogenous levels of BR and BRI [215]. A possible mechanism to explain the relation of BR to plant innate immunity is represented in Figure 8.

The importance of jasmonic acid (JA) and salicylic acid (SA) for the plant innate immune system is well characterized [216,217]. These hormones generate and transmit distinct defense signals which are capable of influencing each other through a complex network of synergistic and antagonistic interactions [218,219], allowing the plant to efficiently create a quick and precise defense reaction to causal agents of many types of biotic stress. Previous studies have already shown a mutually antagonistic activity of JA and SA in plant innate immunity [220–222]. Exogenous application of JA can dramatically decrease the SA content in rice, which suggests that JA can suppress the SA pathway [223]. However, recent studies revealed a diverse and complex interplay between BR, JA, and SA.

A negative role of BR in the defense against brown planthopper (BPH, *Nilaparvata lugens*) was observed in rice (*Oryza sativa*). BPH infestation suppressed the BR pathway, decreasing the expression of signaling genes (*BRI1* and *BZR1*) and the BR concentration, while successively activating SA and JA pathways. Moreover, the application of exogenous BR downregulated the expression of genes related to the SA pathway, such as the biosynthetic genes *ICS1* and *PAL*, and reduced SA content, while it upregulated genes related to the JA pathway, like *MYC2*, *AOS2*, and *LOX1*, and increased the JA content during BPH infestation in WT plants [224]. However, this work also observed that BR-mediated suppression of the SA pathway might be associated with the JA pathway. To further corroborate this fact, JA-deficient mutant *og1* and JA-insensitive mutant *col1-18* were submitted to BR exogenous application. The transcription levels of *ICS1* and *PAL*, two SA biosynthetic genes, were not suppressed and SA levels did not decrease as observed in WT plants upon BPH infestation. A similar response was observed in *coi1-18* mutants, but in this case, the transcription levels of both *ICS1* and

PAL as well as the SA concentration increased. These results collectively suggest that JA might participate in the BR-mediated suppression of the SA pathway, reinforcing this antagonistic response.

Curiously, although BR has been suggested as a negative regulator of innate immunity in plants [132,225,226], it has also been found to positively regulate the defense against the chewing herbivore *Manduca sexta* and the cell-content feeder *Thrips tabaci* [227,228]. These divergent scenarios may be associated with the type of plant tissue affected (root and shoot) and the biotic stressor agent (microbial, virus, insect, necrotrophic, or biotrophic agents); thus, it is very difficult to define a general model of the role of BRs in plant innate immunity and consequently, for BR and JA/SA cross-talk. Furthermore, each plant species, even single plants in the same species, are singular organisms which present different growth–defense trade-offs as a result of resource restriction and these trade-offs are regulated by phytohormone cross-talk in different ways [229].

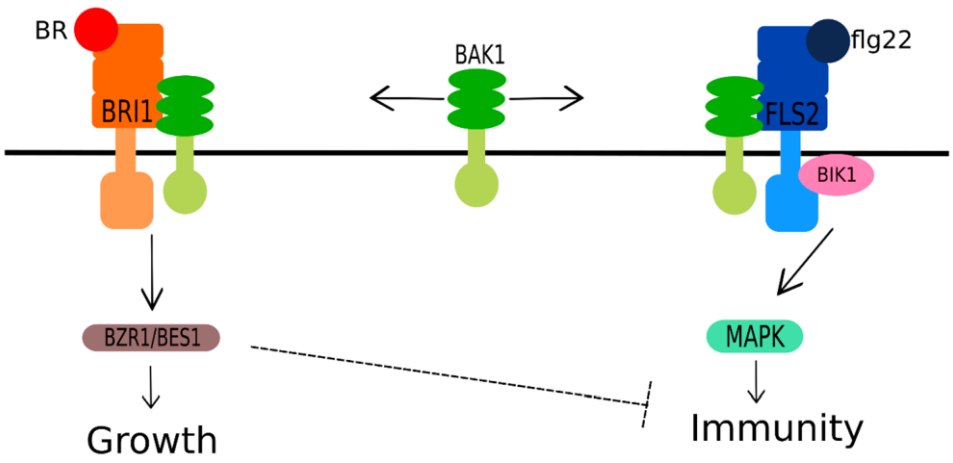

Figure 8. A suggested model of brassinosteroid (BR) regulation of immunity at multiple levels. BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1) is considered to mediate the growth and immunity tradeoff because it serves as a coreceptor for both BR-mediated responses via BRI1 and innate immunity mediated responses via flg-sensing 2 (FLS2). The scheme suggests an inhibition of FLS2-mediated immune signaling by BR, independent of complex formation with coreceptor BAK1, when the inhibition occurs downstream of BAK1. BRI1 (BRASSINOSTEROID-INSENSITIVE 1) represents a BR receptor; flg22 (flagellin 22) is a type of pathogen- and microbe-associated molecule pattern (MAMP/PAMP); FLS2 (flg-sensing2) is a flg22 receptor; BIK1 (BOTRYTIS-INDUCED KINASE 1) is a coreceptor of FLS2; BZR1/BES1 (BRASSINAZOLE RESISTANT 1/BRI1-EMS SUPPRESSOR 1, respectively) are the major transcriptional factors of the BR signaling pathway; MAPKs (mitogen activated protein kinases) are a class of marker proteins which indicate various stress conditions.

3.7. Brassinosteroids and Strigolactones

Strigolactones (SLs) are a recently discovered group of terpenoid phytohormones that are related to the control of shoot branching [230]. One of the most important signaling components discovered in *Arabidopsis* is *MAX2* (More Axillary Growth Locus 2), which functions to inhibit plant shoot branching [231]. MAX2 constantly interacts with BZR1/BES1 through the PEST domain to mediate their degradation in *Arabidopsis*. The exogenous application of SL induced the degradation of both BR transcription factors mediated by MAX2, and consequently, inhibited shoot branching. Thereby, the interaction between SL and BR may control developmental processes by modulating the MAX2-mediated stability of BZR1 and BES1 [231]. Until now, there has been little data on the SL signaling pathway. It is expected that advances in the research of this new class of phytohormone will more clearly explain the hormonal cross-talk between SL and BR.

4. Conclusions and Remarks

As sessile living beings, plants have developed complex mechanisms during their evolution, with phytohormones playing key regulatory roles. The interplay of phytohormones may be used in management and genetic engineering to improve several agricultural traits. In the almost 40 years since the discovery of brassinosteroids as the sixth class of plant hormones, continuous effort has been made to elucidate their role in the multiple aspects of plant physiology. It is known that BRs influence several biological processes, such as growth, protein metabolism, cellular transport and signaling, cell wall biosynthesis, the formation of chromatin and cytoskeleton components, stomatal closure, and environmental responses. Due to the complex network between BRs and other phytohormones and the different physiological effects that this implicates in plant homeostasis, achieving a better understanding of hormonal cross-talk as well as the extensive cross-talk between BRs and other hormones about its role in plant growth and development and responses to stress remains a challenge. This review summarized the previous knowledge about the role of BR cross-talk in plant physiology and compiled the recent findings on these interactions.

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Abbreviations

ABA	Abscisic Acid
ABI	ABA Insensitive
ACS	ACC-SYNTHASE
ACC	1-aminocyclopropane-1-carboxylicacid
ACO	ACC-OXIDASE ABA Insensitive
AOS2	ALLENE OXIDE SYNTHASE 1
ARF	AUXIN RESPONSE FACTOR
AUX	Auxin
AUX/IAA	AUXIN/INDOLE ACETIC ACID
AuxREs	Auxin-responsive DNA Elements
BAK1	BRI1-ASSOCIATED RECEPTOR KINASE 1
BES1/BZR2	BRI1-EMS SUPPRESSOR 1
BIK1	BOTRYTIS-INDUCED KINASE 1
BIN2	BRASSINOSTEROID-INSENSITIVE 2
BL	Brassinolide
BPH	Brown Planthopper
BRI1	BRASSINOSTEROID INSENSITIVE 1
BRs	Brassinosteroids
BRX	BREVIS RADIX
BRZ	Brassinazole
BSKs	BR-SIGNALING KINASE 1
BSU1	BRI1 SUPPRESSOR 1
bZIP	Basic Leucine-Zipper
BZR1	BRASSINAZOLE RESISTANT 1
CDG1	CONSTITUTIVE DIFFERENTIAL GROWTH 1
ChiP	Chromatin Immunoprecipitation
ChiP-seq	Chromatin Immunoprecipitation-sequencing
CKs	Cytokinins
CKXs	Cytokinin oxidases/dehydrogenases
CPD	CONSTITUTIVE PHOTOMORPHISM AND DWARFISM
DWF4	DWARF 4

EGL3	Enhancer of Glabra 3
ET	Ethylene
Fgl22	Flagellin 22
FLS2	Flg-sensing 2
GAs	Gibberellins
GID1	GIBBERELLIN INSENSITIVE DWARF1
GL3	Glabra 3
HDAC19	HISTONE DEACETYLASE 19
HYD1	HYDRA 1
IAA	Indole Acetic Acid
ICS1	ISOCHORISMATE SYNTHASE 1
IPT	Isopentenyltransferases
JA	Jasmonic Acid
KIB1	KINK SUPPRESSED IN BZR1-1D
LOX1	LIPOXYGENASE 1
LRR	Leucine Rich Repeat
MAMPs	Microbial Associated Molecules Patters
MAPKs	Mitogen Activated Protein Kinases
MAX2	More Axillary Growth Locus 2
MTI	MAMP-triggered Immunity
NPF	NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER FAMILY
OA	Okadaic Acid
PAL	PHENYLALANINE AMMONIUM LYASE
PAMPs	Pathogen Associated Molecules Patters
PAP1	Anthocyanin Pigment 1
PP2A	PHOSPHATASE 2A
PP2AB'	PP2AB'α and PP2AB'β subunits
PP2C	PHOSPHATASE 2C
QC	Quiescence Center
qRT-PCR	Real-time Quantitative Reverse Transcription-PCR
RLK	Receptor-like kinase
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAM	Shoot Apical Meristem
SAM^2	S-adenosyl methionine
SBI1	SUPPRESSOR OF BRI1
SLs	Strigolactones
SLR1	SLENDER RICE 1

TIR1/AFB TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX

TPL TOPLESS

WT Wilt-type

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3. CHAPTER 2

Identification of brassinosteroids from sugarcane

3.1 Introduction

Since the discovery of brassinolide (BL) in 1979, several BRs have been detected, as mentioned in chapter 1. However, only a limited fraction was described considering the biological activity in plant. The majority of studies involving the role of BR in growth processes in plants are related to the application of exogenous analogues. Still, there is a lack of works involving the modulation of BR biosynthetic genes. The figure 1 shows the multisteps reactions of BR biosynthesis as well as their steroids precursors (Bajguz *et al*., 2020).

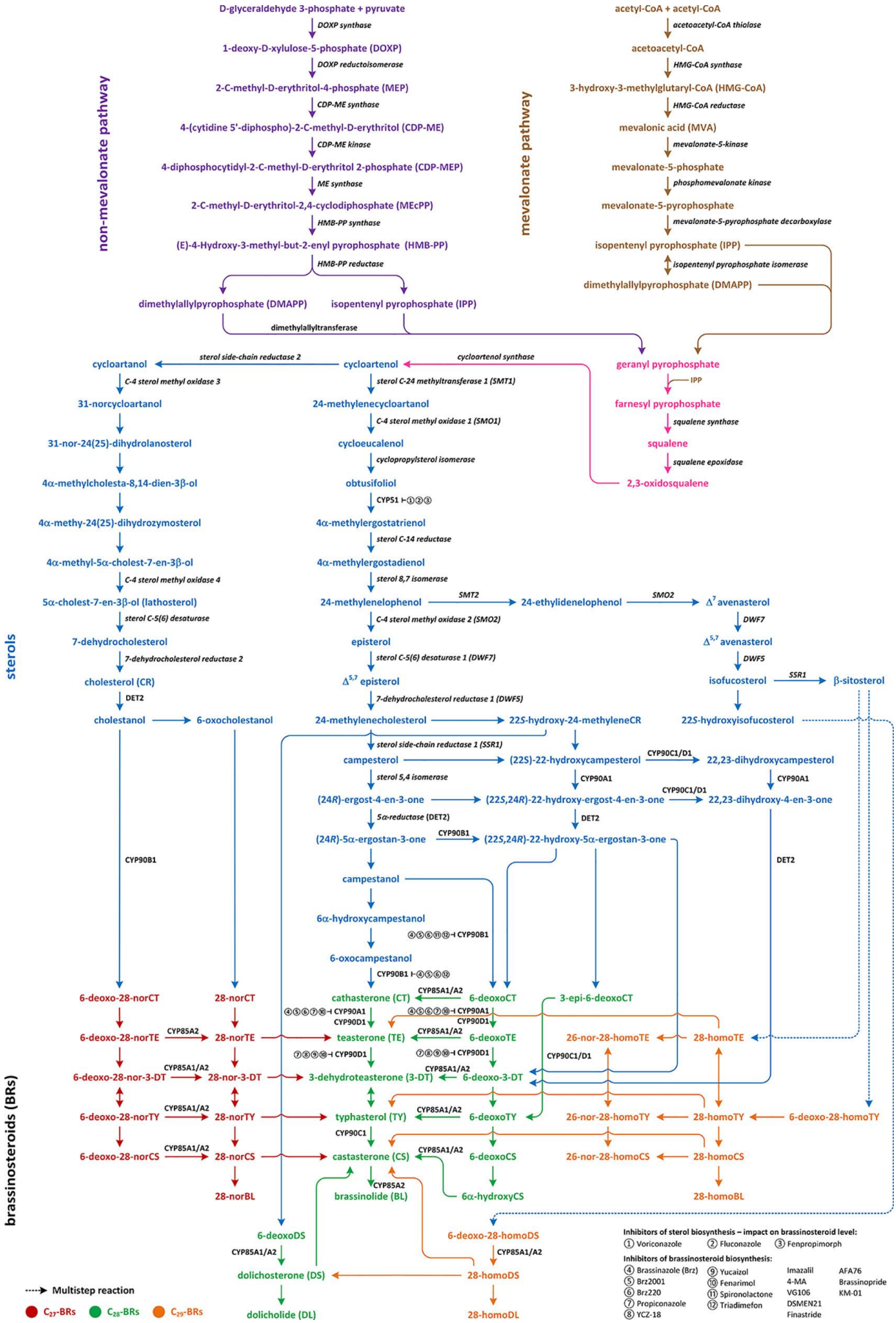
non-mevalonate pathway
D-glyceraldehyde 3-phosphate + pyruvate
DOXP synthase
1-deoxy-D-xylulose-5-phosphate (DOXP)
DOXP reductoisomerase
2-C-methyl-D-erythritol-4-phosphate (MEP)
CDP-ME synthase
4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME)
CDP-ME kinase
4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-MEP)
ME synthase
2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP)
HMB-PP synthase
(E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP)
HMB-PP reductase
mevalonate pathway
acetyl-CoA + acetyl-CoA
acetoacetyl-CoA thiolase
acetoacetyl-CoA
HMG-CoA synthase
3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)
HMG-CoA reductase
mevalonic acid (MVA)
mevalonate-5-kinase
mevalonate-5-phosphate
phosphomevalonate kinase
mevalonate-5-pyrophosphate
mevalonate-5-pyrophosphate decarboxylase
isopentenyl pyrophosphate (IPP)
isopentenyl pyrophosphate isomerase
dimethylallylpyrophosphate (DMAPP)
dimethylallylpyrophosphate (DMAPP)
isopentenyl pyrophosphate (IPP)
dimethylallyltransferase
geranyl pyrophosphate
IPP
farnesyl pyrophosphate
squalene synthase
squalene
squalene epoxidase
2,3-oxidosqualene
cycloartenol
cycloartenol synthase
sterol side-chain reductase 2
cycloartanol
C-4 sterol methyl oxidase 3
31-norcycloartanol
31-nor-24(25)-dihydrolanosterol
4α-methylcholesta-8,14-dien-3β-ol
4α-methy-24(25)-dihydrozymosterol
4α-methyl-5α-cholest-7-en-3β-ol
C-4 sterol methyl oxidase 4
5α-cholest-7-en-3β-ol (lathosterol)
sterol C-5(6) desaturase
7-dehydrocholesterol
7-dehydrocholesterol reductase 2
cholesterol (CR)
DET2
cholestanol
6-oxocholestanol
CYP90B1
sterol C-24 methyltransferase 1 (SMT1)
24-methylenecycloartanol
C-4 sterol methyl oxidase 1 (SMO1)
cycloeucalenol
cyclopropylsterol isomerase
obtusifoliol
CYP51 ⊢①②③
4α-methylergostatrienol
sterol C-14 reductase
4α-methylergostadienol
sterol 8,7 isomerase
24-methylenelophenol
SMT2
24-ethylidenelophenol
SMO2
Δ7 avenasterol
C-4 sterol methyl oxidase 2 (SMO2)
episterol
sterol C-5(6) desaturase 1 (DWF7)
Δ5,7 episterol
7-dehydrocholesterol reductase 1 (DWF5)
24-methylenecholesterol
22S-hydroxy-24-methyleneCR
sterol side-chain reductase 1 (SSR1)
DWF7
Δ5,7 avenasterol
DWF5
isofucosterol
SSR1
β-sitosterol
22S-hydroxyisofucosterol
campesterol
(22S)-22-hydroxycampesterol
CYP90C1/D1
22,23-dihydroxycampesterol
sterol 5,4 isomerase
CYP90A1
(24R)-ergost-4-en-3-one
(22S,24R)-22-hydroxy-ergost-4-en-3-one
22,23-dihydroxy-4-en-3-one
5α-reductase (DET2)
(24R)-5α-ergostan-3-one
CYP90B1
(22S,24R)-22-hydroxy-5α-ergostan-3-one
campestanol
6α-hydroxycampestanol
④⑤⑥⑪⑫⊣ CYP90B1
6-oxocampestanol
CYP90B1 ⊢④⑤⑥⑫
cathasterone (CT)
6-deoxoCT
3-epi-6-deoxoCT
CYP85A1/A2
④⑤⑥⑦⑱⊣ CYP90A1
CYP90D1
teasterone (TE)
6-deoxoTE
26-nor-28-homoTE
28-homoTE
⑦⑧⑨⑱⊣ CYP90D1
3-dehydroteasterone (3-DT)
6-deoxo-3-DT
typhasterol (TY)
6-deoxoTY
26-nor-28-homoTY
28-homoTY
6-deoxo-28-homoTY
castasterone (CS)
6-deoxoCS
26-nor-28-homoCS
28-homoCS
brassinolide (BL)
6α-hydroxyCS
28-homoBL
6-deoxoDS
6-deoxo-28-homoDS
dolichosterone (DS)
28-homoDS
dolicholide (DL)
28-homoDL
6-deoxo-28-norCT
28-norCT
6-deoxo-28-norTE
28-norTE
CYP85A2
6-deoxo-28-nor-3-DT
28-nor-3-DT
6-deoxo-28-norTY
28-norTY
6-deoxo-28-norCS
28-norCS
28-norBL
sterols
brassinosteroids (BRs)
Multistep reaction
C27-BRs
C28-BRs
C29-BRs
Inhibitors of sterol biosynthesis – impact on brassinosteroid level:
① Voriconazole
② Fluconazole
③ Fenpropimorph
Inhibitors of brassinosteroid biosynthesis:
④ Brassinazole (Brz)
⑤ Brz2001
⑥ Brz220
⑦ Propiconazole
⑧ YCZ-18
⑨ Yucaizol
⑩ Fenarimol
⑪ Spironolactone
⑫ Triadimefon
Imazalil
4-MA
VG106
DSMEN21
Finastride
AFA76
Brassinopride
KM-01

Figure 1: Multistep reactions of brassinosteroids biosynthesis and their sterol biosynthetic precursors (Bajguz *et al*., 2020)

In sugarcane, the number of studies involving BR pathways is lower. As we will discuss in chapter 4, the modulation of BR signaling genes may be a feasible tool to improve traits in sugarcane. However, the modulation of genes involved in biosynthetic pathways may be another interesting way to follow. This chapter has the aim to identificaty the endogenous BR in sugarcane as, until now, we did not possess much information about it.

3.2 Material and Methods:

Sugarcane pollen (1g) was collected in the Agronomic Institute (Bahia, Brazil) and submitted to ultra-high performance liquid chromatography (Tarkowská et al., 2016) in collaboration with Dr. Dana Tarkowská of Laboratory of Growth Regulators at the Palacky University, Czech Republic.

In parallel, a second sample (1g) of sugarcane pollen, collected in the same place, was submitted to liquid chromatography followed by mass spectrometry (LC-MS/MS) in collaboration with Prof. Dr. Gustavo Zuñiga at the University of Santiago, Chile. LC-MS/MS analysis was made using a liquid chromatography (LC) system followed by triple quadrupole mass spectrometry (LC-MS-6040, Agilent). The samples were separated in an ACE UltraCore 2.5 SuperC18 (150 x 4.6 mm) column. LC conditions were optimized following: solvent A as 0.1% formic acid and solvent B as 100% acetonitrile. Bomb B gradient program was: 0.01–2 min, 0–40%; 2–5 min, 40–60%; 5–13 min, 100%; e 13–15 min, 20%. The flow was adjusted to 0.5 mL/min and the column temperature to 40ºC. The images were acquired in a positive mode using electrospray ionization and the analyte quantification were conducted in multiple reaction monitoring (MRM) mode. Other operation parameters were: nebulizing gas flow, 3 L min-1; dry gas flow, 15 L min − 1; desolvation line temperature (DL), 250°C, and heat block temperature, 400ºC.

We choose pollen because various indols compounds as cytokinins, gibberellins, abscisic acid and others are very abundant in sexual reproduction parts of the plants (Mitchell, 1971). As the first BR were isolated from pollen of *Brassica napus* L., the sugarcane pollen seemed to be a good clue to extract and determine endogenous BR.

3.3 Results and discussion

To identify the array of BR molecules in sugarcane we used two approaches UHPLC-MS/MS and LC-MS/MS, and the data obtained are shown in figure 1.

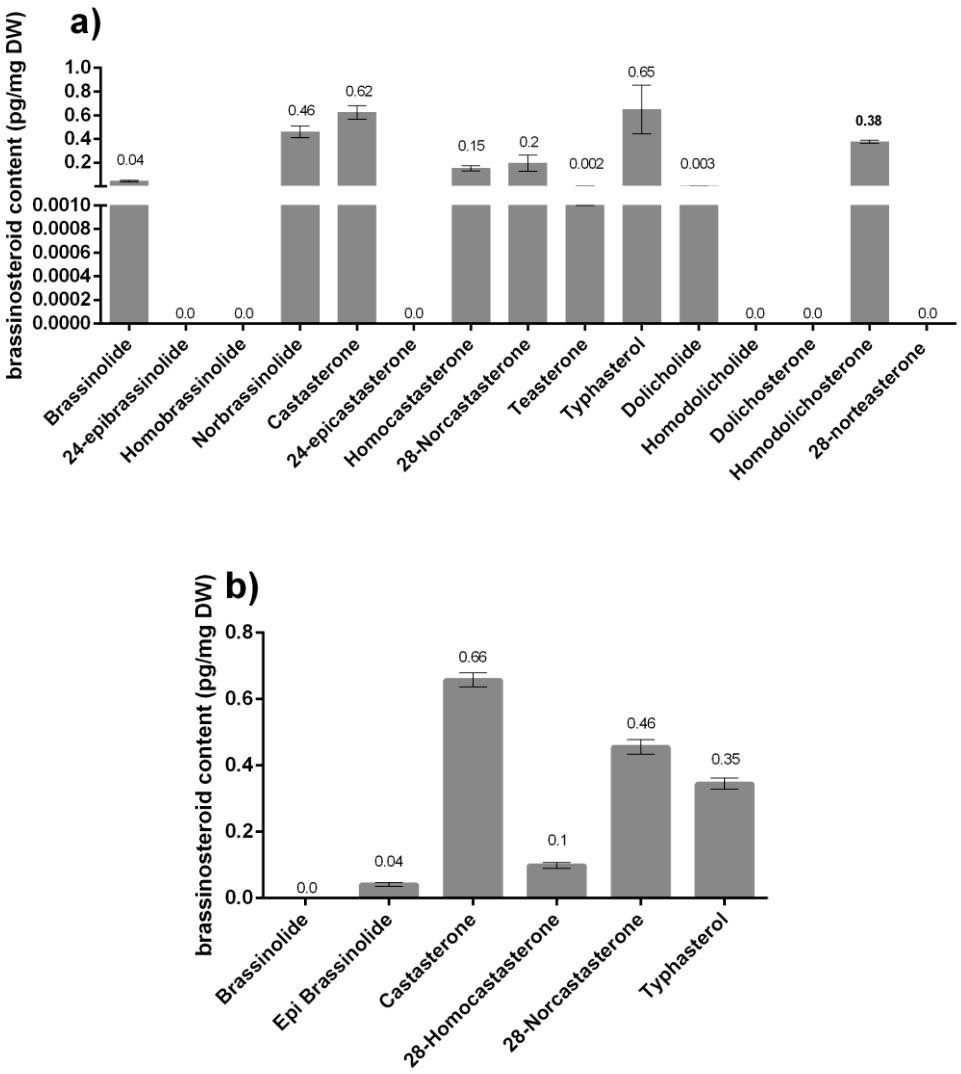

Figure 2: Endogenous BR identified in sugarcane pollen. The plant material was collected at the agricultural station from the Agronomic Institute (Bahia, Brazil) and used for extraction and identification of BR using A) UHPLC / MS-MS and B) LC-MS/MS. Bars indicate the mean ± SE. The numbers above bars indicate the BR amount (pg/mg of dry weight. DW).

Both analyses indicated that the two more abundant components were castasterone and typhasterol. Another compound with significant levels, detected by LC-MS/MS, was norcastasterone, which is originated by two possible ways: cholesterol (originated of campesterol) or through the C-24 castasterone demethylation, which was also found in a previous analyses in sugarcane (Zullo et al., 2018). Among the compounds evaluted using

UHPLC (figure 2A), homobrassinolide, 24-epibrassinolide, 24-epicastasterone, homodolicolide, dolicosterone and 28-norteasterone were not detected, while only trace amounts of brassinolide, teasterone, dilicholide were present. The analysis using LC-MS/MS method (figure 2B) also did not detect brassinolide, and epi brassinolide was barely detected. The absence of detection of the mentioned compounds may be explained by low concentration below detection limits of machinery and/or difference of sensibility of the techniques.

Analysing the BR biosynthetic pathway (Zullo and Adam, 2002), in spite of the final product is characterized as brassinolide, the oxidase which catalyzes the conversion of castasterone into brassinolide does not exist in the grass family, suggesting that castasterone is the bioactive final product of biosynthetic pathway (Kim et al., 2008). Currently, there are six steroid profiles of grass family in the literature (Zullo, 2018): Brachypodium, maize, wheat, barley, ryegrass and rye, as described below.

In the leaves of *Brachypodium distachyon*, castasterone was detected as the biologically active BR by GC/MS (Roh et al., 2020). In maize (*Zea mays*) seeds, castasterone, teasterone, 28-norcastasterone and 6-deoxocastasterone were detected as BR active principles by GC/MS (Kim et al., 1995), while in wheat (*Triticum aestivum* L.) seeds, castasterone, typhasterol and teasterone were detected by GC/MS (Yokota et al., 1994). In barley (*Hordeum vulagre*) seeds, castasterone was the endogenous BR detected by UHPLC-MS/MS (Gruszka et al., 2016). In ryegrass (*Lolium perenne*) pollen, 25-methylcastasterone was identified by HPLC followed by immunocytochemistry with polyclonal antibodies (Taylor et al., 1993). In rye (*Secale cereale*) seeds were identified castasterone, 28-homocastasterone, 28-norcastasterone (brassinone), 6-deoxocastasterone, typhasterol and teasterone as naturally occurring brassinosteroids by chromatography-mass spectral analysis (Schmidt et al., 1995).

Until now, only a few reports describe the steroid profiles of grass plants, as mentioned above. We found for the first time that castasterone is the endogenous and probably the most bioactive BR in sugarcane, in agreement with the data from other grass. In addition, is important to point that the determination of BR in plants presents many challenges as: the concentration of BR in plants tissue is low, ranging from nanograms to picograms, which difficults the detection (Zullo and Adams, 2002); the BR are difficult to extract and purify BR from plants tissue (Li *et al*., 2017); it is difficult to determine the biological activity of BR in different plant tissues and ages due to tha fact that BR are produced in specific tissue and transported to other tissue through vascular system (Jiang *et al*., 2017).

3.4 Conclusions

Due to the role of BRs in plant life, the importance of the elucidation of their biosynthesis might contribute to open new perspective to understand and modulate a range of physiological processes. The present work represents the first analysis of a complete steroid profile of sugarcane plants and due to the significant valuation of this phytohormone as described in chapter 1, this study may open new perspective to regulate BR biossyntethic genes that control agronomic traits. The endogenous BRs found in sugarcane pollen are consistent to previous studies where castasterone is the most bioactive compound in grass plants (Kim et al., 2008). In addition, castasterone and typhasterol are derivate of campesterol, already found in sugarcane wax (Georges et al., 2006).

4. CHAPTER 3

Tropical Plant Biology

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Gene Silencing Using Artificial miRNA in Sugarcane

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Abstract

One of the most common ways to study gene function is to analyze loss-of-function mutants. In polyploid species, although gene redundancy presents some advantages in nature, it offers an extra obstacle for genetic analyses due to the difficulty of obtaining loss-of-function mutants. Sugarcane presents several alleles at the same locus and the strong interaction between them contribute to the variation of phenotype and potential buffering effect, and this is one of the main challenges to study gene function in this species. Although RNA interference (RNAi) is largely used to produce loss-of-function mutants, due to the large fragments used to construct the hairpin structure, it may generate off-targets that can have confounding effect on the phenotype. The artificial micro RNA technique (amiRNA), an improved method of natural miRNA silencing, has been developed in the past years and is an useful tool for gene silencing in plants with high specificity and decreased off-target effects. In the present work we produced 26 transgenic events using amiRNA targeting the *ScPDS* gene, encoding a phytoene desaturase homolog. All events presented reduced expression levels of *ScPDS*. Although some events also showed distinguishable photobleached phenotypes, a high degree of expressivity was observed. Therefore, the use of amiRNA technique can be a good choice to silence genes in sugarcane with higher precision, and, consequently, is also a potential tool to be used in other polyploid species. However, a high number of transgenic events might be needed to achieve high levels of gene silencing.

Keywords Bioenergy · PDS gene · Polyploid species · Gene expression · Chlorophyll content

Introduction

A major challenge of modern biology is to infer gene function from the massive amount of enome sequencing data currently available. Although gene ontology and other *in silico* methodologies may provide insights on the role of a gene, the analysis of loss-of-function alleles is key to understanding gene function. However, functional analysis in plant species with high economic importance and complex genomes, such as sugarcane (*Saccharum* spp.), is much more challenging due to the high ploidy levels. Modern sugarcane varieties have resulted from various crosses and interspecific hybridization and may contain over than 100 chromosomes (Heinz and Tew 1987). A single locus exists in many copies in the genome of these organisms, and due to this, many alleles can coexist in the same plant with different dosages (Glaszmann et al. 1989). RNA interference (RNAi) or genome editing technologies, such as CRISPR Cas9 are useful strategies to infer gene function. However, so far, the use of CRISPR Cas9 in sugarcane is still a challenge, while the use of RNAi has proven to be a straightforward strategy (Bewg et al. 2016; Garcia Tavares et al. 2018; Glassop et al. 2017; Guidelli et al. 2018; Jung et al. 2012, 2016; Khalil et al. 2018; Mohan et al. 2021; Osabe et al. 2009). RNAi is a technique mediated by post-transcriptional gene silencing which acts through the production of double-stranded RNA (dsRNA) replicative intermediates that are cleaved into 21–26 nt shortinterfering RNAs (siRNA) by RNase-III-type enzyme Dicer (Baulcombe 2004). These siRNAs, in turn, cleave specific mRNA-target sequences through sequence complementarity, causing silencing by translation inhibition and/or mRNA degradation (Valencia-Sanchez et al. 2006). Despite several advantages, this technique may have a few drawbacks depending on the gene's function, mode of action, and even on the species being studied, such as partial gene silencing that does not change the phenotype, and off-target effects that can lead to phenotypic changes unrelated to the gene of interest (Liang et al. 2012; Ossowski et al. 2008).

Another silencing gene technique in plants, using microRNAs (miRNAs), can be an useful tool to overcome the disadvantages of RNAi (Bartel 2004; Jones-Rhoades et al. 2006). Like siRNAs, miRNAs are short 21–24 nucleotide RNAs originating from Dicer cleavage of a dsRNA precursor (Baulcombe 2004). However, although miRNAs and siRNAs have shared central biogenesis, these two classes of silencing RNAs present some differences. First, miRNAs are processed from transcripts that form local RNA hairpins while siRNAs are processed from long double-stranded RNA duplexes or extended hairpins. Second, each miRNA hairpin precursor molecule generates only a single miRNA::miRNA* duplex, while multiple duplexes of siRNAs are generated from each siRNA precursor, culminating in producing of many different siRNAs molecules (Bartel 2004). This last consideration may be the mainly cause of RNAi off-targets. Due to the array of different siRNAs duplexes generated, many regions in the genome that present partial identity can be reached, interfering in the expression of mRNAs of unintended genes (Lin et al. 2005; Qiu et al. 2005). The high complementarity between miRNA and its mRNA-target allows fewer mismatches and thus more specific repression of the gene targets, making the miRNA technique a potentially tool for gene-specific silencing.

Many studies have been conducted to improve miRNA silencing techniques in plants and

a new tool for gene silencing known as artificial miRNAs (amiRNAs) was shown to act similar to natural miRNAs in many plant species (Schwab et al. 2006). Gene silencing by amiRNA can be used to study gene function, providing insights into metabolic pathways and has the potential to improve agronomic traits such as pest resistance and others. For example, the silencing of two ProDH genes in potato (Solanum tuberosum L.), a rate-limiting enzyme in the process of proline catabolism, increased tolerance to water deficit (Li et al. 2020). Transgenic tomatoes plants (Solanum lycopersicum L.) using amiRNA to down-regulate the gene HaEcR, encoding an ecdysone receptor or the acetylcholisneterase 1 gene (Ace 1) had increased insect resistance (Faisal et al. 2021).

Despite several reports of using RNAi in sugarcane, however, to our knowledge, there are no reports of the use of amiRNA to silence genes in this polyploid species. In this study, we investigated the viability of using amiRNA in sugarcane as a way to effectively characterize gene function in this species by minimizing the impact of off-targets. For this purpose, we choose phytoene desaturase (PDS) as a marker gene, because it is a rate-limiting enzyme of the carotenoid biosynthesis pathway (Bai et al. 2016; Cazzonelli and Pogson 2010) and is often used in studies of gene silencing due to the easily distinguished phenotypes of photobleaching in transgenic plants. In this study, transgenic sugarcane plants expressing an amiRNA showed decreased expression of the ScPDS gene and reduced levels of chlorophyll content with variable levels of photobleaching. Therefore, amiRNA can be an useful method to investigate gene function in polyploid species such as sugarcane.

Results

Construction of an amiRNA Expression Cassette

The rice PDS protein (access Os03g08570) was used to identify the sugarcane ScPDS gene in two sugarcane sequence databases (Papini-Terzi et al. 2007; Riaño-Pachón and Mattiello 2017). The sequence SP803280_c115863_g1_i1 from (Riaño-Pachón and Mattiello 2017) had the full length ScPDS sequence, and after searching the putative best scored amiRNA and amiRNA* sequences in WMD3 with a local version software, we were able to design a PDS silencing cassette. The ScPDS coding sequence and the corresponding amiRNAPDS are shown in Fig. 1. We used the miR528 from rice to produce a gene silencing cassette, and the Gateway technology was used to transfer this cassette to the pGVG vector, that has been extensively used to transform sugarcane (Guidelli et al. 2018), as shown in Fig. 2.

ATGGACACTGGCTGCCTGTCATCTATGAACATTACTGGAGCTAGCCAAGCAAGACCTTTTGTGGGACAACTTCCTCAGAGATGT
TTTGCGAGTACTCACCATTCGAGCTTTGCCGTGAAAAATCTTGTCTTAAGGAATAAAGGAAGGAGATCACACCATAGACATGCT
GCCTTGCAGGTTGTCTGCAAGGATTTTCCAAGACCTCCACTAGAAAGCACAATAAACTATTTGGAAGCTGGGCAGCTCTCTTCG
TTTTTTAGAAACAGCGAACGCCCCAGTAAACCGTTGCAGGTCGTGATTGCTGGTGCAGGATTGGCTGGTCTATCAACGGCGAAG
TATCTGGCAGATGCTGGCCATAAACCCATATTGCTTGAGGCAAGAGATGTTTTGGGTGGAAAGGTAGCTGCTTGGAAGGATGAA
GATGGGGATTATTACGAGACTGGGCTTCATATCTTTTTTGGAGCTTATCCCAACATACAGAATCTGTTTGGTGAGCTTGGAATT
GAGGATCGTTTGCAGTGGAAAGAACACTCCATGATATTTGCCATGCCAAACAAGCCAGGAGAATTCAGCCGGTTTGATTTCCCA
GAAACTTTGCCAGCCCCTGTAAACGGGATATGGGCCATACTGAGAAACAATGAAATGCTTACCTGGCCGGAGAAGGTGAAGTTT
GCGATTGGACTTCTGCCAGCGATGGTGGGTGGTCAACCTTATGTTGAAGCTCAAGATGGCTTAACCGTTTCAGAATGGATGAAA
AAGCAGGAGAAGCATGGTTCCAAAATGGCATTCTTGGATGGTAATCCACCTGAAAGGCTATGCATGCCTATTGTTGATCACATT
CGGTCTAGGGGTGGAGAGGTCCGCTTGAATTCTCGTATTAAGAAGATAGAGCTGAATCCTGATGGAACTGTAAAACACTTCGCA
CTTAGCGATGGAACTCAAATAACTGGAGATGCTTATGTTTGTGCAACACCAGTTGATATCTTCAAGCTTCTTGTACCTCAAGAG
TGGAGTGAAATTACTTACTTCAAGAAGCTGGAGAAGTTGGTGGGAGTTCCTGTTATCAATGTTCATATATGGTTTGACAGAAAA
CTGAAAAACACATATGACCATCTTCTTTTCAGCAGGAGTTCACTTTTAAGTGTCTATGCGGACATGTCAGTAACCTGCAAGGAA
TACTATGATCCAAACCGTTCAATGCTGGAGTTGGTCTTTGCTCCTGCAGACGAATGGATTGGTCGAAGTGACACTGAAATCATC
GATGCAACTATGGAAGAGCTAGCCAAGTTATTTCCTGATGAAATTGCTGCCGATCAGAGTAAAGCAAAGATTCTTAAGTATCAT
GTTGTGAAGACACCGAGATCGGTTTACAAAACTGTCCCAAACTGTGAACCTTGCCGACCTCTCCAAAGGTCACCGATCGAAGGT
TTCTATCTGGCTGGTGATTACACAAAGCAGAAATACTTGGCTTCCATGGAAGGTGCAGTTTTATCCGGGAAGCTTTGCGCCCAG
TCTATAGTGCAGGATTATAGCAGGCTCGCTCTCAGGAGCCAGAAAAGCCTACAATCCGAAGGAGTTCCTGTCCCATCTTAG

Fig. 1 CDS region of the *ScPDS* gene and location of the amiRNAPDS. The underlined part comprises the location where the amiRNAPDS

Photobleaching Phenotypes

As shown in Fig. 3, a visual inspection on the majority of transgenic events did not show phenotypic difference in relation to WT, since they presented mostly green leaves. However, some transgenic events such as ev. 5, ev. 9, ev. 12 and ev. 26 showed both green and white leaves in the same plant (mixed events), while ev. 7 showed photobleaching in all leaves. It is worth noting that wild type (WT) plants were cultivated in vitro in the same way transgenic plants, and we did not observed white leaves among WT regenerated plants. Therefore, we can discard that the phenotypes observed in the transgenic events are due to in vitro culture conditions.

Expression Analysis of amiRNAScPDS

To further evaluate the silencing of the ScPDS gene due to the amiRNA expression, the total RNA was extracted from leaves and used in a qRT-PCR. Most

transgenic events align to the *ScPDS* gene presented ScPDS expression lower than WT plants, consistent with effective amiRNA expression. For the most affected event (ev.7) ScPDS expression was reduced approximately 8.5-fold relative to WT. Other events also had significant reductions of ScPDS expression compared to WT: ev. 9 (sixfold reduction), ev. 5 (4.7-fold reduction), ev. 26 (7.3fold reduction) and ev. 12 (4.5-fold reduction) (Fig. 4). In addition, the levels of ScPDS expression in most of the transgenic events are statistically different from WT plants (Fig. 4), corroborating to the fact that amiRNA technique does present silencing effects. We also used the Mann–Whitney U Test to compare the two populations and the result showed a p-value 0.0048, indicating a significant result at $p < 0.05$.

A)

CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTAC
GTTTATTACTAAAATAAAACTGACTATCACTGGACAAGCAACGTTGTTTAACTACTCGTTACGAAAAAATATTACGGTTGAAACATG

AAAAAAGCAGGCTGGATCCCAGCAGCAGCCACAGCAAAATTTGGTTTGGGATAGGTAGGTGTTATGTTAGGTCTGGTTTTTTGGCTG
TTTTTTCGTCCGACCTAGGGTCGTCGTCGGTGTCGTTTTAAACCAAACCCTATCCATCCACAATACAATCCAGACCAAAAAACCGAC

TA*tacgta*GCAGCAGCAGTTAATCCTGCACTATACACTGCAGGAGATTCAGTTTGAAGCTGGACTTCACTTTTGCCTCTCTCAGTGA
AT*atgcat*CGTCGTCGTCAATTAGGACGTGATATGTGACGTCCTCTAAGTCAAACTTCGACCTGAAGTGAAAACGGAGAGAGTCACT
SnaBI amiRNAPDS

ATACTGCAGGATTAATTCCTGCTGC*gacgtc*TAGGCTGTTCTGTGGAAGTTTGCAGAGTTTATATTATGGGTTTAATCGTCCATGGC
TATGACGTCCTAATTAAGGACGAC*ctgcag*ATCCGACAAGACACCTTCAAACGTCTCAAATATAATACCCAAATTAGCAGGTACCG
amiRNAPDS* *AatII*

ATCAGCATCAGCAGCGGTACCCTGCAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGCA
TAGTCGTAGTCGTCGCCATGGGACGTCTGGGTCGAAAGAACATGTTTCAACCGTAATATTTTTTATTAACGAGTAGTTAAACAACGT

ACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG
TGCTTGTCCAGTGATAGTCAGTTTTATTTTAGTAATAAAC

B)

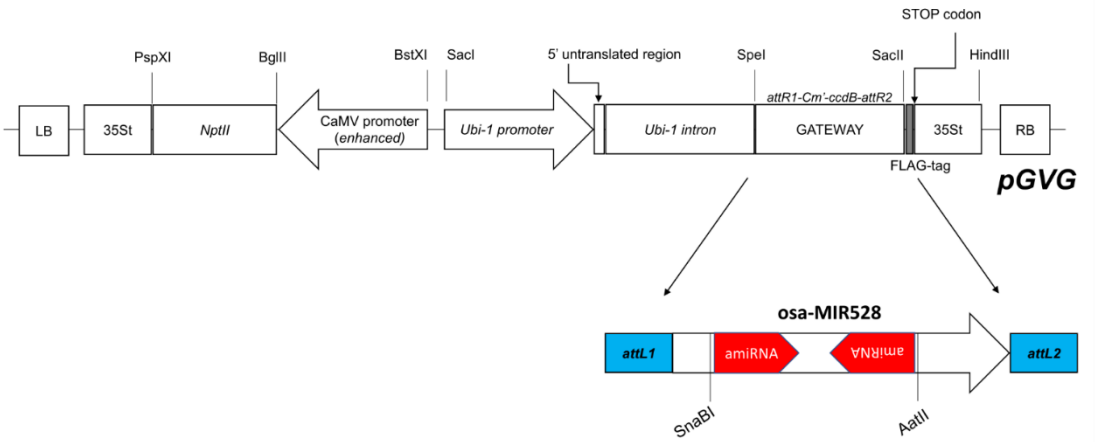

Fig. 2 Vector for amiRNA silencing of the *ScPDS* gene in sugarcane. **A**) synthetic fragment, containing the *attL1* and *attL2* gateway recombination sites (blue) and the rice miR528 (black), in which the natural miRNA and miRNA* were replaced by the *ScPDS* sequence (red). The restriction sites of SnabI and AatII (indicated in italics) were added to facilitate future cloning reactions and design of new amiRNA vectors. **B**) Cloning strategy, where the synthetic fragment indicated in (**A**) was recombined with the pGVG vector (Guidelli et al. 2018)

Chlorophyll Content Decreases in *ScPDS*-Silenced Plants

The quantification of chlorophyll *a* and *b* is an important parameter to be evaluated in PDS-silenced plants. This is because carotenoids are intimately linked to chlorophyll stability due to their role in photoprotection of the reaction center of chlorophylls, especially under stress conditions (Ramel 2013). We observed that the chlorophyll a and b content decreased in most transgenic plants (Fig. 5). The most evident difference of decreasing of this pigment content is presented in total chlorophyll (Fig. 5c) where the majority of the events presented significant difference in the ratios in relation to WT plants.

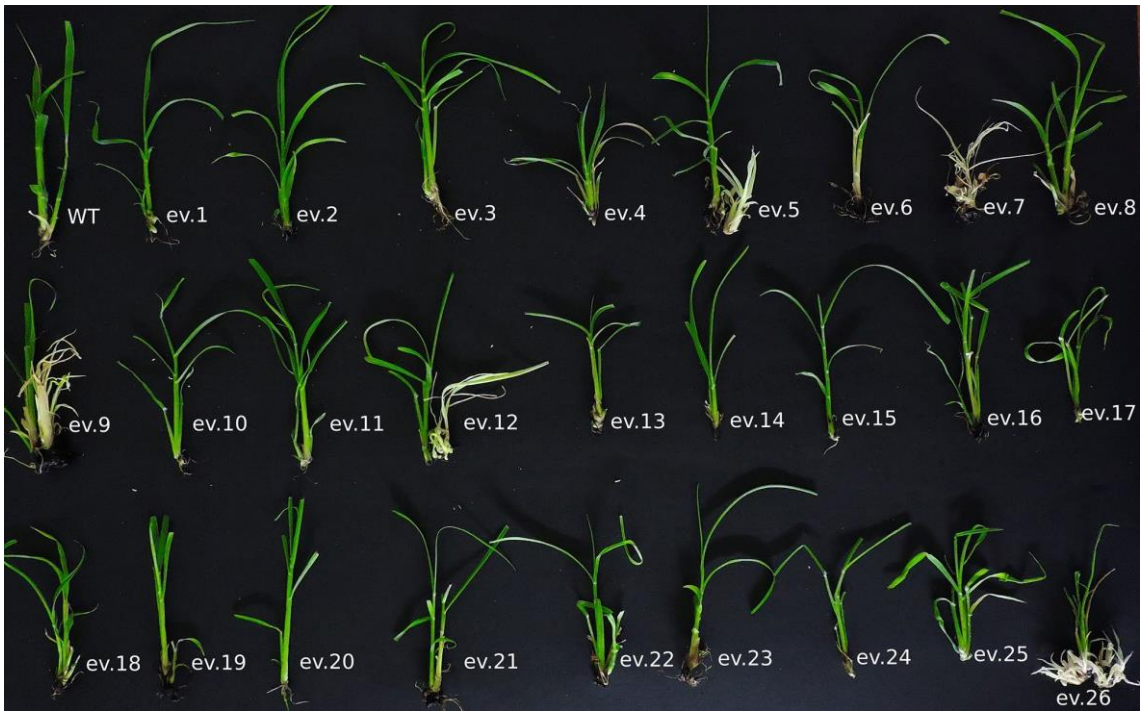

Fig. 3 WT plants and transgenic events with silenced *ScPDS* gene

The gene expression levels had a significant correlation ($p < 0.0001$) with the total chlorophyll content, showing that the decreased chlorophyll content is related to reduction of *ScPDS* gene expression (Fig. 6).

Discussion

Classically, penetrance is defined as the percentage of individuals with determined genotype

that exhibit an associated phenotype, whereas expressivity measures the extent to which determined genotype is expressed in an individual at the phenotypic level. In this study, we may consider penetrance as the percentage of transgenic plants which exhibit a phenotype of reduced chlorophyll content and expressivity as the expression levels of ScPDS gene detected by qRT-PCR.

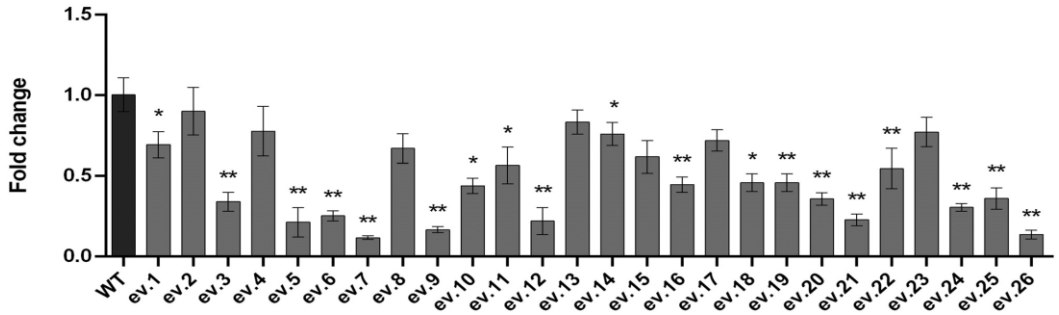

Fig. 4 Gene expression analysis of fold change of WT plants (dark than 0.05 (*) or 0.001 (**), according to a Student test comparing grey bar) and transgenic events (light grey bars) via qRT-PCR. Data expression levels in the transgenic events with WT plants (n = 3) are presented as the mean ± SE. Asterisks indicated a p-value lower

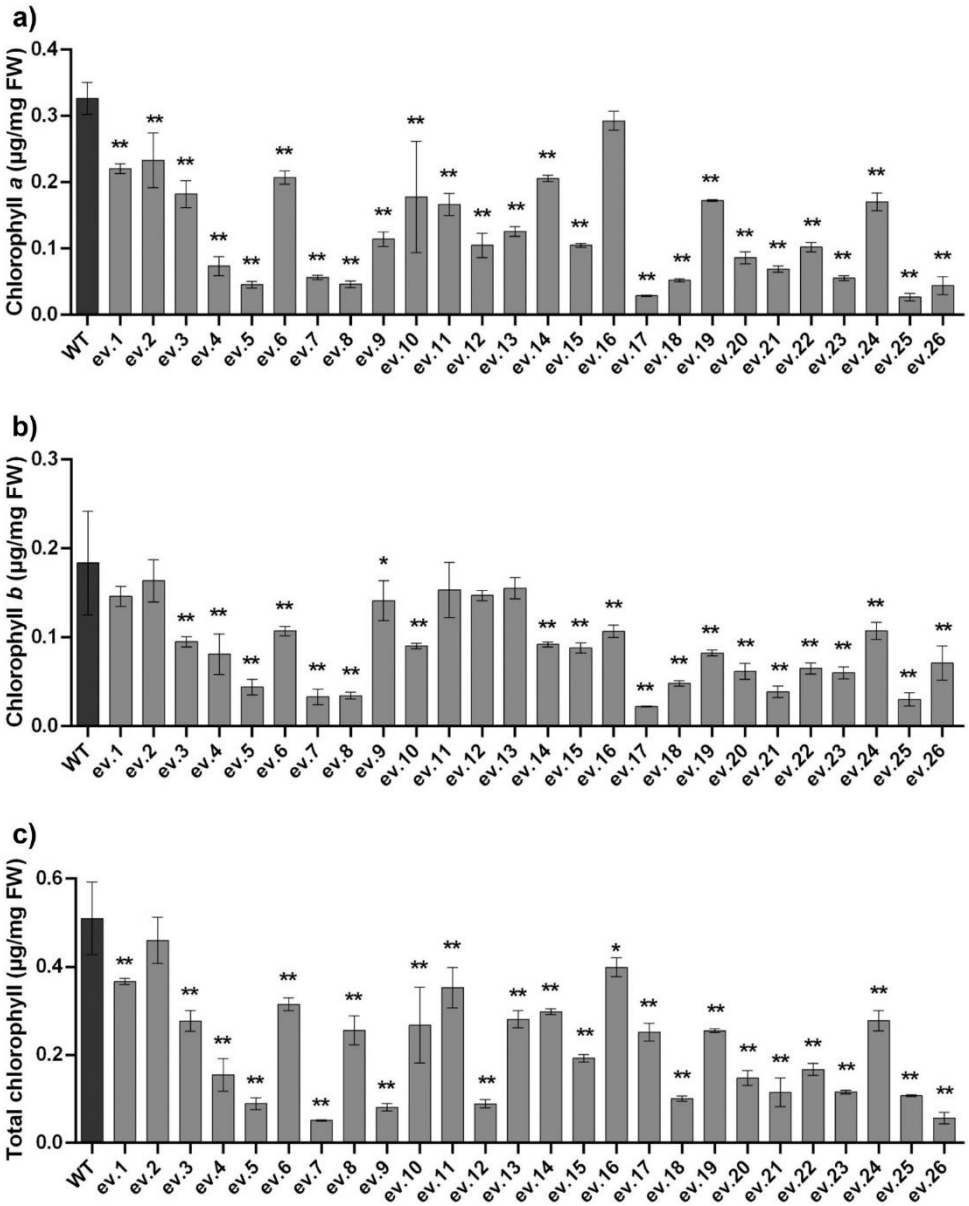

Fig. 5 Chlorophyll content in WT (dark grey bars) plants and trans- mean chlorophyll content of an event is significantly different from genic events (light grey bars). (**a**) chlorophyll *a*; (**b**) chlorophyll *b*; (**c**) WT according to Dunnett's multiple comparison test. Asterisks inditotal chlorophyll. Bars indicate the mean ± SE. Asterisks indicate that cated a p-value lower than 0.05 (*) or 0.001 (**)

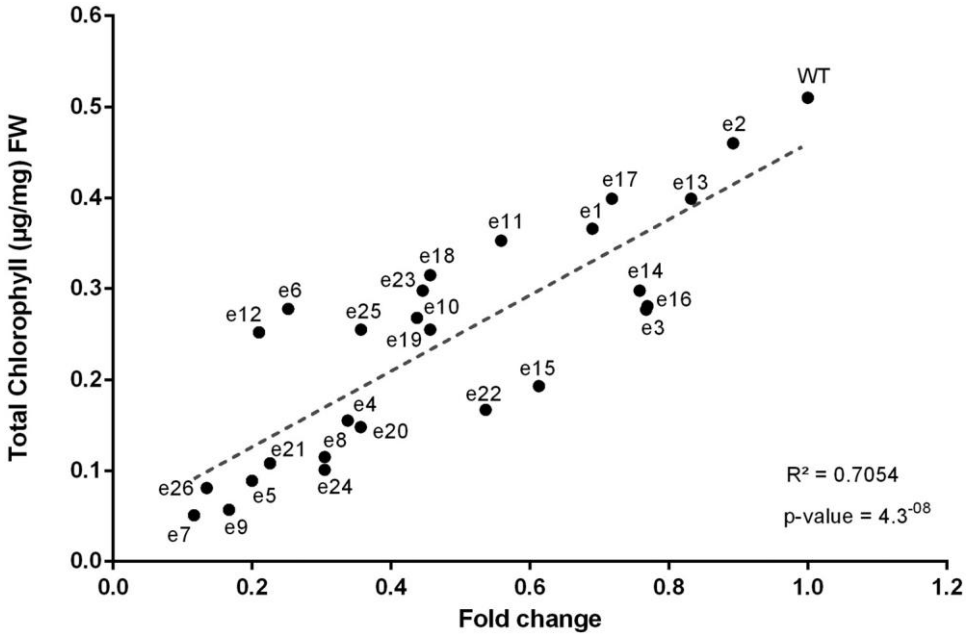

Fig. 6 Correlation between total chlorophyll and gene expression levels. Data from gene expression (Fig. 4) and chlorophyll content (Fig. 5) were plotted to evaluate their correlation

If comparing the results showed by Figs. 3, 4 and 5, ev.7, the only event completely photobleached, presented the lowest expression level of *ScPDS* and the lowest chlorophyll content. The mixed events, ev.9, ev.5, ev.26 and ev.12, are followed with intermediary silencing levels of *ScPDS* gene and chlorophyll content, after ev.7. The other events, even did not showing visible photobleaching phenotypes, still present variable levels of silencing of *ScPDS* and chlorophyll content in relation to WT plants.

Even though most of the transgenic events did not show photobleached leaves, the correlation between *ScPDS* gene expression and chlorophyll content indicated that the gene silencing technique was effective in producing a range of silencing levels in sugarcane. This can be useful in cases where a fine tuning of gene expression levels are desired. The absence of photobleached leaves in these events can be explained by others studies, as we can see further. Experiments with arabidopsis (85%) (Wang et al. 2005) showed a high ratio of photobleaching phenotypes. This phenomenon can also be observed in monocot species as rice (92.9%) (Warthmann et al. 2008). However, the rate of photobleached plants decreases in polyploid species such as banana (59%) (Kaur et al. 2018) and bread wheat (25%) (Travella et al. 2006). In our work, the main reason of the lack of a visible phenotype may be related to the polyploidy nature of the sugarcane genome. In polyploid species, most traits are multigenic and/or multiallelic and, therefore, are quantitatively inherited (Casu et al. 2005). Because of this, many traits present genetic redundancy where any gene locus and its allelic complement is probably represented multiple times due to the large number of homologous chromosomes. In this case, a compensatory effect of the multiple alleles in the sugarcane genome may be responsible for the varying degree of gene silencing and chlorophyll content among independent events. This may also explain the presence of photobleached sectors in some events, where a certain threshold is achieved, leading to a lower content of chlorophyll. This

hypothesis does not rule out other mechanisms, such as the need of a stabilization of RNAi mechanisms, as suggested by Travella et al. (2006) to explain their observation of wheat leaves containing sectors with photobleached phenotype. Further work is need to explain these hybrid phenotypes in PDSsilenced plants.

The ability to engineer traits by modulating expression levels of crop species genes is likely to be a powerful tool to molecular improvement and creation of new cultivars with favorable agronomic qualities. Sugarcane is an important crop and despite much study of its large genome, it still presents many challenges. One of them is the ability to silence genes due to the large number of alleles at the same locus. The amiRNA technique uses the natural miRNA silencing machinery, presenting advantages in relation to other conventional silencing techniques, such as high specificity and reduced off-targets effects. We can conclude that the use of amiRNA can be an useful tool for gene silencing in sugarcane, showing high penetrance, since all transgenic events showed decreased of chlorophyll content, and variable expressivity, with all transgenic event presenting different levels of silencing of the target gene. Probably due to sugarcane polyploid genome, the number of transgenic plants needed to obtain events with high levels of silencing is higher than those from diploid species.

Methods

amiRNA Design

We performed a BLASTp analysis with the rice PDS protein sequence (Os03g08570; Oryza sativa Niponbare japonica) to search for a putative sugarcane PDS protein and the corresponding gene sequences in two sugarcane genome databanks: CTBE (http:// 186. 249. 222. 29/ ctbeb last/) (RiañoPachón and Mattiello 2017) and SUCEST-fun (http:// sucest- fun. org) (Papini-Terzi et al. 2007). The complete coding sequence (ScPDS) with the highest match score was used as a target to design suitable amiRNA sequences for silencing ScPDS gene using the web application Web MicroRNA Designer 3 (WMD3) (http:// wmd3. weige lworld. org; (Ossowski et al. 2008). This program identifies the best amiRNA candidates based on good hybridization properties to the target mRNA while minimizing possible off-targets effects to other genes in the genome (Ossowski et al. 2008). Since the reference genome sequence we were interested in surveying was not available in the WMD3 web server, we set up a local version of this software with our sugarcane transcriptome sequences and searched for good amiRNA targets using the Designer (http:// www. plant cell. org/ conte nt/ 18/5/ 1121 and (https://o nlinelibra ry .wiley.c om /doi/f ull /10.1 111 /j.1365-3 13 X. 2007. 03328.x) application (minimum number of targets was set to 1, and accepted off-targets to 0). Then, we used the Target Search application of WMD3 (http://w md3.weigel world .org /) to select the highest scoring amiRNA (called amiRNAPDS) targeting only our gene of interest, and to design its reverse complement sequence (amiRNAPDS*).

Plasmid Construction

The original construct of an amiRNA involves several overlapping PCR reactions where oligonucleotides primers replace the original miRNA and miRNA* regions with the artificial selected sequences (Schwab et al. 2006). However, due to time constraints and the possibility of base mutation during cloning processes we decided to synthesize the amiRNA expression cassette (GenOne Biotechnologies, Brazil).

The engineered expression cassette contains the stemloop region (named as amplicon) from osa-MIR528, a natural miRNA of Oryza sativa, monocotyledonous model plant and closely related to sugarcane. We replaced the 21 bases of the natural miRNA osa-MIR528 (miR528), as well as the partially complementary region (miR528*), by the sequences amiRNAPDS and amiRNAPDS*, respectively. In addition, we add the restriction sites of SnabI and AatII flanking the sequence of amiRNAPDS and amiRNAPDS*, respectively, allowing this expression cassette to be used as a template for future cloning processes and facilitating the design of new amiRNA vectors. The amiRNAPDS expression cassette was flanked with the Gateway recombination sites attL1 and attL2 to optimize gene transfer between vectors.

The entry vector (attL1::amiRNAPDS::attL2) was recombined with the destination vector (pGVG, (Guidelli et al. 2018)) using the Gateway LR Clonase II Enzyme (Life Technologies, Brazil), transferred to competent DH5α E. coli cells and confirmed by digestion with restriction enzymes and DNA sequencing. The construct was then used to transform competent EHA105 strain of A. tumefaciens (Hood et al. 1993) cells by the freeze-thaw method (Chen et al. 1994).

Sugarcane Transformation

Transformed Agrobacterium cell cultures were sent to PangeiaBiotech (Campinas, Brazil) to produce transgenic sugarcane plants (SP80-3280 cultivar). Briefly, A. tumefaciens cultures were incubated with sugarcane call uses under vacuum pressure for five minutes and transferred to co-cultivation medium (4.33 g/L MS salts, 1 mL/L MS vitamins, 3 mg/L 2,4- D, 0.15 g/L citric acid, 25 g/L sucrose and 3.5 g/L phytagel) at 22 °C, in the dark for 3 days. Then, calli were kept in resting medium (4.33 g/L MS salts, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0.15 g/L citric acid, 25 g/L sucrose, 100 mg/L proline, 2.8 g/L phytagel and 200 mg/ mL timentin) at 26 °C, in the dark for 6 days. Following the resting phase, the transformed calli were transferred to a selective regeneration medium [4.33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 5 mg/mL CuSO4, 1 mg/mL benzylaminopurine (BAP), 7 g/L agar, 200 mg/mL timentin and 40 mg/L geneticin] at 26 °C, during 14 days with 16 h photoperiod.

The transgenic events were kept in medium without phytohormones (4.33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 7 g/L agar, 200 mg/mL timentin and 40 mg/L geneticin) to induce growth and rooting. Twenty-six independent transgenic events were obtained. As a control, we used three WT plantlets, obtained from calluses that passed throughout all the in vitro regeneration process as the transgenic events, except for the absence of antibiotic. For each of the 26 transgenic three clones were produced by splitting the branches formed during the in vitro culture and they were used as biological replicates. Therefore, in the experiments described below three biological replicates of WT plants and of each transgenic event were used.

Total RNA Extraction

All the leaves from the sugarcane plantlets, including those with photobleached phenotype, were frozen using liquid nitrogen and ground with a Mini-BeadBeater-96 (Biospec, USA). Total RNA was extracted using guanidine extraction buffer (Logemann et al. 1987). After a DNase I (Fermentas, USA) treatment, the RNA was reverse transcribed with iScript® (BioRad, USA), following the manufacturer´s instructions.

Gene Expression by Quantitative Real-Time PCR (qRT-PCR)

We performed qRT-PCR to evaluate the expression levels of ScPDS gene using GoTaq qPCR Master Mix (Promega). The reactions (12 μL final volume) contained 1 μL of cDNA (diluted 1:20) and 200 nM of the forward (5' CTGG TGCAG GAT TGG CTG GT 3') and the reverse (5' GGG GAT TGG TAC GAG ACT GGGC 3') primers of the ScPDS gene. As internal control we used primers of the polyubiquitin gene (NCBI accession CA179923.1) (Papini-Terzi et al. 2005) for data normalization. A 7500 Real Time PCR System (Applied Biosystems, USA) with the following conditions was used: -50 °C (2 min.) and 95 °C (10 min.), followed by 40 cycles of 95 °C (15 s.) and 60 °C (1 min.). The expression data represent the mean of three biological replicates, and three technical replicates were performed for each biological replicate.

The $2-\Delta\Delta CT$ method was used to calculate relative gene expression according to Livak and Schmittgen (2001), with the expression in WT plants being considered as having a fold change of 1.

Chlorophyll Quantification

Chlorophyll (a and b) levels were quantified using an ethanol solution, according to Cross et al. (2006), using three biological replicates (two technical replicates were done for each biological replicate). In all cases, even when photobleached leaves were observed, all the leaves were collected, as described for the total RNA extraction. A microplate spectrophotometer (SpectraMax M3, Molecular Devices, USA) was used for absorbance readings taken at wavelengths of 665 and 645 nm for chlorophyll a and b, respectively.

Statistical Analysis

Quantitative real-time PCR data were analyzed as described by Tournayre et al. (2019), using a Student test to compare data from each transgenic event and WT plants. For chlorophyll content analyses, the data were evaluated using ANOVA (p-value < 0.05), followed by Dunnett´s multiple comparison test to compare means using the GraphPad Prism 8 software (GraphPad Software, Inc.). For correlation between chlorophyll content and ScPDS gene expression, the analyses were conducted using Microsoft Excel software (p-value < 0.05).

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Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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5. CHAPTER 4

The BR signaling pathway in sugarcane: consequences of the modulation of *ScBZR1* and *ScLIC* in development and stress response

5.1 Introduction

5.1.1 The differences of BR signaling pathway in eudicotyledonous and monocotyledonous

Molecular studies have uncovered several aspects of the BR signaling pathway in eudicotyledonous, as described in chapter 1. However, recently studies using reverse genetics identified a high number of conserved and specific components of BR signaling pathway in rice, a monocotyledonous model plant, indicating some differences between eudicots and monocots (Zhang et al., 2014).

The rice genes *OsBRI1*, *OsBAK1* and *OsBZR1* and the orthologs from Arabidopsis show highly conserved functions in both species (Zhang et al., 2014). Besides, the nucleocytoplasmic localization of *OsBZR1* is resulted by action of 14-3-3 proteins, as observed in Arabidopsis (Bai et al., 2007). In relation to the specificities founded in the signaling pathway in monocots, *OsBZR1* is the closest orthologue of BZR1 in Arabidopsis. There is no specific orthologue for *BES1* in rice, and orthologues of genes encoding PP2A, BSKs and BSU1 were not identified until now. Other components, such as *OsDLT* (repressed by *BZR1*, involved in the gibberellin pathway) and *OsTUD1* (interacts with the co-receptor *BAK1*), were only identified in rice, with no corresponding orthologues in Arabidopsis (Tong and Chu, 2012). In rice, *GSK3* is the orthologue of BIN2 from Arabidopsis, the main negative regulator of BR signaling pathway (Koh et al., 2007). *ILI1* and *IBH1* are involved in leaf architecture determination: while the overexpression of *ILI1* increases leaf angle, the overexpression of *IBH1* leads to erect leaves. *ILI1* interacts with the bHLH IBH1 protein, which is a negative regulator of the BR signaling pathway. *OsBZR1* activates *ILI1*, that downregulates *IBHI* gene expression. Then, is presumable that ILI1 and IBH1 possesses antagonistic action downstream to *OsBZR1*, mediating BR signaling (Zhang et al., 2009).

In rice, LIC (LEAF AND TILLERING INCREASED CONTROLLER) acts as an antagonist of the BZR1 transcriptional factor to attenuate BR signaling pathway. While BZR1

actives the pathway, LIC is responsible to slow down the amplification cascade (Zhang et al., 2012).

However, the mechanism of mutual repression between *BZR1* and *LIC* depends on the levels of BR. Experiments in rice using exogenous BR (24-eBL) showed that the treatment with low concentration of 24-eBL induced *BZR1* expression, promoting dephosphorylation and activation of the BR pathway. In contrast, the treatment with high concentrations of BR induced LIC expression, repressing BR signaling pathway. These facts suggest a possible feedback mechanism between BZR1 and LIC, preventing unnecessary activation of BR cascade (Zhang et al., 2012).

If comparing with Arabidopsis, the role of BR signaling pathway in monocots still lacks several information. Previous works showed that BR mutants in Arabidopsis and rice presented modifications in the size of plants, leaf curvature, tillering and productivity (Clouse, 2011; Li and Jin, 2007; Yang et al., 2011 and Zhang et al., 2014). In this context, the manipulation of biosynthetic and signaling pathways of BR is capable to modify plant architecture, which may be a feasible approach to improve their productivity (Divi and Krishna, 2009).

5.1.2 Agronomic traits mediated by BR

Since 1980, BR has been used as a plant growth modulator (Khripach, 2000). Once the genes of the BR signaling pathway were identified, a large number of studies verified a great potential of the modulation of these genes in plant breeding (Divi and Krishna, 2009). Studies in rice showed a strong influence of BR in agronomic traits, as will be discussed below.

Plant Height: The reduction in plant height in semi-dwarf varieties led to improved harvest index (grain:straw ratio) and enhanced biomass production (Khush, 1999). *OsBRI1* seems to be involved in internode elongation, since deficient/insensitive BR mutants show dwarfism with specific patterns of internode elongation (Zhang et al., 2014). Moreover, *OsBZR1* can bind to the promoters to at least three metabolic genes of gibberellin biosynthesis, promoting cell elongation and, consequently, increasing plant height (Tong et al., 2014).

Leaf angle: Leaf angle is an important agronomic trait associated with photosynthesis. Plants with erect leaves can capture more light and provide more dense plantings with a higher leaf area available for photosynthesis, which contribute to increase yields (Sinclair and Sheehy, 1999). In addition, the lamina joint could be one of the most sensitive tissues in response to BR

fluctuation in rice. Transgenic rice lines obtained by partial suppression of *OsBRI1* (low sensitivity to BR) present erect leaves. The estimated grain yield of these transformants is 30% higher than wild type at high density (Morinaka et al., 2006). The suppression of *OsBAK1*, co-receptor of BRI1, also improved rice grain and yield by decreasing the leaf angle (Li et al., 2009). The bHLH transcription factor *OsIBH1* binds to *OsILI1* protein promoting its inactivation, causing the inhibition of cellular elongation and decreasing the lamina joint, which promotes higher growth density and grain yield. While *OsLIC* represses transcription of *OsILI1*, *OsBZR1* represses *OsIBH1*. Therefore, *ILI1* and *IBH1* are good potential candidate genes for breeders hoping to design optimal rice architecture (Zhang et al., 2012).

Tiller number: BRs are involved in tillering and branching in rice and Arabidopsis. Although there is no genetic evidence for an association between hight and tillering, in rice plants its known that tiller number and height are usually negatively related (dwarf plants generally present more tillers and vice-versa) (Zhang et al., 2014). *OsDLT* is a GRAS family member that encodes a transcription factor whose members SLR1, MOC1 and SCR play critical roles in gibberellins (GA) signaling, tillering and root development, respectively (Di Laurenzio et al., 1996). It is possible that BR regulates the expression of *OsDLT* trough *BZR1*, which binds to the CGTG(C/T)G elements found in the *DLT* promoter, inhibiting *DLT* expression and thereby controls rice tillering (Tong et al., 2009). As mentioned before, *BZR1* has a positive effect on cell elongation, increasing plant heigh, and we can speculate that *BZR1* controls tillering by suppressing *DLT* expression.

Stress response: Loss-of-function of *OsGSK3* (a kinase that downregulates BR responses) improved tolerance to cold, heat, salt and drought stresses in rice, showing that BR plays a positive role in various stress responses (Koh et al., 2007). In addition, BR might contribute to enhance stress responses by influencing other hormone effects: ABA and BR crosstalk promote stomatal closure, process mediated by nitric oxide (NO), suggesting an interplay between BR and abiotic stress tolerances (Choudhary et al., 2012; Cui et al., 2011; Haubrick et al., 2006 and Zhang et al., 2011).

Biomass, grain size, filling and yield: *OsBZR1* interacts with genes responsible for sugar partitioning in pollen and seed, increasing grain weight and number in rice, indicating that BR promotes grain filling (Wu et al., 2008). Sterol C-22 hydroxylase is an important enzyme which participate of *DFW-11* synthesis, one of the major gene of BR biosynthesis, and its overexpression in maize enhanced seed size and biomass (Wu et al., 2008). Overexpression of *D11* (a BR biosynthetic gene) and *OsBZR1* resulted in high sugar accumulation in seeds and

higher grain yield (Zhu et al., 2015²). The treatment of rice plantlets with 24-epibrassinolide, a synthetic BR, increased seed fresh weight by 22% and seed dry weight by 31.5%. BL treatment also increases plant growth rate, root size and dry weight of root and stem (Zullo and Adam, 2002). In addition, field grown sugarcane sprayed with ethephon, an ethylene-releasing compound, has high levels of BR transcripts in the internodes, when comparedc to plants treated with AVG, an ethylene inhibitor (Cunha et al., 2017).

Considering the research in the literature describing the importance of BR in many physiological processes, in this chapter we aim to analize how the modulation of two key transcriptional factors, *BZR1* and *LIC*, interfere with sugarcane growth, development and stress response, as well as provide a molecular analysis of BR signaling pathway in this specie. Due to the lack of information of how BR works in sugarcane, the present work will open new perspective to understand and modulate agronomic traits by modifying BR levels.

5.2 Material and methods

5.2.1 Identification of sugarcane genes involved in BR biosynthesis and signaling

To identify sugarcane genes, the coding sequences (CDS) and protein sequences from rice and sorghum were used to perform a BLAST in the CTBE database (http://bce.bioetanol.cnpem.br/ctbeblast/) (Mattiello et al., 2015) and in another sugarcane genome database, the SUCEST-FUN (https://sucest-fun.org/). All sequences used as baits in the search of the sugarcane homologs and all designed vectors used in this project are listed in session 8.1 of "Attachments".

The selected genes, as well as their main characteristic, are listed in the table 1.

Gene	Main characteristic
ScBZR1	Positive transcriptional factor of BR signaling pathway
ScLIC	Negative transcriptional factor of BR signaling pathway
ScBRI	BR receptor
ScGSK3	Negative regulator of BR signaling pathway
ScDLT	Acts on tillering effect
ScILI1	Leaf architeture
ScIBH1	Leaf architeture
ScDWF4	BR biosynthetic gene

Table 1: Genes of the BR biosynthetic and signaling pathways used in RT-qPCR analyses.

Another gene used in this project was *ScPDS*, coding a limiting step of carotenoid biosynthesis, an important marker gene involved in carotene biosynthesis pathway. We choose this gene to be a positive control for gene silencing in sugarcane, since its silencing causes an albino phenotype in leaves, making easy the identification of successful silencing (Fraser et al., 1994). The identification of this gene followed the same steps of BR genes.

5.2.2 Design of DNA constructs

For overexpressing analysis, the CDS regions from *ScBZR1* (1,004 bp) and *ScLIC* (761 bp) were used to design the vectors. For silencing constructions, we used different approaches for each gene. For *ScBZR1*, which possesses a highly specific domain named BZR1 (141 bp), we choose the RNAi technique (RNA interference) using a region of 250 bp flanking the domain to design the silencing vector. For *ScLIC*, which did not present any specific domain, we choose the amiRNA (artificial micro RNA) method to minimize off-targets. AmiRNA acts similarly to natural micro RNA in plants (Bartel, 2004 and Jones-Rhoades et al., 2006). The Web MicroRNA Designer (WMD) (http://wmd3.weigelworld.org) was designed to analyze the target sequence and choose the most promissory amiRNA affording hybridization properties, minimizing off-targets (Ossowski et al., 2008). Based on this, we used the CDS sequence of *ScLIC* to search the best two scored amiRNA (named amiRNALIC1 and amiRNALIC2) and designed the reverse complement, amiRNA*, (named amiRLIC1* and amiRNALIC2*, respectively). Both pairs of amiRNAs were designed in separated vectors compatible with Agrobacterium. We sought that transformed calli containing the two amiRNA precursors would have a higher efficiency in silencing the *ScLIC* gene.

At this stage of the experiment, we were not aware of any work with gene silencing by amiRNA in sugarcane. So, as mentioned before, the *ScPDS* gene was used as a control, due to the easy identification of the silenced phenotype (plants with silenced *PDS* gene do not produce carotenoids efficiently and present photobleached, white leaves).

The amiRNA silencing vector were designed containing the stem-loop region (amplicon) of the natural miRNA MIR528 from rice, a model monocotyledonous plant and close related to sugarcane. In this template, the original sequences of miRNA (mi528) and miRNA* (mi528*) were replaced by the designed sequences by WMD: amiRNALIC1 e

amiRNALIC1*, amiRNALIC2 e amiRNALIC2*, respectively (for the *ScLIC* silencing vector) and amiRNAPDS e amiRNAPDS*, respectively (for the *ScPDS* silencing vector).

Moreover, in the *ScPDS* vector, we added restrictions sites of SnaB and AattII enzymes flanking the amiRNA and amiRNA* regions, respectively, which allows this vector to be used as a template, facilitating the design of vectors with new amiRNAs. The proof-of-concept of the use of amiRNA for silencing sugarcane genes was described in chapter 2.

The insert containing the miRNA precursors were chemically sinthesized and cloned in the Entry vector pBKS (GenOne Biotechnologie, Brazil) between the gateway recombination sites *attL1* and *attL2*. Then, the miRNA precursor was recombined with the final vector, pGVG, designed for sugarcane transformation (Guidelli et al., 2018). To this end, we used the Gateway LR Clonase II Enzyme, (Life Technologies). The final vectors (listed in the section 8.2 and 8.3 of the "Attachments") were used for chemical transformation of competent cells of DH5α *E. colli*. DNA minipreparations were confirmed to the presence of transgene through double digestion with single site restriction enzymes, where one enzyme cuts into the transgene and the other cuts in a region of destination vector. All cut sites were mapped by SnapGene Viewer and Double Digest (Thermo Fisher Scientific, USA) (http://www.thermoscientificbio.com/webtools/doubledigest). After the digestion, the products were submitted to electrophoresis gel (agarose 1%) to confirm the expected band patterns. The minipreparations were also sent to gene sequencing to confirm the correct sequence of the transgenes.

5.2.3 *Agrobacterium* transformation and tissue culture

After confirmation of recombination reaction by double digestion and gene sequencing, a DNA positive minipreparation of each transgenic construction was used to perform EAH105 *Agrobacterium tumefaciens* heat chock transformation. In the case of the amiRNA vectors, each vector was transferred independently to Agrobacterium and a mix of the two cultures were used in co-transformation of sugarcane calli by PangeiaBiotech (Campinas, Brazil). Briefly, *A. tumefaciens* were incubated with SP803280 sugarcane cultivar calli under vacuum condition for 5 minutes and then transferred to a sequence of media as described bellow (Table 2):

	Medium description	**temperature**	**conditions**	**time**

1	4.33 g/L MS salt, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.15 g/L citric acid, 25 g/L sucrose e 3,5 g/L phytagel	22ºC	darkness	3 days
2	4.33 g/L MS salt, 1 mL/L MS vitamins, 3 mg/L 2,4-D, 0.5 g/L casein hydrolysate, 0,15 g/L citric acid, 25 g/L sucrose, 100 mg/L prolin, 2,8 g/L phytagel e 200 mg/mL timentin	26ºC	darkness	6 days
3	4,33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 5 mg/mL $CuSO_4$, 1 mg/mL benzylaminopurine (BAP), 7 g/L agar, 200 mg/mL timentin e 40 mg/L geneticin	26ºC	16h photoperiod	14 days
4	4,33 g/L MS salts, 1 mL/L MS vitamins, 25 g/L sucrose, 7 g/L agar, 200 mg/mL timentin e 40 mg/L geneticin	26ºC	16h photoperiod	+

Table 2: Media used in sugarcane tissue culture.

Five transgenic constructions resulted after transformation (Table 3). We obtained 25 transgenic events of each transgenic construction and 25 wild type plants (WT), which were used as control for all experiments. The plantlets were kept in tissue culture to multiply the tillers until transferring to the greenhouse.

Construct	**Gene**	**Technique**
pScBZR1_OE	*BZR1*	Overexpression
pScLIC_OE	*LIC*	Overexpression
pScBZR1_HP	*BZR1*	Silencing by RNAi
pScPDS_amiRNA	*PDS*	Silencing by amiRNA
pScLIC_amiRNA (co-transformation of ScLIC1_amiRNA and ScLIC2_amiRNA)	*LIC*	Silencing by amiRNA

Table 3: Constructs used in the project.

5.2.4 Greenhouse acclimation and morphological analyses of sugarcane plants

Three biological replicates of each transgenic event and WT, obtained from tillers of in vitro grown seedlings, were acclimatized in a greenhouse at the Functional Genome Laboratory, Institute of Biology, Campinas, Brazil (22°49'09.2"S, 47°04'15.4"W). Sugarcane plantlets were transferred to a 500 mL plastic pots containing a mix of soil Tropstrato HT Hortaliça (VidaVerde, Brazil) and vermiculite (1:1 v/v) under daily irrigation. A plastic cup were used to cover the plantlets during 15 days, to keep high humidity and prevent dehydration (figure 3).

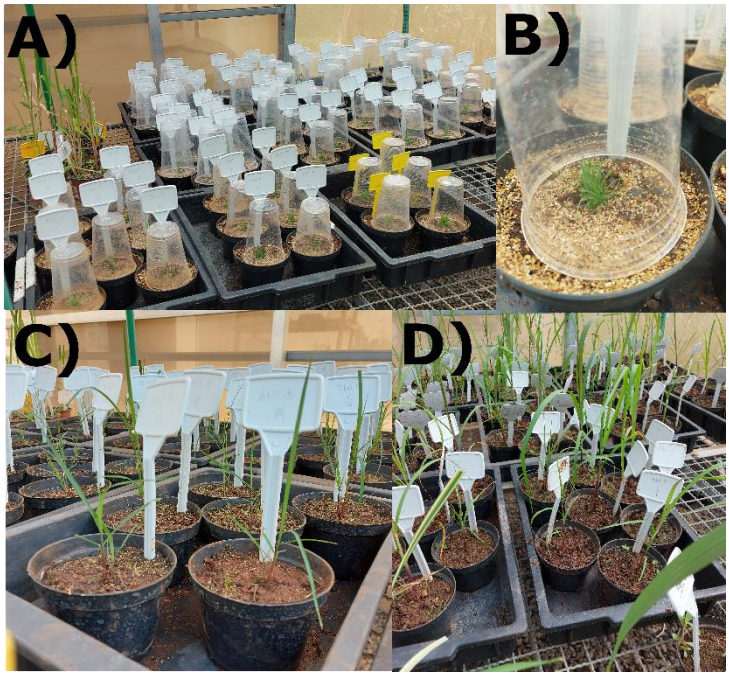


Figure 3: Acclimation of sugarcane plantlets from tissue culture under greenhouse conditions. A) Each plastic pot corresponds to a biological replicate of each transgenic event. B) Close-up of a plastic cup used to keep high humidity of plantlets. C) Plantlets after 15 days of acclimation. D) Plants after 1 month of acclimation.

After 1 month, one single tiller of each plant was transferred to a 5L plastic pot containing a mix of soil Tropstrato HT Hortaliça (VidaVerde, Brazil), sand and latosol (1:1:1 v/v) under daily irrigation (figure 4).

Figure 4: 1 month old plants after transfer to 5 L plastic pots.

The plants were kept with daily irrigation in the greenhouse for 3 month until we collected the leaves for molecular analyses. The transgenic events were selected based in the RT-qPCR data (see below) and transferred to 20 L plastic pots containing the same substrate and kept under automatic irrigation for further morphological analyses. Sugarcane plants were organized using a randomized block design.

We measured three biometric factors (stalk number, main stalk height and diameter) at two different stages of sugarcane development: 3 month old (before we collect the leaves to perform RT-qPCR) and 10 month old (before start the water deficit stress test). The diameter were measured at the base of the plant using a caliper rule and the heights were measured considering the space from the base until the insertion of the leaf +1.

5.2.5 Total RNA extraction

Leaves from three biological replicates from 3 month-old plants of each transgenic events and WT were collected, frozen in liquid nitrogen and macerated with a Mini-BeadBeater (Biospec Product, USA) for 1 minute. Total RNA were extracted using a guanidine-based protocol (Logemann, 1987). Samples were treated with DNaseI (Fermentas) and cDNAs were synthesized using cDNA Synthesis Kit with RNAse Inhibitor (Cellco), according manufacturer's recommendations. Integrity of the RNA samples were confirmed by electrophoresis on a denaturation gel (1%) before cDNA synthesis. The cDNAs were quantified after synthesis by electrophoresis of agarose gel 1%.

5.2.6 Gene expression analyses by RT-qPCR

Gene expression in leaf tissue were confirmed by RT-qPCR (three biological and three technical replicates of each transgenic events and WT), using GoTaq qPCR Master Mix (Promega), on 7500 Real Time PCR System (Applied Byosistems), following manufacture's recommendations. A polyubiquitin gene were used for data normalization (PUB) (SCCCST2001G02.g; NCBI accession CA179923.1) (Papini-Terzi, 2005). The sequences of primers used in qPCR reactions were listed in Table 4.

Gene	Seq FW 5'-3'	Seq RV 3'-5'
poliubiquitina	CCGGTCCTTTAAACCAACTCAGT	CCCTCTGGTGTACCTCCATTTG
ScBZR1	CCGAAAGGGATGCAAGCCG	GACCACAGCAGCGGCA
ScLIC	ACCAGCAGCCCACACCTC	GCTCACCTCAGGAGTGGTCC
ScPDS	CTGGTGCAGGATTGGCTGGT	GGGGATTGGTACGAGACTGGGC
ScBRI	CGCCATCCCGGAGTCAATCT	ACCGAGCATTCCGAGGGATG
ScGSK3	AGGACAAGCGCTACAAGAATAG	GCTTGTGGTAGAGAAGAAGCA
ScDLT	GAAGACGTCCGCCTGTGGAT	ACGAGGACGCACTCAACTCC
ScILI1	GACACGTGCAGCTACATCA	CTGGTCGCTGGTGACATC
ScIBH1	CGCCGAGATGGAGTACTGCA	TGTTGGGCGGAGAAGAGGTC
ScDWF4	CTCCCCAAGATCGACGCCTT	CATCAGAGAGTGGGCCAGCA

Table 4: Sequences of the primers used in the RT-qPCR reactions.

Relative quantification of genes were made by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). WT plants were used as control (fold change = 1) in $^{-\Delta\Delta CT}$ calculation. The analyses were conducted using GraphPad Prism version 6.01.

In relation to *ScBZR1* and *ScLIC* expression levels, the best four independent events of each transgenic line (the best four overexpressing events of pScBZR1_OE and pScLIC_OE and the best four silencing events of pScBZR1_HP and pScamiRNA_LIC) were selected to remain in the greenhouse to perform water deficit stress assay.

5.2.7 Drought stress assay and biomass analyses

Drought stress assay was performed using the best four independent events of each transgenic construction, i.e., the lowest *ScLIC* and *ScBZR1* expression in silenced lines and highest expression in overexpressing lines. We used four biological replicates of each transgenic event, as well as of WT plants, used as control group. The plants were kept in the same pots and reorganized using a randomized block design.

To evaluate the physiological response of sugarcane to water deficit we measured the following gas exchange parameters using a portable photosynthesis system (LCi ADC BioScientific Ltd) with ambient CO_2 and artificial light system: photosynthesis (A), stomatal conductance (gs) and transpiration rate (E). The parameters measuring were taken following the sequence: day zero (D0) all plants were soaked in water and then the irrigation was suspended. The drought condition was evaluated at days two (D2), four (D4), eight (D8), when the plants presented severe signals of dehydration and/or showed photosynthesis rate close to

zero. Then, the plants were rehydrated and monitored at days ten (D10) and twelve (D12). All measuring were taken in the leaf +1, from 8:00 am to 11:00 am. One month after the rehydration period, the fresh weight of stalk and leaves were evaluated. Data were analyzed using ANOVA (p-value < 0.05), followed by a T-*Student* test (T-test, p-value < 0.05) to compare means, using GraphPad Prism version 6.01.

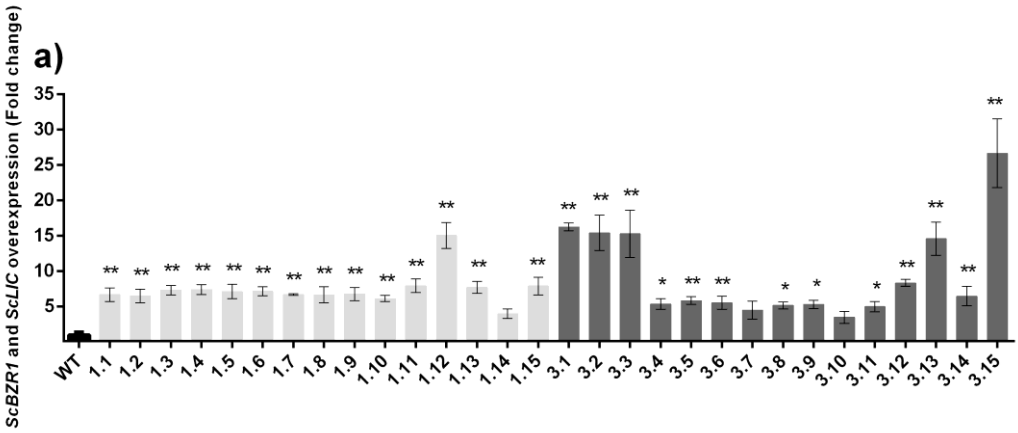
5.2.8 Evaluation of oxidative stress

Foliar discs with 0.5 cm diameter from four independent events from transgenic lines and WT were incubated during 36 hours in Petri dishes containing 1 mL of H_2O_2 in the following concentrations: 0 M, 0.5 M and 1 M. We used three biological replicates of each independent event and WT. After oxidative stress, we chlorophyll was extracted by maceration in absolute ethanol and quantified as described by (Arnon, 1949). Data were analyzed as described above.

5.3 Results and discussion

5.3.1 Expression analysis of *ScBZR1* and *ScLIC* in transgenic sugarcane plants

The expression levels in each transgenic line and WT were quantified (Figure 5).



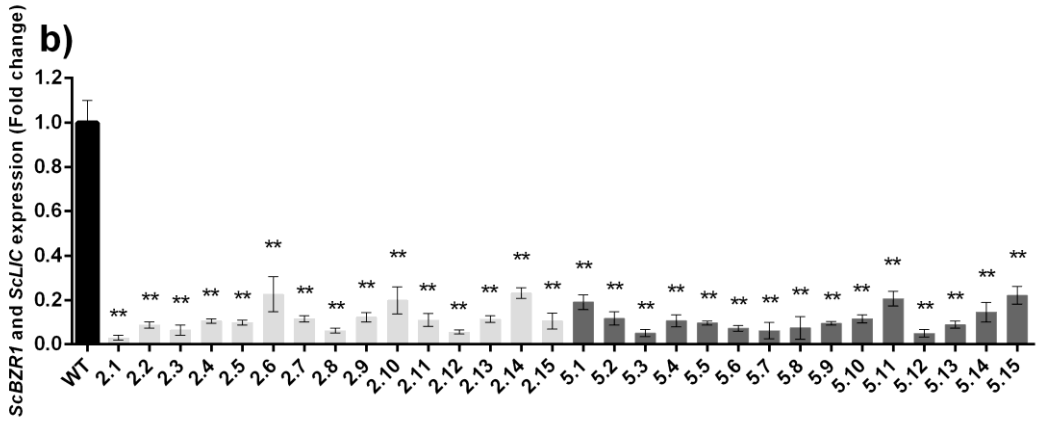


Figure 5: Gene expression analyses (fold change) of WT plants (black bar) and transgenic events (gray bars) via RT-qPCR. A) Gene expression of independent events of pScBZR1_OE line (light gray bars) and pScLIC_OE line (dark gray bars). B) Gene expression of independent events of pScBZR1_HP line (light gray bars) and pScLIC_amiRNA line (dark gray bars). The X axis numbers correspond to 15 independent events analyzed of each transgenic line. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=3).

In the pScBZR1_OE line, all independent event except 1.14 were statistically different to WT. In pScLIC_OE line, all independent events except 3.7 and 3.10 were statistically different to WT (figure 5A). For both pScBZR1_HP and pScLIC_amiRNA lines, all 15 independent events were statistically different to WT (figure 5B).

We selected the best four transgenic events of each transgenic line for the subsequent analysis in greenhouse, i.e: events with the highest expression for overexpressing lines and events with the lowest expression for silencing lines. The selected independent events are listed in decreasing order of expression (Table 5).

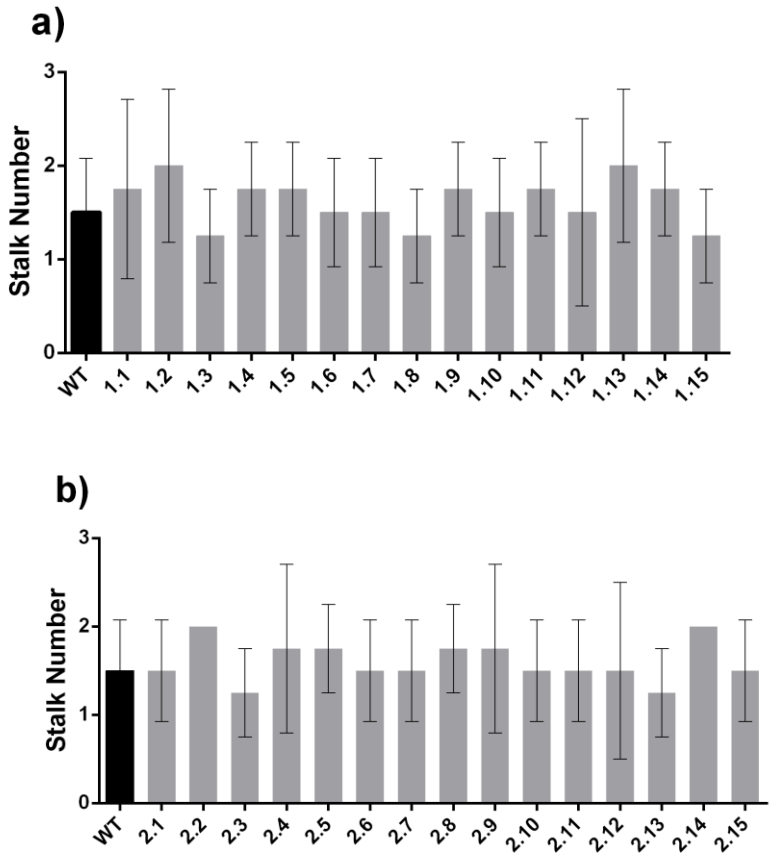
Constructions	Independent event	Expression level related to WT
pScBZR1_OE	1.12	15-fold increase
	1.11	8-fold increase
	1.15	8-fold increase
	1.13	7.5-fold increase
pScBZR1_HP	2.3	15.5-fold reduction
	2.8	16.5-fold reduction
	2.12	20-fold reduction
	2.1	33-fold reduction
pScLIC_OE	3.15	26.7-fold increase
	3.1	16-fold increase
	3.2	15.5-fold increase

	3.3	15-fold increase
pScLIC_amiRNA	5.6	14-fold reduction
	5.7	17-fold reduction
	5.3	20-fold reduction
	5.12	20-fold reduction

Table 5: Selected independent events of each transgenic line for the analyses in greenhouse. Expression levels were measured by RT-qPCR using WT plants as a control.

5.3.2 Biometric analyses

Biometric parameters (tiller number, main stalk height and diameter) were taken from 3 month old plants (before the sampling of leaves for RT-qPCR analyses) (figures 5, 6 and 7) and from 10 month old plants (before the start of the water deficit stress) (figures 8, 9 and 10).



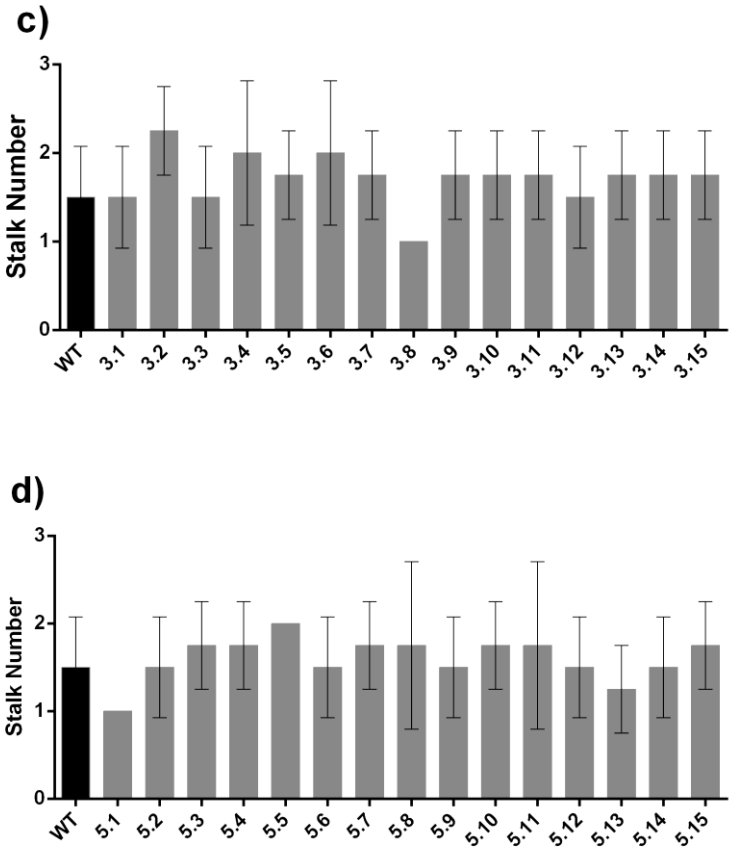
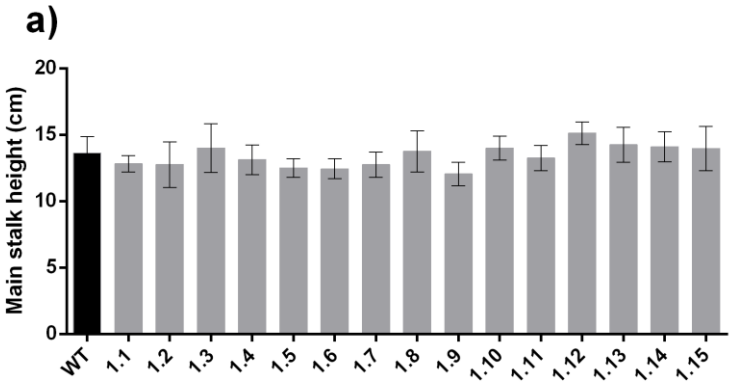


Figure 6: Stalk number of 3-month-old sugarcane plants in greenhouse. A) pScBZR1_OE line. B) pScBZR1_HP line. C) pScLIC_OE line. D) pScLIC_amiRNA line. In all cases, black bars are WT plants and gray bars are the independent events of respectively transgenic lines. Data are presented as the mean ± SE plants (n=4).



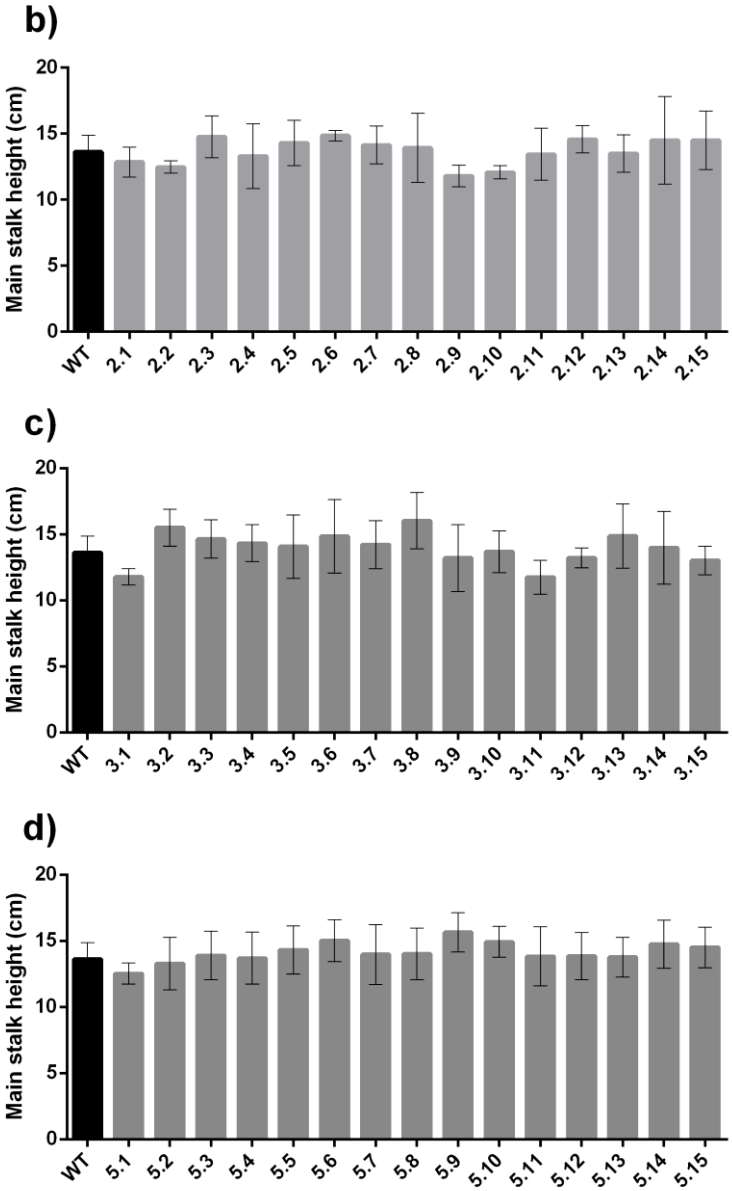


Figure 7: Main stalk height of 3-month-old sugarcane plants in greenhouse. A) pScBZR1_OE line. B) pScBZR1_HP line. C) pScLIC_OE line. D) pScLIC_amiRNA line. In all cases, black bars are WT plants and gray bars are the independent events of respectively transgenic lines. Data are presented as the mean ± SE plants (n=4).

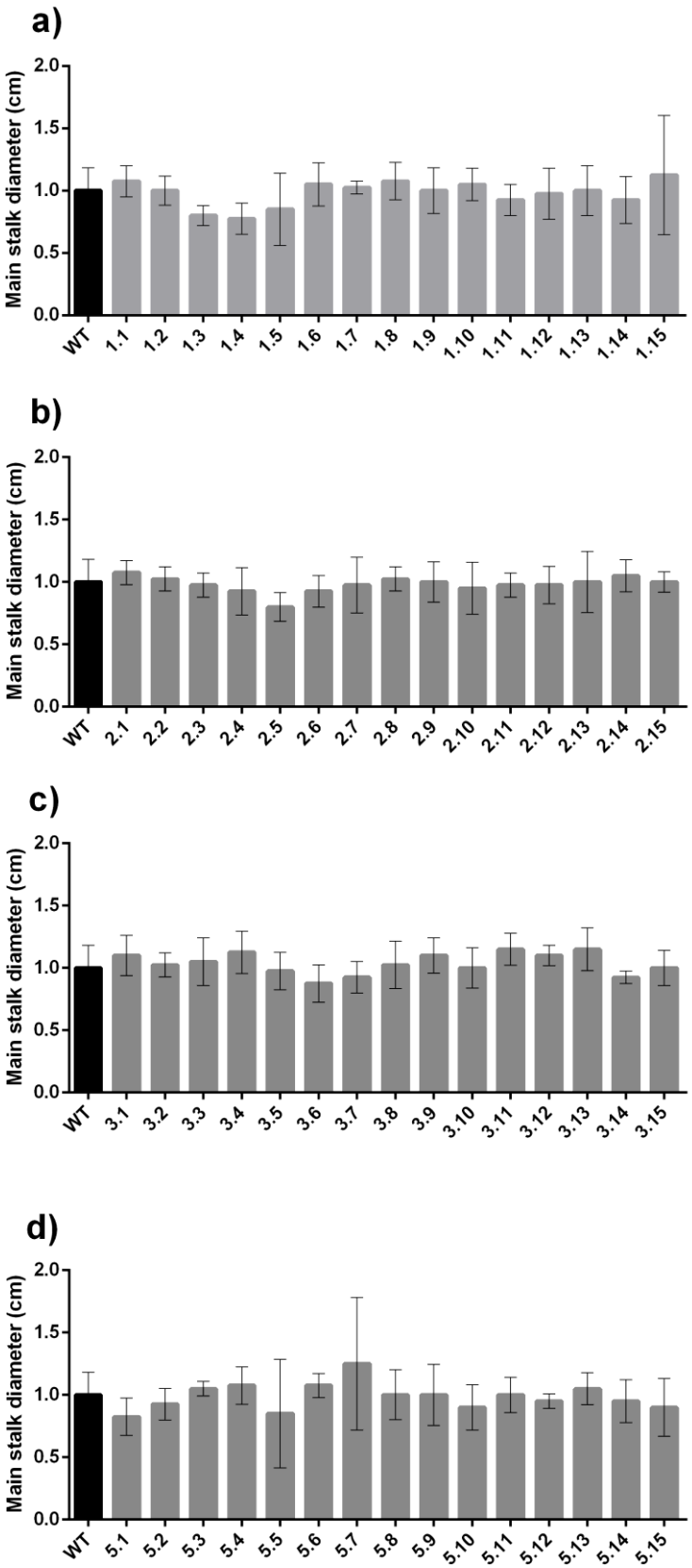

a)
Main stalk diameter (cm)
2.0
1.5
1.0
0.5
0.0
WT
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
1.10
1.11
1.12
1.13
1.14
1.15
b)
Main stalk diameter (cm)
2.0
1.5
1.0
0.5
0.0
WT
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
2.9
2.10
2.11
2.12
2.13
2.14
2.15
c)
Main stalk diameter (cm)
2.0
1.5
1.0
0.5
0.0
WT
3.1
3.2
3.3
3.4
3.5
3.6
3.7
3.8
3.9
3.10
3.11
3.12
3.13
3.14
3.15
d)
Main stalk diameter (cm)
2.0
1.5
1.0
0.5
0.0
WT
5.1
5.2
5.3
5.4
5.5
5.6
5.7
5.8
5.9
5.10
5.11
5.12
5.13
5.14
5.15

Figure 8: Main stalk diameter of 3-month-old sugarcane plants in greenhouse. A) pScBZR1_OE line. B) pScBZR1_HP line. C) pScLIC_OE line. D) pScLIC_amiRNA line. In all cases, black bars are WT plants and gray bars are the independent events of respectively transgenic lines. Data are presented as the mean ± SE plants (n=4).

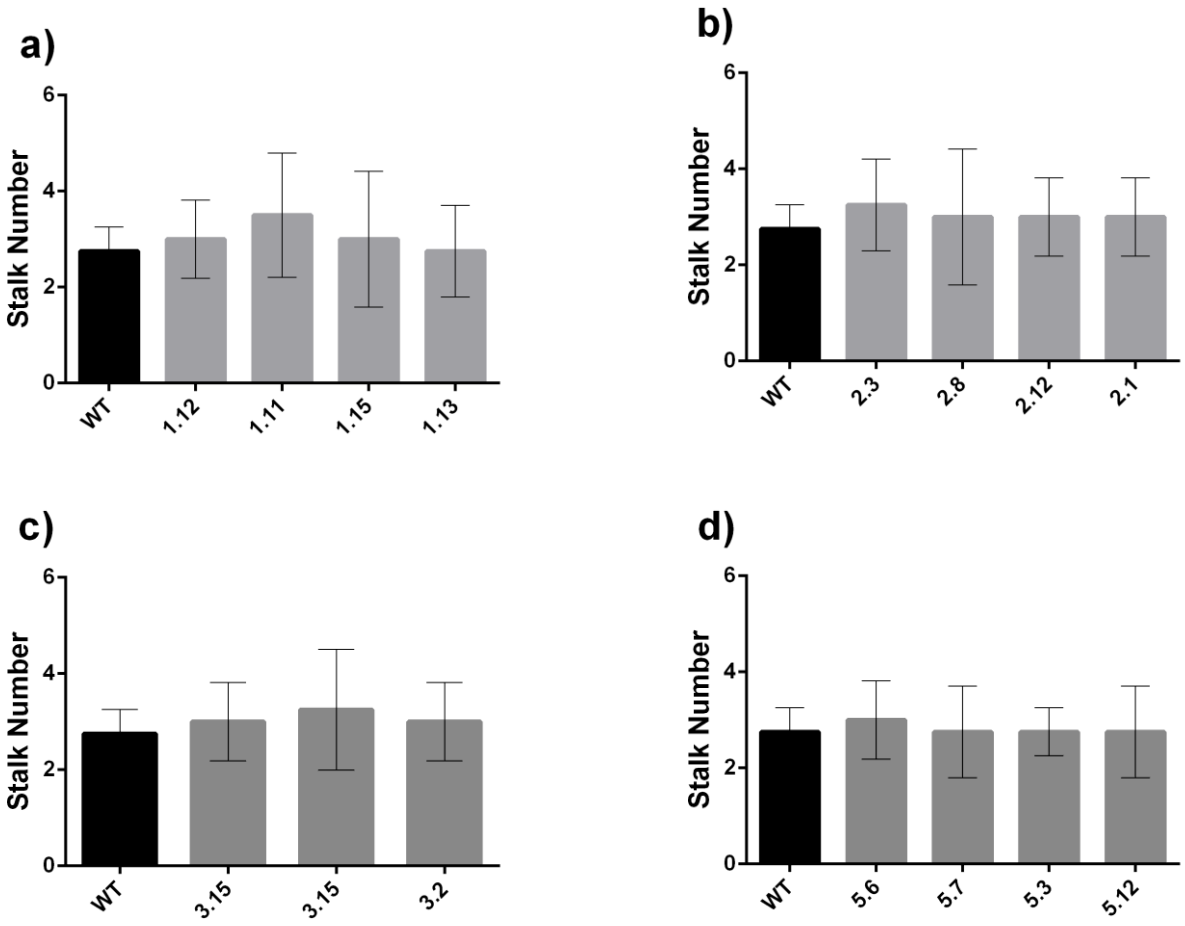


Figure 9: Stalk number of 10-month-old sugarcane plants in greenhouse. A) pScBZR1_OE line. B) pScBZR1_HP line. C) pScLIC_OE line. D) pScLIC_amiRNA line. In all cases, black bars are WT plants and gray bars are the independent event of respectively transgenic lines. Data are presented as the mean ± SE plants (n=4).

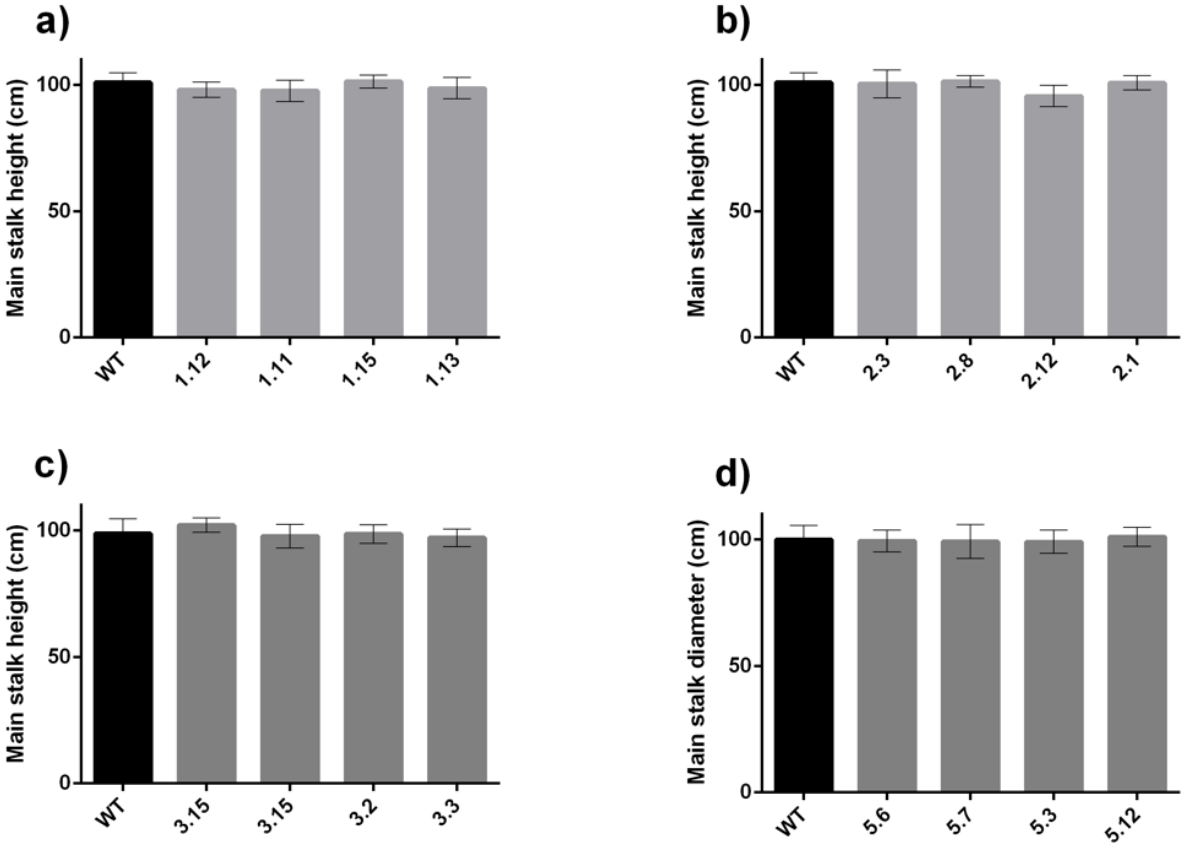
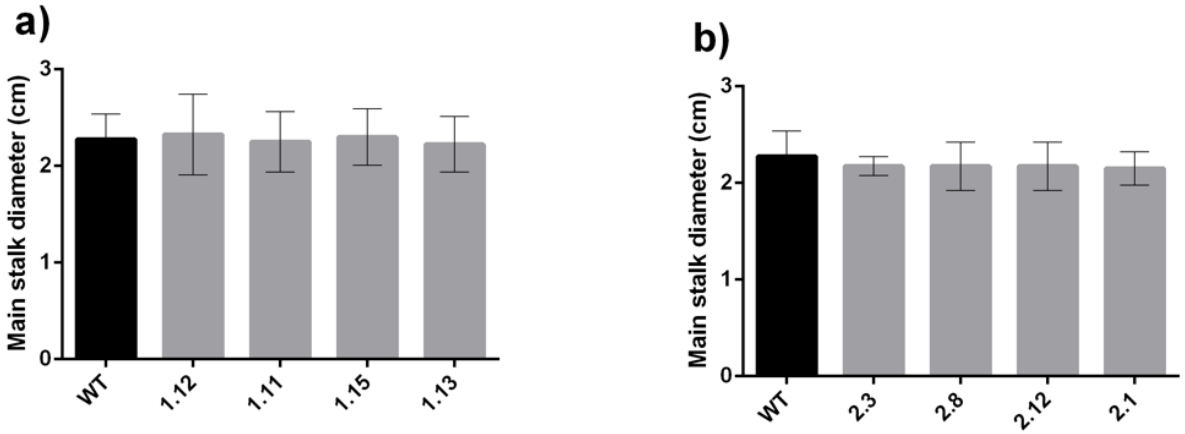


Figure 10: Main stalk height of 10-month-old sugarcane plants in greenhouse. A) pScBZR1_OE line. B) pScBZR1_HP line. C) pScLIC_OE line. D) pScLIC_amiRNA line. In all cases, black bars are WT plants and gray bars are the independent event of respectively transgenic lines. Data are presented as the mean ± SE plants (n=4).



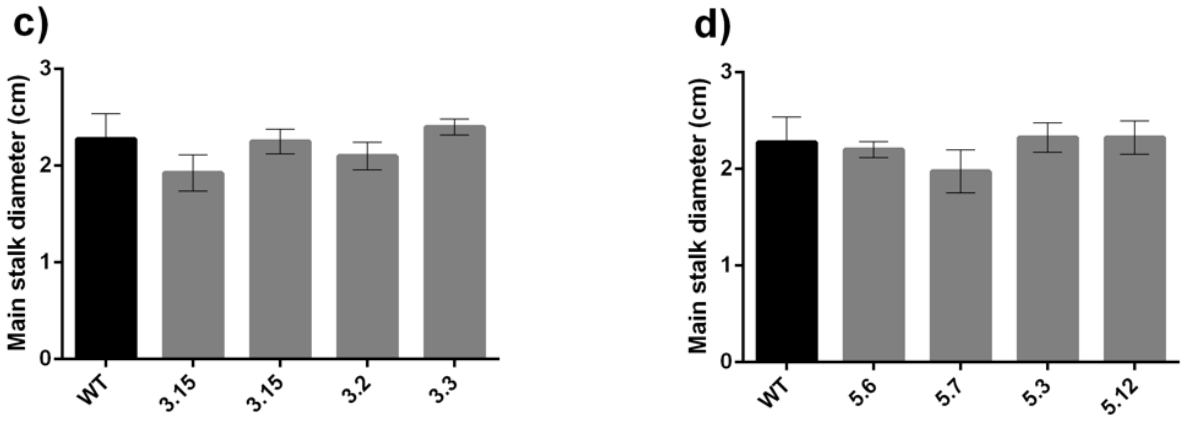


Figure 11: Main stalk diameter of 10-month-old sugarcane plants in greenhouse. A) pScBZR1_OE line. B) pScBZR1_HP line. C) pScLIC_OE line. D) pScLIC_amiRNA line. In all cases, black bars are WT plants and gray bars are the independent event of respectively transgenic lines. Data are presented as the mean ± SE plants (n=4).

As mentioned previously, we expected that the lines with higher *ScBZR1* expression (pScBZR1_OE) and those silenced for *ScLIC* (pScLIC_amiRNA) would present higher growth rate due to the fact the BZR1 transcriptional factor regulates the promoter of several genes associated growth and development processes by activating BR signaling cascade. On the other hand, we expected that the lines with lower *BZR1* expression (pScBZR1_HP) and higher *LIC* expression (pScLIC_OE) would result in the opposite due to suppression of the BR signaling pathway. However, all biometric parameters analyzed in 3 and 10-month-old transgenic plants did not present any statistic difference in relation to WT plants.

Little is known in the literature about BR signaling pathway of monocots, compared to the knowledge in Arabidopsis. Considering the number of different components in the two pathways, we can spectulate that monocots present other genes that also play a role on the action of *BZR1* and *LIC*, and this would contribute to the lack of phenotype observed in transgenic sugarcane plants.

5.3.3 Expression profile of genes of BR signaling and biosynthesis pathways in transgenic sugarcane

Besides *ScBZR1* and *ScLIC*, we decide to evaluate how other genes of BR signaling and biosynthesis pathways were affected in transgenic sugarcane lines if comparing to WT. The selected genes and their main characteristic were already listed in Table 1.

First, we measured *ScBZR1* expression levels in LIC transgenic lines (pScLIC_OE and pScLIC_amiRNA) and *ScLIC* expression levels in BZR1 transgenic lines (pBZR1_OE and pBZR1_HP) (figure 12). The independent events are listed in decreasing order of gene expression in order to evaluate putative correlations.

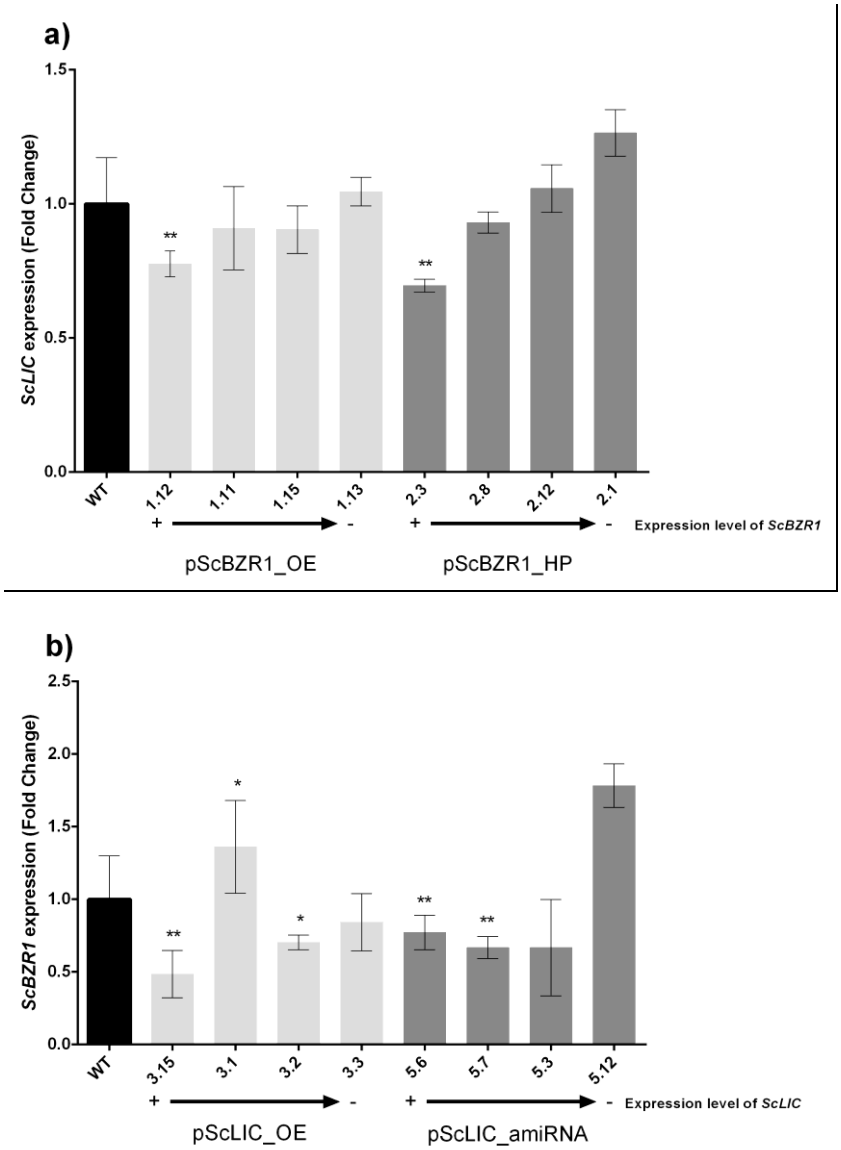


Figure 12: A) Gene expression levels analysis (fold change) of *ScLIC* in BZR1 transgenic lines (LIC *versus* BZR1) by RT-qPCR. The light gray bars are *ScLIC* expression in independent events of pScBZR1_OE and dark gray bars are *ScLIC* expression in independent events of pScBZR1_HP. The black bars are *ScLIC* expression in WT plants. B) Gene expression levels analysis (fold change) of *ScBZR1* in LIC transgenic lines (BZR1 *versus* LIC) by RT-qPCR. The light gray bars are *ScBZR1* expression in independent events of pScLIC_OE and dark gray bars are

ScBZR1 expression in independent events of pScLIC_amiRNA. The black bars are *ScBZR1* expression in WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

According to the literature, it was expected that the highest levels of *ScBZR1* would be related with the lowest levels of *ScLIC* and *vice-versa*, regarding the fact that both genes present antagonist actions in BR signaling pathway. Nevertheless, in general, the present data did not showed a clear opposite effect between these two genes. The feedback mechanism between them is important to prevent unnecessary activation of BR cascade as observed in rice (Zhang et al., 2012). We suspect that due to the complexity of the sugarcane genome, other transcriptional factors might be the responsible of the lack of contrasting results in the expression analyses. In any case, our data indicated sugarcane does not follow the same pattern observed in other species so far.

Analyzing *ScBRI1* expression, the major receptor of BR, we observed that most events overexpressing either *ScBZR1* or *ScLIC* had higher expression of *ScBRI1* compared to WT plants (Figure 13). According with previous data in the literature presented in chapter 1, the activation of BR signaling pathway depends on the BR content. In the lines with high amounts of *ScBZR1* (pScBZR1_OE) the signaling cascade would be already activated, so *ScBRI1* would not be active. On the contrary, in the events with high amounts of *ScLIC*, when the signaling cascade is being repressed, there would be an attempt to re-activate BR signaling pathway, increasing the levels of *ScBRI1* expression. In fact, these plants had a trend of higher expression of *ScBRI1*. In the events silenced for either of these two genes (Figure 13), the expression pattern was highly variable among the transgenic events and no clear conclusion could be drawn.

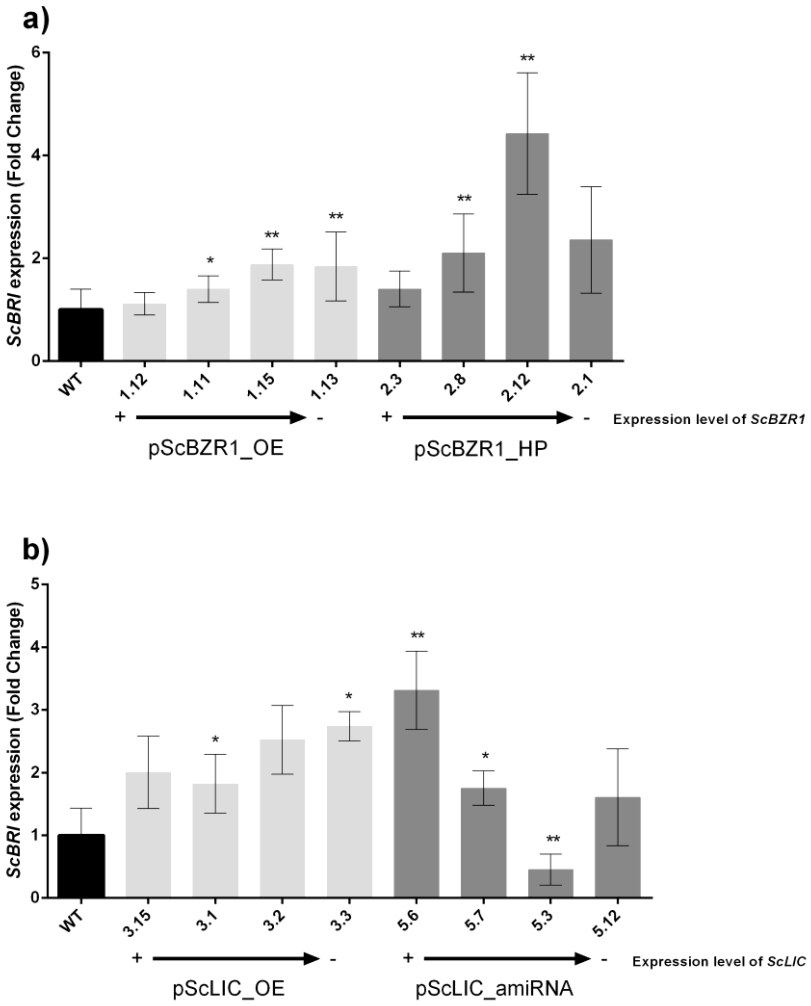


Figure 13: A) Gene expression levels analysis (fold change) of *ScBRI1* in BZR1 transgenic lines by RT-qPCR. The light gray bars are *ScBRI* expression in independent events of pScBZR1_OE and dark gray bars are *ScBRI* expression in independent events of pScBZR1_HP. B) Gene expression levels analysis (fold change) of *ScBRI1* in LIC transgenic lines by RT-qPCR. The light gray bars are *ScBRI* expression in independent events of pScLIC_OE and dark gray bars are *ScBRI* expression in independent events of pScLIC_amiRNA. In both cases, black bars are *ScBRI* expression in WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

In the figure 14, we can observe the expression of *ScGSK3*, the major repressor of BR signaling pathway. It was expected that higher levels of *ScBZR1* are related to the lowest levels of *ScGSK3* (Koh et al., 2007 and Li and Nam, 2002), but this was observed only in the event 1.12, with the highest level of *ScBZR1* expression. The silencing of *ScBZR1* had no clear effect on *ScGSK3* expression.

No relation was observed between *ScGSK3* and *ScLIC* overexpression. The main cause might be associated to the fact that *ScGSK3* and *ScLIC* are both repressors of BR signaling pathway, suggesting that the repressing of signaling cascade it is already being done by the higher *ScLIC* content. However, the silencing of *ScLIC* did not cause any changes in *ScGSK3*. Clearly, the regulation of these genes in sugarcane does not mimick the regulation in other species.

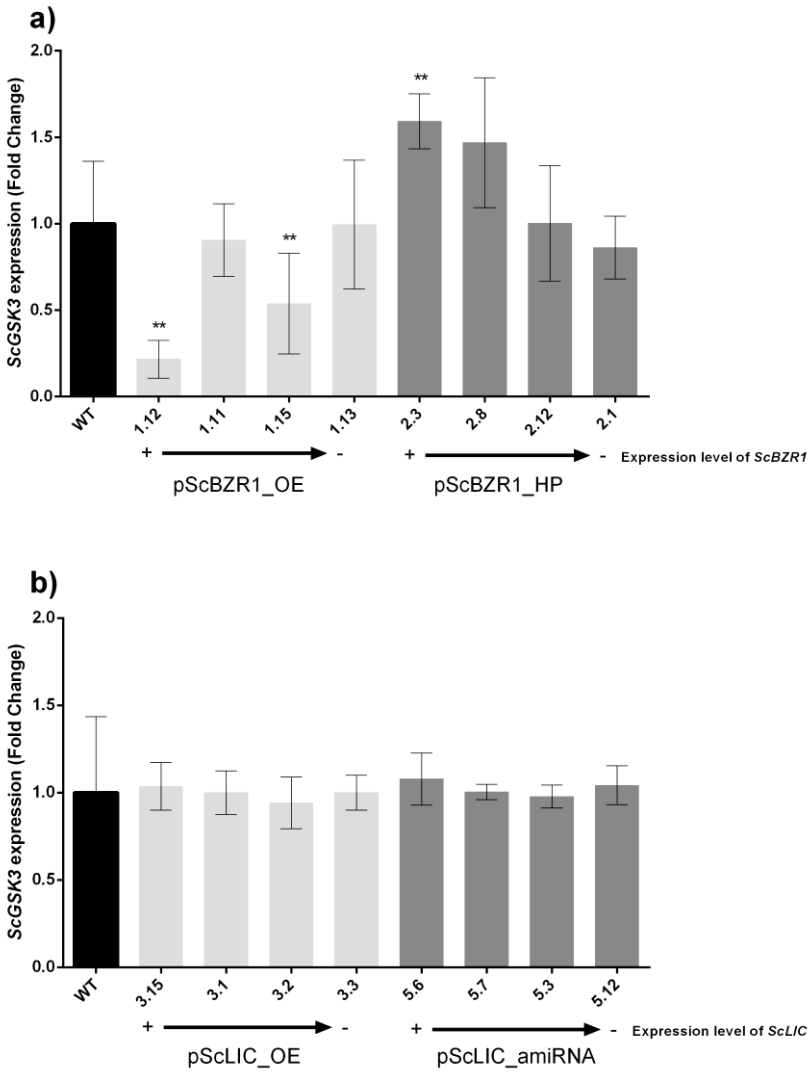


Figure 14: A) Gene expression levels analysis (fold change) of *ScGSK3* in BZR1 transgenic lines by RT-qPCR. The light gray bars are *ScGSK3* expression in independent events of pScBZR1_OE and dark gray bars are *ScGSK3* expression in independent events of pScBZR1_HP. B) Gene expression levels analysis (fold change) of *ScGSK3* in LIC transgenic lines by RT-qPCR. The light gray bars are *ScGSK3* expression in independent events of pScLIC_OE and dark gray bars are *ScGSK3* expression in independent events of pScLIC_amiRNA. In both cases, black bars are *ScGSK3* expression in WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

The next gene evaluated was *ScDLT*, which is involved in tillering process. Although there is no studies in the literature providing a direct comparison of *LIC* and *DLT*, it is known that *dlt* mutants have a dwarf phenotype, similar to BR-deficient or -signaling mutants in rice, because *BZR1* binds to the *DLT* promoter, inhibiting its expression (Tong et al., 2009). This model was confirmed in the events overexpressing *ScBZR1*, which showed lower levels of *ScDLT*. Interestingly, there was a trend of higher levels of *ScDLT* in *ScBZR1*-silenced plants (figure 15a). Moreover, higher levels of *ScDLT* were also observed in *ScLIC* overexpressing lines, and particularly in the event 3.15, which presented the lowest level of *ScBZR1* (see figure 5). In addition, the two transgenic events with the lowest expression levels of *ScLIC* (5.3 and 5.12) had the lowest level of *ScDLT* (event 5.3 had the highest level of *ScBZR1*; figure 5). These data support the the opposite role of *BZR1* and *DLT* in the signaling pathway.

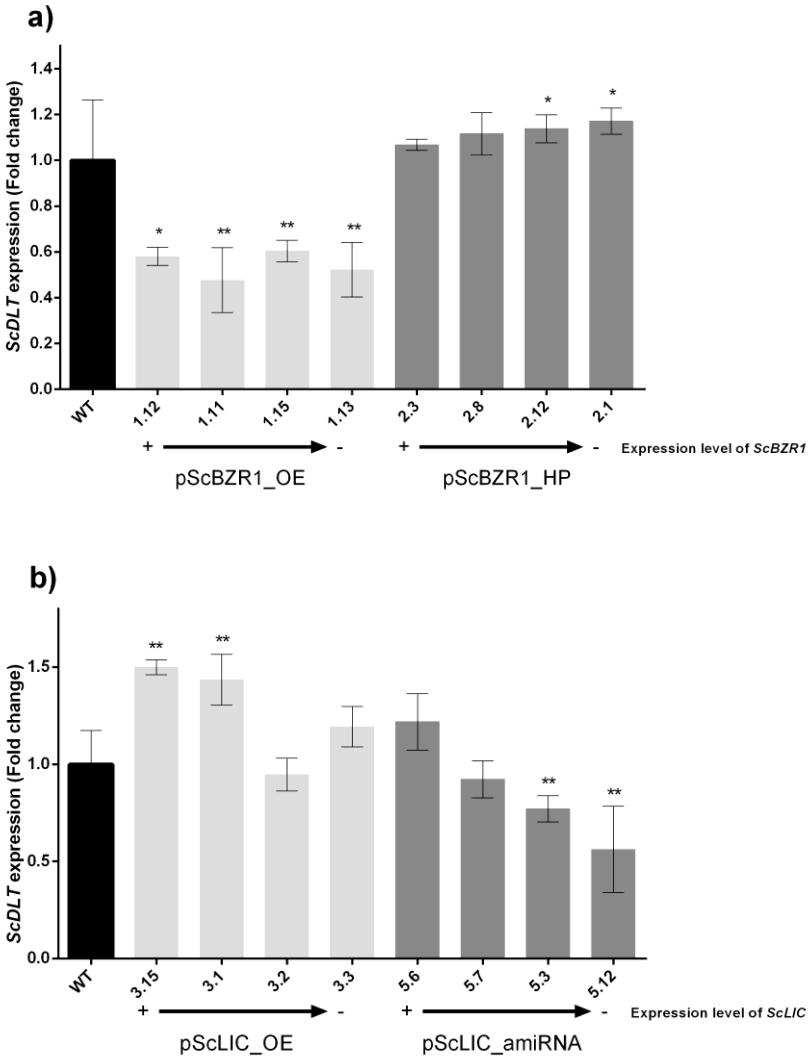
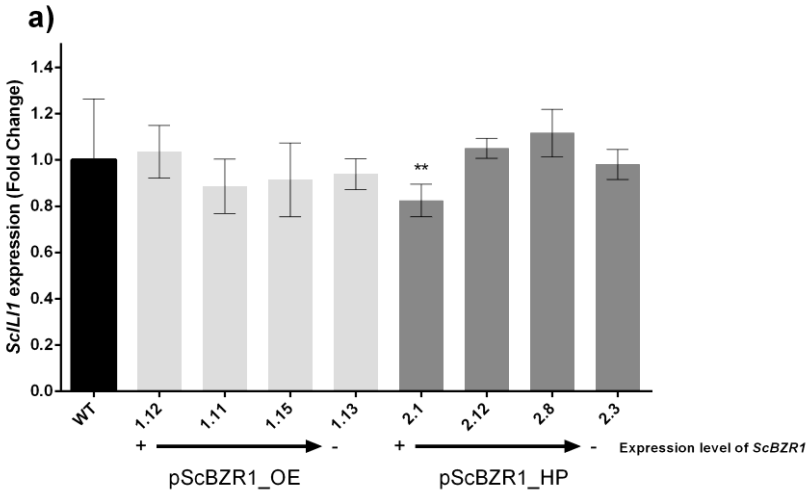


Figure 15: A) Gene expression levels analysis (fold change) of *ScDLT* in BZR1 transgenic lines by RT-qPCR. The light gray bars are *ScDLT* expression in independent events of pScBZR1_OE and dark gray bars are *ScDLT*

expression in independent events of pScBZR1_HP. B) Gene expression levels analysis (fold change) of *ScDLT* in LIC transgenic lines by RT-qPCR. The light gray bars are *ScDLT* expression in independent events of pScLIC_OE and dark gray bars are *ScDLT* expression in independent events of pScLIC_amiRNA. In both cases, black bars are *ScDLT* expression in WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

The lasts signaling genes evaluated were *ILI1* and *IBH1*, exclusive of monocots, which play an important role in leaf angle. Previous studies show that *ILI1* and *IBH1* suppress each other, while *BZR1* inhibits *IBH1* and *LIC* inhibits *ILI1* in rice (Zhang et al., 2012). We expected that the events overexpressing *ScBZR1* (and consequently presented lower levels of *ScLIC*) would show a suppression of *ScIBH1*. In the same way, the transgenic lines overexpressing *ScLIC* (and consequently presenting lower levels of *ScBZR1*), would show a suppression of *ScILI1*. However, none of the expression data showed any relation between the feedback mechanisms involving these four genes (figure 16). The main cause which might explain these results is the presence of more genes at this signaling point which controls leaf angle that are not already completely elucidated, added to the lack of information about BR signaling pathway in monocots.



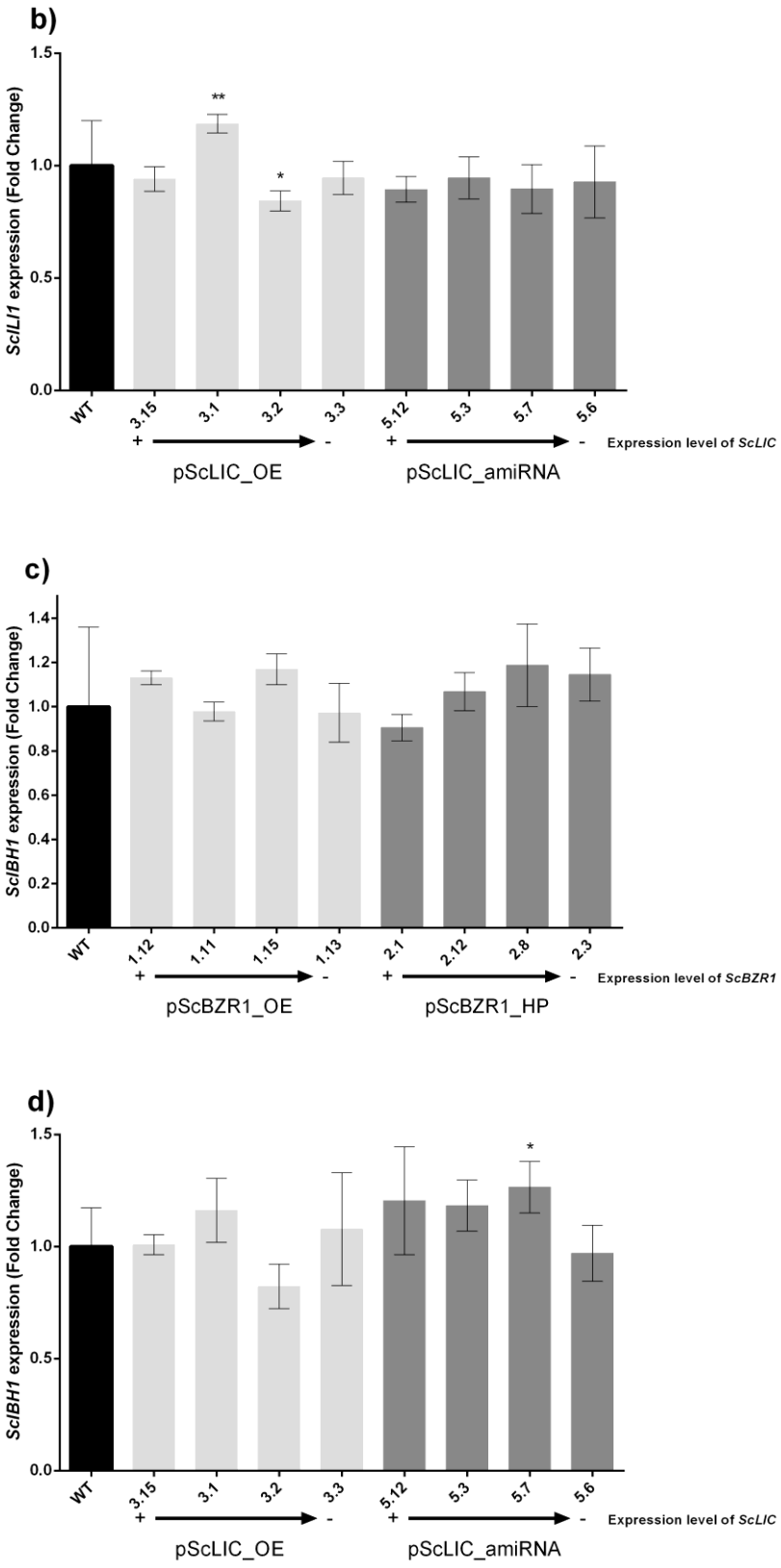


Figure 16: A) Gene expression levels analysis (fold change) of *ScILI1* in BZR1 transgenic lines by RT-qPCR. The light gray bars are *ScILI* expression in independent events of pScBZR1_OE and dark gray bars are *ScILI1* expression in independent events of pScBZR1_HP. B) Gene expression levels analysis (fold change) of *ScILI1* in LIC transgenic lines by RT-qPCR. The light gray bars are *ScILI1* expression in independent events of pScLIC_OE

and dark gray bars are *ScILI1* expression in independent events of pScLIC_amiRNA. In both cases, the black bars are *ScILI1* expression in WT plants. C) Gene expression levels analysis (fold change) of *ScIBH1* in BZR1 transgenic lines by RT-qPCR The light gray bars are *ScIBH1* expression in independent events of pScBZR1_OE and dark gray bars are *ScIBH1* expression in independent events of pScBZR1_HP. D) Gene expression levels analysis (fold change) of *ScIBH1* in LIC transgenic lines by RT-qPCR. The light gray bars are *ScIBH1* expression in independent events of pScLIC_OE and dark gray bars are *ScIBH1* expression in independent events of pScLIC_amiRNA. In both cases, the black bars are *ScIBH1* expression in WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

The major BR biosynthetic gene evaluated was *DFW4*. The biosynthesis and signaling BR pathways present feedback mechanism. When the BR biosynthesis pathway is activated, BR signaling is inactivated and vice-versa. The main fact that justified this mechanism is the ability that *BZR1* presents to bind to the promoter of biosynthetic genes causing their inhibition and preventing unnecessary pathway activation (He et al., 2005). The gene expression data showed that all overexpressing events of *ScBZR1* exhibited suppression of *ScDFW4* and the same was true for the silencing lines of *ScLIC* . Moreover, the overexpressing of *ScLIC* and silencing of *ScBZR1* showed higher levels of *ScDFW4*, confirming the antagonistic role of biosynthetic and signaling pathways.

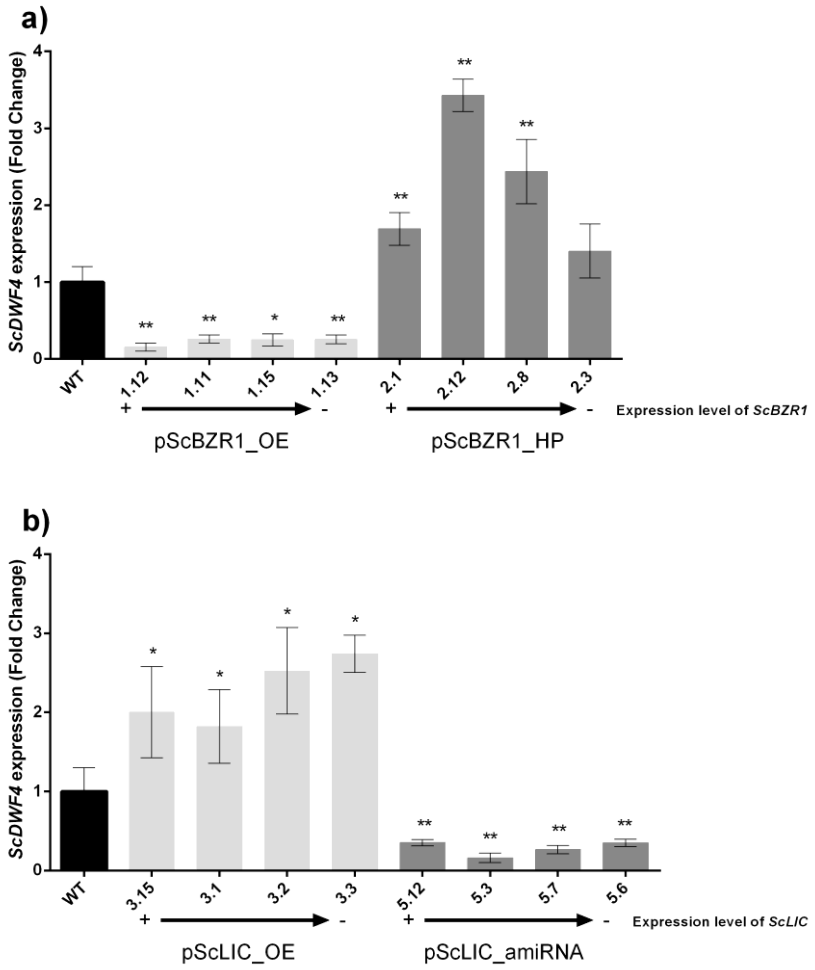


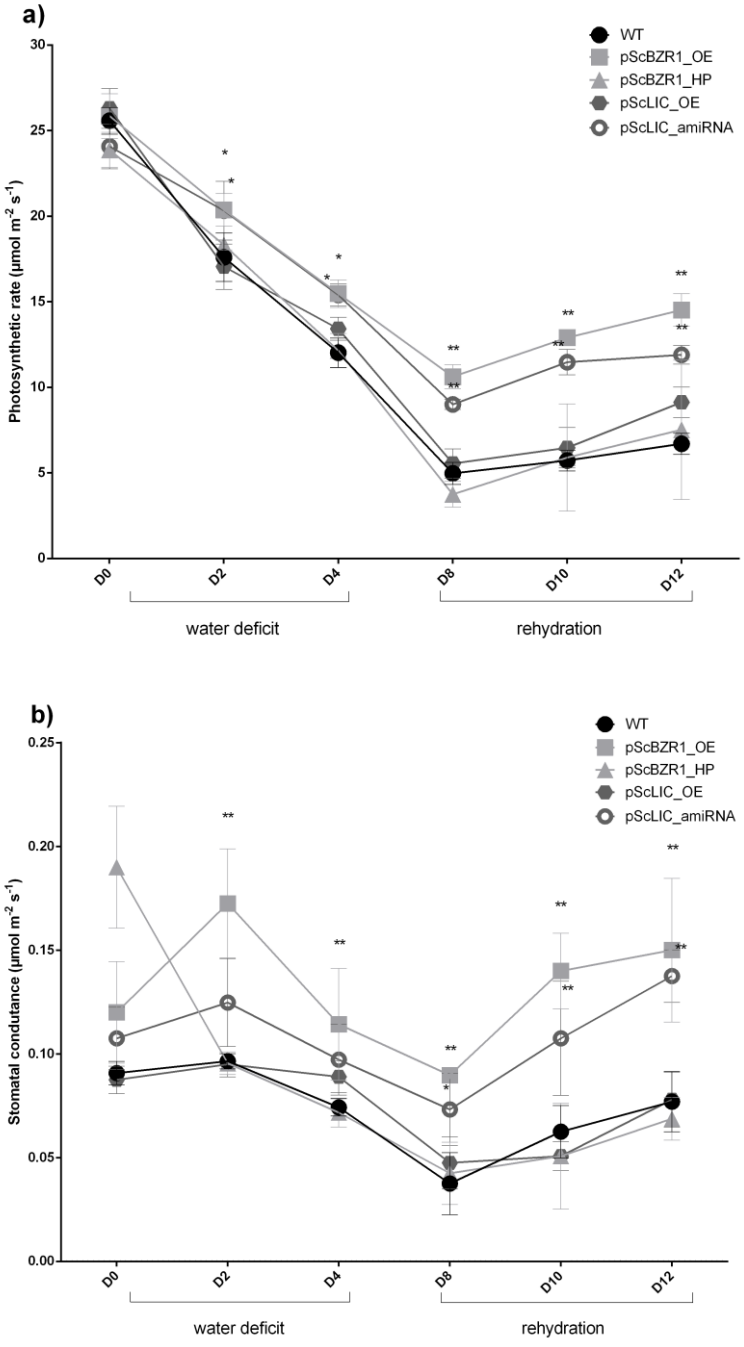
Figure 17: A) Gene expression levels analysis (fold change) of *ScDFW4* in BZR1 transgenic lines by RT-qPCR. The light gray bars are *ScDWF4* expression in independent events of pScBZR1_OE and dark gray bars are *ScDWF4* expression in independent events of pScBZR1_HP. B) Gene expression levels analysis (fold change) of *ScDWF4* in LIC transgenic lines by RT-qPCR. The light gray bars are *ScDWF4* expression in independent events of pScLIC_OE and dark gray bars are *ScDWF4* expression in independent events of pScLIC_amiRNA. In both cases, black bars are *ScDWF4* expression in WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

5.3.4 Effect of brassinosteroid in drought stress response and biomass

Is well documented in the literature that the exogenous application of BR in several plants as tomato (Yuan et al., 2010), maize (Anjum et al., 2011), Arabidopsis (Planas-Riverola et al., 2019) and others increase drought tolerance by activation of BR signaling pathway. The mechanism is specific in each case, but seems to be related to the ability of *BZR1* modulates the expression of oxidative enzymes, mobilization of osmoprotectant metabolites and other

phytohormones associated to stress response as ABA (Cui et al., 2019; Feng et al., 2022; Saha et al., 2015 and Sun et al., 2020). However, until now, there was no data that elucidate how BR works in sugarcane drought stress.

All the four independent event of each transgenic line and WT were submitted to drought stress conditions and monitored by gas exchange parameters (figure 18).



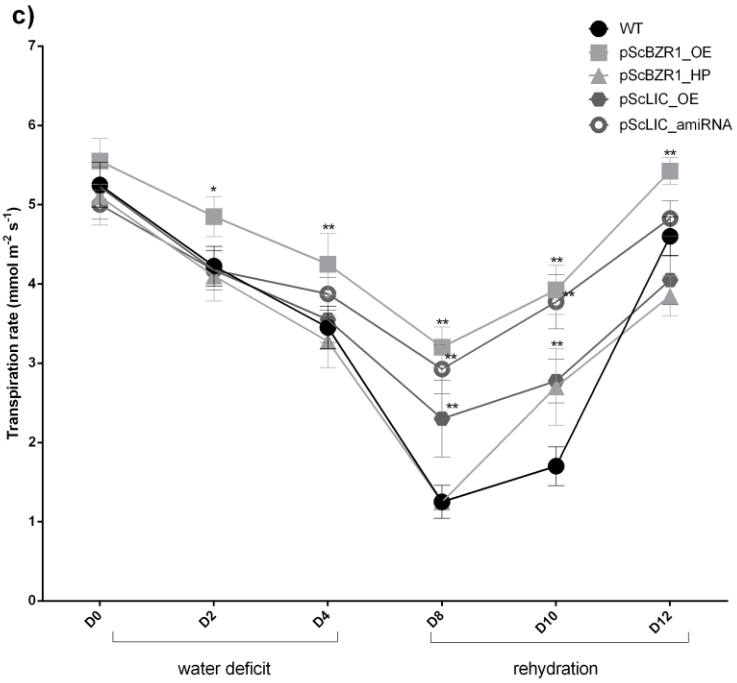


Figure 18: A) Photosynthesis rate (A), B) stomatal conductance (gs) and C) transpiration rate (E) in drought and rehydration conditions. The water deficit period correspond to D2 and D4 and the rehydrated period correspond to D8 to D12. In all cases light gray squares correspond to independent events of pScBZR1_OE, light gray triangle correspond to independent events of pScBZR1_HP, dark gray hexagon correspond to independent events of pScLIC_OE and dark gray empty circles correspond to independent events of pScLIC_amiRNA. Black circles correspond to WT plants. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

The two lines with higher *ScBZR1* expression (pScBZR1_OE and pScLIC_amiRNA, respectively) presented better photosynthetic rate, stomatal conductance and transpiration rate after D2, if comparing with WT plants. On the opposite, the transgenic lines with lower *ScBZR1* expression (pScBZR1_HP and pScLIC_OE, respectively) did not presented statistic difference to WT plants. Moreover, pScBZR1_OE and pScLIC_amiRNA lines presented better recovering after rehydration and presented less severe damage caused by drought condition (figure 19).

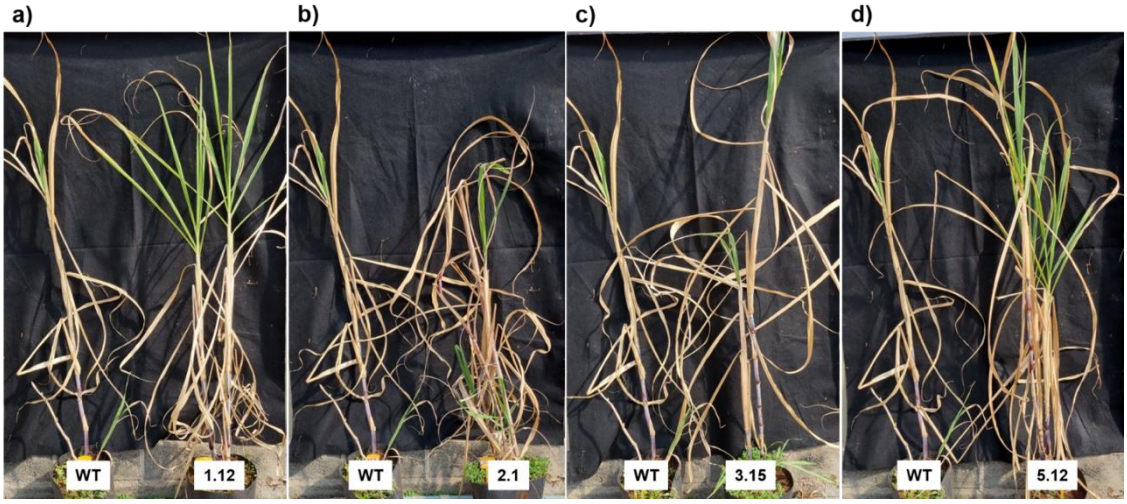
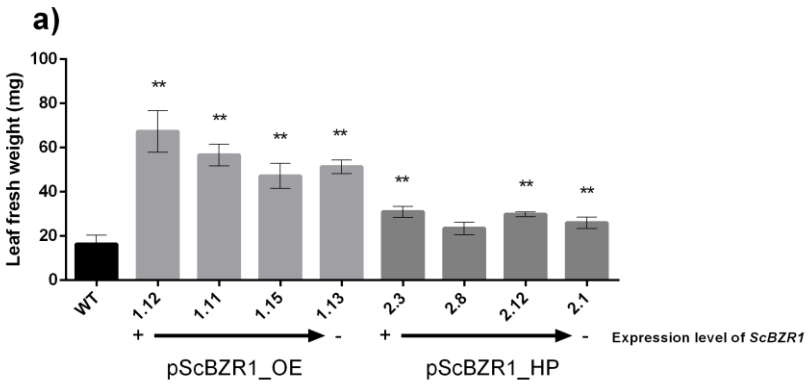


Figure 19: Sugarcane plants in D12 of drought stress assay. A) WT and pScBZR1_OE, B) WT and pScBZR1_HP, C) WT and pScLIC_OE and D) WT and pScLIC_amiRNA.

One month after the end of drought assay, we perform the destructive analyses of sugarcane plants to measure the biomass from leaves and stalk (figure 20).



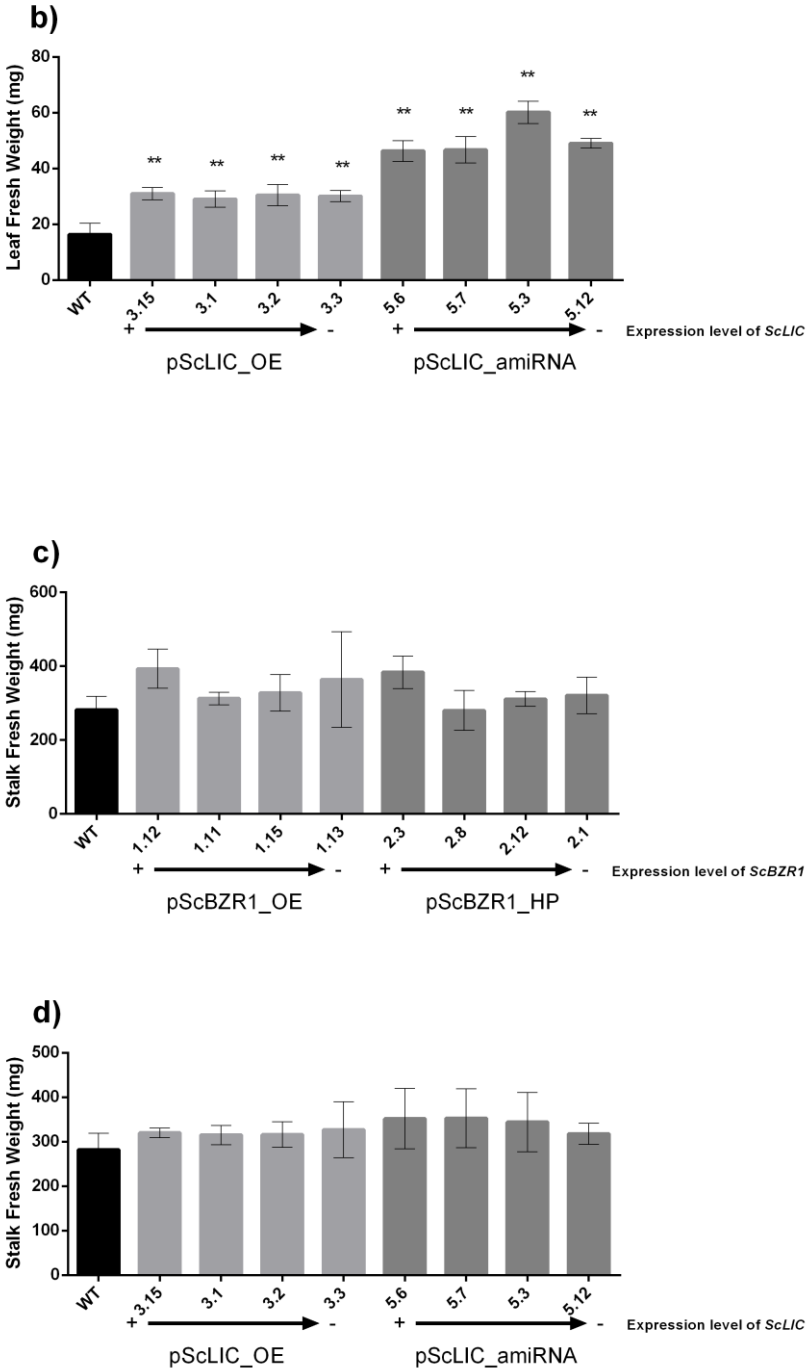


Figure 20: Sugarcane biomass analyses. A) Leaf fresh weight (mg) of *ScBZR1* lines. B) Leaf fresh weight (mg) of *ScLIC* lines. C) Stalk fresh weight (mg) of *ScBZR1* lines. D) Stalk fresh weight (mg) of *ScLIC* lines. Black bars correspond to WT plants, light gray bars to the overexpressing lines and dark gray bars to the silencing lines. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

The mainly difference was observed in the leaves where most of events presented higher biomass in relation to WT. It is presumable that the events with better performance in the drought assay (figure 18) would have leaves more preserved with less signs of dehydration (dry leaves), and therefore, presenting greater biomass. As the stalk did not presented differences in the transgenic lines in relation to WT, it seems that in this case, the BR cascade were more relevant on stress recovery on the leaves. Also, the short period between the stress and the biomass quantification certainly was not enough to allow the increased photosynthesis rates to cause an increase in the stalk biomass.

Regarding the fact that we did not observed any difference in the plant height and stalk diameter before the drought assay (figure 8, 9 and 10), in the present study, the modulation of BR signaling pathway did not present visible signs in increasing sugarcane biomass in the stalks in the absence of water deficit.

5.3.5 Effect of BR gene deregulation on oxidative stress response

Reactive oxygen species (ROS), as H_2O_2, are normally producted under normal condition due to the important role in cell signaling. However, many abiotic stresses, as drought stress, are related to increase H_2O_2 concentration in cells, providing cell death (Impa *et al*., 2012). As *ScBZR1* played positive role in drought stress, we aim to understand how BR transgenic lines acts in oxidative stress. Leaf discs were incubated in different concentrations of H_2O_2 and, in general, the pScBZR1_OE lines presented less chlorophyll degradation if comparing with WT at the concentration of 0.5M. At 1M of H_2O_2, both pScBZR1_OE and pScLIC_amiRNA lines presented less chlorophyll degradation if comparing to WT plants (figure 21). The appearance of the leaf disks can be observed in Figure 22.

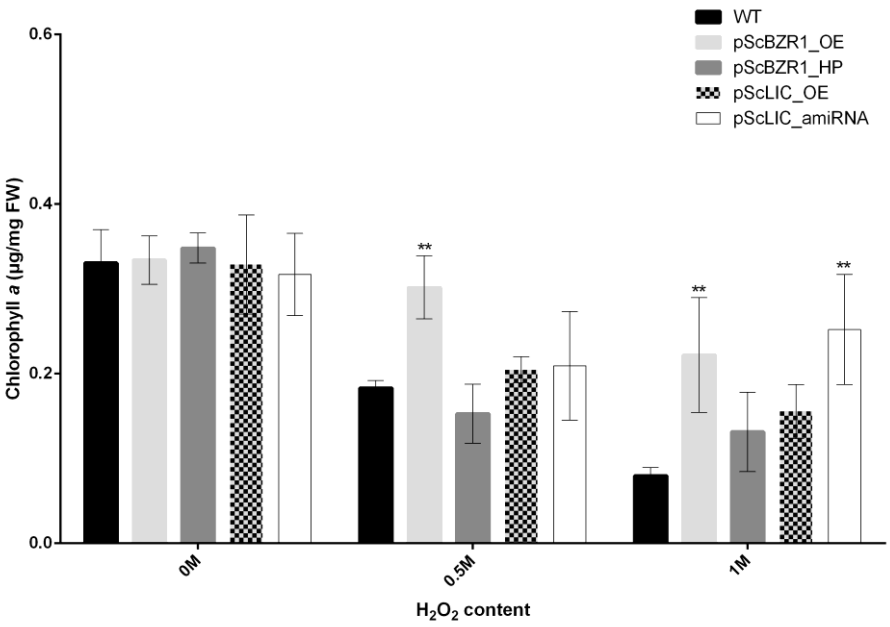


Figure 21: Chlorophyll *a* content in µg/mg of fresh weight after oxidative stress assay in 0M, 0.5M and 1M concentrations of H_2O_2. Data are presented as the mean ± SE. Asterisks indicated a P value lower than 0.05 and double asterisks indicated a P value lower than 0.001, according to a T-*Student* test comparing expression levels in the transgenic events with WT plants (n=4).

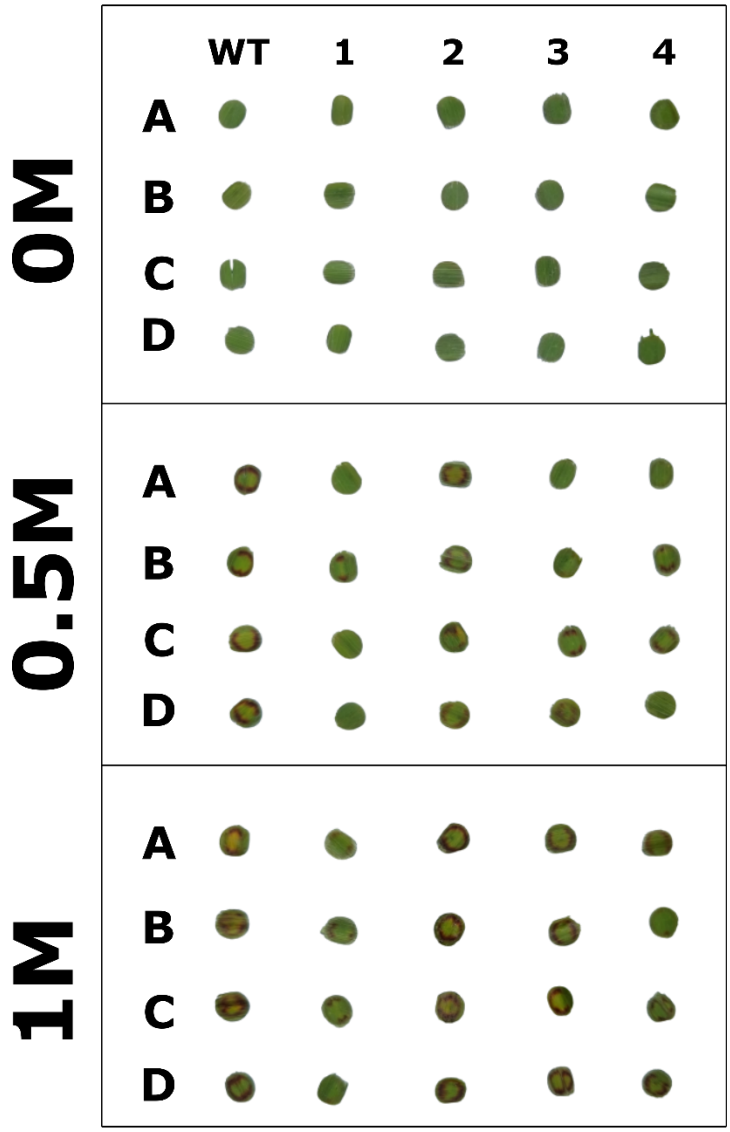


Figure 22: Analysis of foliar disc of sugarcane in three different concentrations of H_2O_2 at 0M, 0.5M and 1M. The numbers 1 to 4 in the horizontal axis correspond to pScBZR1_OE, pScBZR1_HP, pScLIC_OE and pScLIC_amiRNA, respectively. The letters A to D in the vertical axis correspond to biological replicates of the same independent event.

The present work showed that higher levels of *ScBZR1* in the pScBZR1_OE and pScLIC_amiRNA lines provided better tolerance to oxidative stress and less chlorophyll degradation. In the opposite, higher levels of *ScLIC* and lower levels of *ScBZR1* in the lines pScLIC_OE and pScBZR1_HP, respectively, provided less tolerance to oxidative stress and more chlorophyll degradation, similar to WT plants. One explanation to these effects may be

because *ScBRZ1* can bind to the promoter of specific genes that increases the tolerance response to various stresses, including the oxidative, as previous discussed.

5.4 Conclusions on the gene regulatory network

Many genes are involved in BR signaling and biosynthesis pathways. The regulation of the expression of these genes is extremely important to activate signaling or biosynthesis pathway according to the plant needs.

The first objective of this chapter is evaluate how the modulation of *ScBZR1* and *ScLIC* interferes in sugarcane growth and development. The biometric analyses of height, tiller number and stalk diameter did not indicate significant differences in relation to WT plants. It seems to be justify by the existence of a fine regulation between the two transcriptional factors and the possible existence of more genes involving in the signaling pathway of monocots, which might control growth processes. When subjected to water deficit stress, we observed higher leaf biomass in the transgenic lines in relation to WT, namely in the lines overexpressing *ScBZR1* and silenced for *ScLIC*. However, no significant difference was observed in stalk biomass, probably due to the short period between the stress and the biomass evaluation.

The second objective of this chapter was to elucidate how key genes of the BR signaling pathway are influenced by *ScBZR1* and *ScLIC,* which work as antagonistic genes. *ScBRI* is required to activate the signaling cascade, but the expression of *ScBRI1* did not present a clear pattern in the sugarcane transgenic events. *ScGSK3* is active at low BR content, being down regulated only in the event with the highest overexpression of *ScBZR1*. Therefore, ScGSK3 regulation by *ScBZR1* and *ScLIC* in sugarcane do not follow the observed in other species. *ScDLT* was repressed by *ScBZR1*, being down regulated in the events with high *ScBZR1* and up regulated in the events with high *ScLIC* expression, reinforcing the opposite role of these two genes. No clear patterns were observed in the expression of *ScILI1* and *ScIBH1*, probably due to the feedback mechanism between *ScBZR1* and *ScLIC* in the transgenic lines and the possibility of the existence of more genes downstream the transcriptional factors, which regulate leaf angle. Lastly, *ScDWF4* is active in the absence of BR, being down regulated in the events with high *ScBZR1* levels and up regulated in the eventos with high *ScLIC* levels. This is in line with a balance between the two pathways involved in biosynthesis of BR an its signaling.

The final objective of this chapter is evaluate the role of BR in stress response. We evaluated how the transgenic lines responded to drought and oxidative stresses. In both cases, lines with high *ScBZR1* or low *ScLIC* levels presented better tolerance to droght stress, higher recovering capacity after water deficit and less chlorophyll degradation, confirming the posivite role of *ScBZR1* in stress response.

6. FINAL CONCLUSIONS

Until know, there were no data in the literature that brings a detailed analysis of bioactive BR in sugarcane. The complete steroid profile was provided and the results were in agreement to prior studies in the literature, indicating castasterone is the main endogenous and consequently the most bioactive BR in sugarcane.

Despite of BR enhancement of plant growth, high amounts of this phytohormone can present the opposite effect. The carefully modulation of BR levels is essential to have optimal plant growth and development. Exogenous application of BR is a feasible tool to modulate important agronomic traits. However, with genetics advancing, the molecular modulation of BR levels can be a more interesting way to produce homogeneous results.

Regardless the fact that all elements of BR signaling pathway are not completely elucidated, it is well known that the BR cascade is modulated by the two major transcriptional factors, *BZR1* and *LIC*. We expected that the modulation of these two genes would influence sugarcane growth and development. However, in the present work, the modulation of the *ScBZR1* and *ScLIC* did not modulate sugarcane growth and stalk fresh weight. We can infer different causes for these results:

1. We cannot rule out the existence of other genes involved in the control of BR pathway that have not been discovered until now. Also, there are recent evidences on the existence of differences in the BR signaling pathway between eudicots and monocots.

2. Finally, the BR signaling cascade presents a careful modulation involving a feedback mechanism between *BZR1* and *LIC*. The overexpressing or silencing of one of them may produce a compensatory effect, refining the control of the pathway. Although we cannot observe this at molecular levels, it may interfere in the expected phenotype.

Molecular analyses of BR biosynthetic and signaling genes by RT-qPCR presented some results in line with those observed in other species, but also presented results that were not conclusive. These last, may be justified by a summing up factors where the BR signaling pathway presents multifactorial control in which many genes regulate each other in a refine way. The modulation of their expression may imply in the expression of the others, dificulting the observation of a consistent pattern in all provided analyses, which influence in the molecular analyses as well as in the morphological phenotype.

Literature data already show that BR are related to many stress responses in different ways: inducing other phytohormones synthesis and regulating the promoter activities of stress-related genes, for example. In the present work, plants with higher levels of *ScBZR1* and lower levels of *ScLIC* presented better drought tolerance and response to oxidative stress, confirming the fact that BR plays a positive regulation in drought and oxidative stresses by activating BR signaling pathway.

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8. ATTACHMENTS

8.1 Sequences used to perform BLAST in sugarcane databases

>BZR1 rice CDS_LOC_Os07g39220:

ATGACGTCCGGGGCGGCGGCGGCGGGGAGGACGCCGACGTGGAAGGAGAGGGAGAACAACAAGAGGCGGGAGCGGCGGCGGCGTGCCATCGCCGCCAAGA
TCTTCACGGGGCTCCGGGCGCTCGGGAACTACAACCTCCCCAAGCACTGCGACAACAACGAGGTGCTCAAGGCGCTCTGCCGCGAGGCCGGCTGGGTTGT
CGAGGACGACGGCACCACCTACCGCAAGGGATGTAAGCCGCCGCCATCGTCGGCTGGGGGAGCGTCGGTGGGGATGAGCCCCTGCTCGTCAACGCAGCTG
CTGAGCGCGCCGTCGTCGTCGTTCCCGAGCCCGGTGCCGTCGTACCACGCGAGCCCGGCGTCGTCGAGCTTCCCGAGCCCCAGCCGGATCGACAACCCGA
GCGCCTCCTGCCTCCTCCCGTTCCTCCGGGGGCTCCCCAACCTCCCGCCGCTCCGCGTCTCCAGCAGCGCGCCCGTCACGCCGCCGCTCTCGTCGCCGAC
GGCGTCGCGGCCGCCCAAGATCAGGAAGCCGGACTGGGACGTCGACCCGTTCCGGCACCCCTTCTTCGCGGTCTCCGCGCCGGCGAGCCCCACCCGCGGC
CGCCGCCTCGAGCACCCGGACACGATACCGGAGTGCGACGAGTCCGACGTCTCCACGGTGGACTCCGGCCGGTGGATCAGCTTCCAGATGGCCACGACGG
CGCCGACGTCGCCCACCTACAACCTCGTCAACCCGGGCGCCTCCACCTCCAACTCCATGGAGATAGAAGGGACGGCCGGCCGAGGCGGCGCGGAGTTCGA
GTTCGACAAGGGGAGGGTGACGCCATGGGAGGGCGAGAGGATCCACGAGGTCGCCGCCGAGGAGCTCGAGCTCACGCTCGGCGTCGGCGCGAAATGA

>BZR1 rice protein_LOC_Os07g39220:

MTSGAAAAGRTPTWKERENNKRRERRRRAIAAKIFTGLRALGNYNLPKHCDNNEVLKALCREAGWVVEDDGTTYRKGCKPPPSSAGGASVGMSPCSSTQLLSAPSSS
FPSPVPSYHASPASSSFPSPSRIDNPSASCLLPFLRGLPNLPPLRVSSSAPVTPPLSSPTASRPPKIRKPDWDVDPFRHPFFAVSAPASPTRGRRLEHPDTIPECDE
SDVSTVDSGRWISFQMATTAPTSPTYNLVNPGASTSNSMEIEGTAGRGGAEFEFDKGRVTPWEGERIHEVAAEELELTLGVGAK*

>BZR1 sorghum CDS_Sb02g037500:

ATGACGTCGGGGGCGGCCGCAGCGGCGGCGGCGGCGGGAGCGCTGGGTCGGACGCCGACGTGGAAGGAGCGGGAGAACAACAAGCGCCGGGAGCGCCGGC
GGAGGGCCATCGCCGCCAAGATCTTCACGGGCCTCCGCGCGCTCGGCAACTACAAGCTGCCCAAGCACTGCGACAACAACGAGGTGCTCAAGGCGCTCTG
CCGCGAGGCGGGCTGGGTCGTCGAGGACGACGGCACCACCTACCGAAAGGGATGCAAGCCGCCGCCGGGGATGATGAGCCCGTGCTCGTCCTCGCAGCTG
CTGAGCGCGCCGTCGTCGAGCTTCCCGAGCCCGGTGCCGTCCTACCACGCCAGCCCGGCGTCATCCAGCTTCCCGAGCCCGACGCGCCTCGACCACAGCA
GCGGCAGCAACCACCACCACCACCACAACCCGGGCCCCACTGCTGCCGCTGCCGCGGCGGCGGCCTCCTCCCTGCTCCCGTTCCTCCGGAGCCTGCCGAA
CCTCCCGCCGCTCCGCGTCTCCAGCAGCGCGCCCGTCACGCCGCCGCTCTCCTCGCCCACGGCGGCGTCGCGGCCGCCCACCAAGGTCCGCAAGCCCGAC
TGGGACGCCGCCGTCGCCGACCCGTTCCGGCACCCCTTCTTCGCGGTCTCCGCCCCCGCCAGCCCCACCCGCGCACGCCGGCGCGAGCACCCGGACACCA
TCCCCGAGTGCGACGAGTCCGACGTCTGCTCCACCGTCGACTCCGGCCGCTGGATCAGCTTCCAGGTGGGCGCGGCGACCACGGCGCCCGCGTCGCCCAC
CTACAACCTCGTCCACCCGGCCGGCGGCGGCGCGTCCGCCTCCAACTCCATGGAGCTGGACGGGATGGCGGCCGCGGACATCGGCGGCAGGGGCGGCGGT
CCCGCGGAGTTCGAGTTCGACAAGGGCCGTGTCACGCCGTGGGAAGGCGAGCGCATCCACGAGGTCGCCGCCGAGGAGCTCGAGCTCACGCTTGGCGTCG
GCGCCAAGTGA

>BZR1 sorghum protein_Sb02g037500:

MTSGAAAAAAAAGALGRTPTWKERENNKRRERRRRAIAAKIFTGLRALGNYKLPKHCDNNEVLKALCREAGWVVEDDGTTYRKGCKPPPGMMSPCSSSQLLSAPSSS
FPSPVPSYHASPASSSFPSPTRLDHSSGSNHHHHHNPGPTAAAAAAAASSLLPFLRSLPNLPPLRVSSSAPVTPPLSSPTAASRPPTKVRKPDWDAAVADPFRHPFF
AVSAPASPTRARRREHPDTIPECDESDVCSTVDSGRWISFQVGAATTAPASPTYNLVHPAGGGASASNSMELDGMAAADIGGRGGGPAEFEFDKGRVTPWEGERIHE
VAAEELELTLGVGAK*

>BZR1 sugarcane CDS (ScBZR1):

ATGACGTCGGGGGCGGCCGCGGCGGCGGCGGCCGGAGCGCTGGGGCGGACGCCGACGTGGAAGGAGCGGGAGAACAACAAGCGCCGGGAGCGCCGGCGGAGGGC
CATCGCCGCCAAGATCTTCACGGGCCTCCGCGCGCTCGGCAACTACAAGCTGCCCAAGCACTGCGACAACAACGAGGTGCTCAAGGCGCTGTGCCGCGAAGCGGGCT
GGGTCGTCGAGGACGACGGCACCACCTACCGAAAGGGATGCAAGCCGCCGCCGGGGATGATGAGCCCGTGCTCGTCCTCGCAGCTGCTGAGCGCGCCGTCCTCGAGC
TTCCCGAGCCCGGTGCCGTCCTACCACGCCAGCCCGGCGTCGTCGAGCTTCCCGAGCCCGACGCGCCTCGACCACAGCAGCGGCAGCAACCACCACAACCACAACCC
GGGCCCCGCCGCCGCCGCCGCTGCCTCGTCCCTGCTCCCGTTCCTCCGGGGCCTGCCGAACCTCCCGCCGCTCCGCGTCTCCAGCAGCGCGCCCGTCACGCCGCCGC

TCTCCTCGCCCACGGCGGCGTCGCGGCCGCCCACCAAGGTCCGCAAGCCGGACTGGGACAACGCCGTCGCCGACCCGTTCCGCCACCCCTTCTTCGCGGTCTCCGCC
CCCGCCAGCCCCACCCGGGCCCGCCGGCGCGAGCACCCGGACACCATCCCCGAGTGCGATGAGTCCGACGTCTGCTCCACCGTCGATTCCGGCCGCTGGATCAGCTT
CCAGGTGGGCGCGGCGACCACGGCACCCGCGTCGCCCACGTACAACCTCGTCAACCCGGCCGGCGGCGCGTCCGCCTCCAACTCCATGGAGCTGGACGGGATGGCGG
CCGCGGACATCGGCGGCAGGGGCGGCGGCCCCGCGGAGTTCGAGTTCGACAAGGGCCGTGTTACGCCGTGGGAAGGCGAGCGGATCCACGAGGACTCTGGGTCAGAC
GACCTGGAGCTCACGAGCTCACGCTCGGCGTCGGCGCCAAGTGA

>BZR1 sugarcane protein (ScBZR1):

MTSGAAAAAAAAGALGRTPTWKERENNKRRERRRRAIAAKIFTGLRALGNYKLPKHCDNNEVLKALCREAGWVVEDDGTTYRKGCKPPPGMMSPCSSSQLLSAPSSS
FPSPVPSYHASPASSSFPSPTRLDHSSGSNHHNHNPGPAAAAAASSLLPFLRGLPNLPPLRVSSSAPVTPPLSSPTAASRPPTKVRKPDWDNAVADPFRHPFFAVSA
PASPTRARRREHPDTIPECDESDVCSTVDSGRWISFQVGAATTAPASPTYNLVNPAGGASASNSMELDGMAAADIGGRGGGPAEFEFDKGRVTPWEGERIHEDSGSD
DLELTSSRSASAPS*

>LIC rice CDS_ LOC_Os06g49080.1:

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GCAGCAGAAGCCCAATCCTTTTGGTTTTGGGGTACAAGGTGCCAATGCCCAGTCACGTAATGCTCCTGGTCCTGCGAAGCCTTTTCAAAATAAGTGGGTA
AGGGACCCCTCGGCCCCGACGAAGCAAACAGAGGCGGTGCAGCCACCGCAGGCGCAGGCTGCCCACACCTCCTGTGAAGACCCTCAGTCATGCAGACAAC
AAATTTCTGAGGATTTTAAGAACGAGGCTCCTATTTGGAAGCTTACTTGTTATGCTCATCTCAGAAATGGCCCTTGCAATATTAAGGGAGACATTAGCTT
CGAGGAACTAAGAGCTAAAGCCTATGAGGAAGGAAAGCAAGGGCATTCTCTGCAATCAATAGTTGAGGGAGAAAGAAATCTACAAAACGCAAAGTTAATG
GAGTTTACTAATCTGCTCAATAGTGCACGTCCATCACAAACTCCAAGCTTTCCTACTATGAGTTCCTTCCCTGAAGTGAAAAACAACTCATCATTCGGGG
CTTCTCAAACCAATGGACCACCTGTGTTCAGTAGTTTCAGTCAAATTGGAGCAGCAACTAATATTGGACCTGGGCCAGGAACCACCGCACCAGGAATGCC
AGCCAGTAGTCCTTTTGGTCATCCAAGCTCTGCACCACTTGCTGCCCCTACTTTTGGCAGTTCTCAAATGAAGTTTGGAGTTTCGAGCGTATTTGGAAAC
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CACAAGAGTTGCTTAATGGGATGGTAACTCCTCCTAGTGTGATGTTTGAGGAATCTGTTGGGAACAACAAAAATGAAAATCAGGATGATAGCATCTGGTT
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>LIC rice protein_ LOC_Os06g49080.1:

MSRRQEICRNFQRGSCKYGAQCRYLHASPHQQQQQQQAKPNPFGFGTGSRQQQQPSFGSQFQQQQQQQQKPNPFGFGVQGANAQSRNAPGPAKPFQNKWVRDPSAPT
KQTEAVQPPQAQAAHTSCEDPQSCRQQISEDFKNEAPIWKLTCYAHLRNGPCNIKGDISFEELRAKAYEEGKQGHSLQSIVEGERNLQNAKLMEFTNLLNSARPSQT
PSFPTMSSFPEVKNNSSFGASQTNGPPVFSSFSQIGAATNIGPGPGTTAPGMPASSPFGHPSSAPLAAPTFGSSQMKFGVSSVFGNQGSGQPFGSFQAPRFPSSKSP
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>LIC sorghum CDS_ Sb10g029250:

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AGTCGAAGCCCAACCCGTTTGGGTTCGGCTCTCAGCAGCAGCAGGCGAATACCAACCCGTTTGGGTTCGGCTCTGGGAGCAGGCAGCAGCAGCAGTCTTC
GTTCGGTGCGCAGTTCCAGCAGCAGCAGCAGAAGCCCAATCCGTTTGGATTTGGAGTGCAAGGGGGTGCGGCCGCGCAGTCCAGAAACGCCTCTGGCACG
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CATCACTGGGGATATTAGCTTTGAAGAGCTGAGAGCTAAAGCGTATGAGGAAGGCAGGCAGGGGCATCCTCTGCCGTCAATAGTCGAGGGTGAAAGAAAT
TTGCAAAATGCAAAGCTGACAGAGTTTAATAATTTTCTGAACAATCCACATCTATCGGTATCACAAACTCCAAGCTTTCCAACTGTGGCTTCCTTCCCTG
AAGTGAAAAATAACTCGCCATTTGGGGTTTCTCAAAATAACGGACCACCAGTGTTTAGTAGTTTCAGTCAAATTGGAGCAGCCAATAATATTGGACCTGG
GTCCAGAACTACACCAGGATTCCCAACCAATAGTATTTTTGGTCAGTCTAACCAGCCGAGCCATCCAGGATTTCCTACACCTACTTTTGGCCGTTCTGAT
ATGAAATTTGGAGTTTCAGGTTCATTTGGAAGCCAGATATCACAACAATCATCTGGGAGTTTGCAAGGCTCAAACATGTCCAGTGTTGGCAATTTCCCAA
AGCCTCATGCTAGCTACCAGCAGTCTCCTTCCTCCTCTAGCCATCATAGAGACATTGATAGGCAGTCACAAGATTTACTTAGTGGAATCGTGGCTCCTAC
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>LIC sorghum protein_ Sb10g029250:

MSRRQELCRNFQRGSCKYGAQCRFVHASSQQQQQSKPNPFGFGSQQQQANTNPFGFGSGSRQQQQSSFGAQFQQQQQKPNPFGFGVQGGAAAQSRNASGTAKPFQNK
WVRDPSAPTKQQEAAQPAPAAHTSCTDPESCRHQIAEDFKNETPLWKLTCYAHLRSGPCDITGDISFEELRAKAYEEGRQGHPLPSIVEGERNLQNAKLTEFNNFLN
NPHLSVSQTPSFPTVASFPEVKNNSPFGVSQNNGPPVFSSFSQIGAANNIGPGSRTTPGFPTNSIFGQSNQPSHPGFPTPTFGRSDMKFGVSGSFGSQISQQSSGSL
QGSNMSSVGNFPKPHASYQQSPSSSSHHRDIDRQSQDLLSGIVAPTSATNQAPVEDNKNENQDDSIWLKEKWSIGEIPLGEPPQRHISHVF*

>LIC sugarcane CDS (ScLIC):

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CGCAGTTCCAGCAGCAGCAGAAGCCCAATCCGTTTGGATTTGGAGTACAAGGCGGTGCGGCTGCGCAGTCCAGAAACGCTCCTGGCCCGGCAAAGCCATTTCAA
AATAAGTGGGTAAGGGACCCCTCAGCCCCGACGAAGCAACAGGAAGCGGCACAGACACCACCAGCAGCCCACACCTCCTGTACAGATCCTGAGTCATGCAGGCACCA
GATTGCTGAGGATTTTAAGAATGAAACTCCACTCTGGAAGCTTACGTGTTATGCTCATCTCAGGAGTGGTCCTTGTGACATCACTGGGGATATTAGCTTTGAAGAGC
TGAGAGCTAAAGCGTATGAGGAAGGCAGGCAAGGGCATCCTCTGCAGTCAATAGTCGAGGGTGAAAGAAATCTGCAAAATGCAAAGCTGATGGAGTTTAATAATTTT
CTGAACACTCCACGTGTATCAGTATCACAAAGTCCAAACTTTCCAACTGTCACTTCCTTCCCTGAAGTGAAAACTAATTCATCATTTGGGGTTTCTCAAACTAATGG
ACCACCAGTGTTTAGTAGTTTCAGTCAAGTTGGAGCAGCAGCTAATATTGGGCCTGGGTCAAGAACTGCACCAGGAGTCTCAACCAATAGTATTTTTGGTCAATCTA
GCCAGCCGAACCATCCAGGATTTCCTGCACCTACCTTTGGCCGTTCTGATATGAAATTCGGAGTTTCAGGTTCATTTGGAAGCCAGACATCACAACAACCATCTGGG
AGTTTACAAGGCTCAAGCATGTCCAGCTTTGGCAATTTTCCGAAGTCCCATGCAGGCTATCAGCAGCCCCCTGCCTCCTCTAGCCATCATAGAGACATTGATAGGCA
GTCACAAGATTTACTTAGTGGAATCGTGGCTCCTACAAGTGCCATAAACCAAGCTCCTGTTGAGGACAATAAAAATGAAAATCAGGATGATAGTATTTGGTTGAAGG
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>LIC sugarcane protein (ScLIC):

MSRRQELCRNFQRGSCKYGAQCRFVHASSQQQQQAKPNPFGFGSQQQQQAKPNPFGFGSGSRQQQQQSSFGAQFQQQQQKPNPFGFGVQGGAAAQSRNAPGPAKPFQ
NKWVRDPSAPTKQQEAAQTPPAAHTSCTDPESCRHQIAEDFKNETPLWKLTCYAHLRSGPCDITGDISFEELRAKAYEEGRQGHPLQSIVEGERNLQNAKLMEFNNF
LNTPRVSVSQSPNFPTVTSFPEVKTNSSFGVSQTNGPPVFSSFSQVGAAANIGPGSRTAPGVSTNSIFGQSSQPNHPGFPAPTFGRSDMKFGVSGSFGSQTSQQPSG
SLQGSSMSSFGNFPKSHAGYQQPPASSSHHRDIDRQSQDLLSGIVAPTSAINQAPVEDNKNENQDDSIWLKEKWSIGEIPLGEPPQRHISHVF*

>PDS rice CDS_ LOC_Os03g08570.1:

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ACAGGTTGTTTGCCAGGACTTTCCAAGACCTCCACTAGAAAACACAATAAACTTTTTGGAAGCTGGACAACTATCTTCATTTTTCAGAAACAGTGAACAA
CCCACTAAACCATTACAGGTCGTGATTGCTGGAGCAGGATTAGCTGGTTTATCAACGGCAAAATATCTGGCAGATGCTGGTCATAAACCCATATTGCTTG
AGGCAAGGGATGTTTTGGGTGGAAAGATAGCTGCTTGGAAGGATGAAGATGGAGATTGGTATGAAACTGGGCTTCATATCTTTTTTGGAGCTTATCCCAA
CATACAGAACTTGTTTGGCGAGCTTGGTATTAATGATCGGTTGCAATGGAAGGAACACTCCATGATATTTGCCATGCCAAACAAGCCAGGAGAATTCAGC
CGGTTTGATTTTCCTGAAACATTGCCTGCACCCTTAAATGGAATATGGGCCATACTAAGAAACAATGAAATGCTAACTTGGCCAGAGAAGGTGAAGTTTG
CTCTTGGACTTTTGCCAGCAATGGTTGGTGGCCAAGCTTATGTTGAAGCTCAAGATGGTTTTACTGTTTCTGAGTGGATGAAAAAGCAGGGTGTTCCTGA
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TTTCTTCAGGAGAAGCATGGTTCTAAGATGGCATTCTTGGATGGTAATCCTCCTGAAAGGTTATGCATGCCTATTGTTGACCATGTTCGCTCTTTGGGTG
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TGCTTATGTTTTTGCAACACCAGTTGATATCTTGAAGCTTCTTGTACCTCAAGAGTGGAAAGAAATATCTTATTTCAAGAAGCTGGAGAAGTTGGTGGGA
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AATCATCGAAGCAACTATGCAAGAGCTAGCCAAGCTATTTCCTGATGAAATTGCTGCTGATCAGAGTAAAGCAAAGATTCTGAAGTATCATGTTGTGAAG
ACACCAAGATCTGTTTACAAGACTATCCCGGACTGTGAACCTTGCCGACCTCTGCAAAGATCACCGATTGAAGGGTTCTATCTAGCTGGTGACTACACAA
AGCAGAAATATTTGGCTTCGATGGAGGGTGCAGTTCTATCTGGGAAGCTTTGTGCTCAGTCTGTAGTGGAGGATTATAAAATGCTATCTCGTAGGAGCCT
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>PDS rice protein_ LOC_Os03g08570.1:

MDTGCLSSMNITGTSQARSFAGQLPTHRCFASSSIQALKSSQHVSFGVKSLVLRNKGKRFRRRLGALQVVCQDFPRPPLENTINFLEAGQLSSFFRNSEQPTKPLQV
VIAGAGLAGLSTAKYLADAGHKPILLEARDVLGGKIAAWKDEDGDWYETGLHIFFGAYPNIQNLFGELGINDRLQWKEHSMIFAMPNKPGEFSRFDFPETLPAPLNG
IWAILRNNEMLTWPEKVKFALGLLPAMVGGQAYVEAQDGFTVSEWMKKQGVPDRVNDEVFIAMSKALNFINPDELSMQCILIALNRFLQEKHGSKMAFLDGNPPERL
CMPIVDHVRSLGGEVRLNSRIQKIELNPDGTVKHFALTDGTQITGDAYVFATPVDILKLLVPQEWKEISYFKKLEKLVGVPVINVHIWFDRKLKNTYDHLLFSRSSL
LSVYADMSVTCKEYYDPNRSMLELVFAPAEEWVGRSDTEIIEATMQELAKLFPDEIAADQSKAKILKYHVVKTPRSVYKTIPDCEPCRPLQRSPIEGFYLAGDYTKQ
KYLASMEGAVLSGKLCAQSVVEDYKMLSRRSLKSLQSEVPVAS*

>PDS sorghum CDS_ Sb06g030030:

MDTGCLSSMNITGASQARPFAGQLPQRCFATTHHSSFSVRNLILRNKGRRSHRRHAALQVVCKDFPRPPLESTINYLEAGQLSSFFRNSERPSKPLQVVIAGAGLAG
LSTAKYLADAGHKPILLEARDVLGGKVAAWKDEDGDWYETGLHIFFGAYPNIQNLFGELGIEDRLQWKEHSMIFAMPNKPGEFSRFDFPETLPAPVNGIWAILRNNE
MLTWPEKVKFAIGLLPAMVGGQPYVEAQDGLTVSEWMKKQGVPDRVNDEVFIAMSKALNFINPDELSMQCILIALNRFLQEKHGSKMAFLDGNPPERLCMPIVDHIR
SRGGEVRLNSRIKKIELNPDGTVKHFALSDGTQITGDAYVCAAPVDIFKLLVPQEWSEITYFKKLEKLVGVPVINVHIWFDRKLKNTYDHLLFSRSSLLSVYADMSV
TCKEYYDPNRSMLELVFAPADEWIGRSDTEIIDATMEELAKLFPDEIAADQSKAKILKYHIVKTPRSVYKTVPNCEPCRPLQRSPIEGFYLAGDYTKQKYLASMEGA
VLSGKLCAQSIVQDYSRLALRSQKSLQSEEVPVPS*

>PDS sorghum protein_ Sb06g030030:

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ACCTCCACTAGAAAGCACAATAAACTATTTGGAAGCTGGGCAGCTCTCTTCGTTTTTTAGAAACAGCGAACGCCCCAGTAAACCGTTGCAGGTCGTGATT
GCTGGTGCAGGATTGGCTGGTCTATCAACGGCGAAGTATCTGGCAGATGCTGGCCATAAACCCATATTGCTTGAGGCAAGAGATGTTTTGGGCGGAAAGG
TAGCTGCTTGGAAGGATGAAGATGGGGATTGGTACGAGACTGGGCTTCATATCTTTTTTGGAGCTTATCCCAACATACAGAATCTGTTTGGTGAGCTTGG
AATTGAGGATCGTTTGCAGTGGAAAGAACACTCCATGATATTTGCCATGCCAAACAAGCCAGGAGAATTCAGCCGGTTTGATTTCCCAGAAACTTTGCCA
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AGAAGATAGAGCTGAATCCTGATGGAACTGTAAAACACTTCGCACTTAGCGATGGAACTCAAATAACTGGAGATGCTTATGTTTGTGCAGCACCAGTTGA
TATCTTCAAGCTTCTTGTACCTCAAGAGTGGAGTGAAATTACTTACTTCAAGAAGCTGGAGAAGTTGGTGGGAGTTCCTGTTATCAATGTTCATATATGG
TTTGACAGAAAGCTGAAAAACACATATGACCATCTTCTTTTCAGCAGGAGTTCACTTTTAAGTGTCTATGCAGACATGTCAGTAACCTGCAAGGAATACT
ATGATCCAAACCGTTCAATGCTGGAGTTGGTCTTTGCTCCTGCAGATGAATGGATTGGTCGAAGTGACACTGAAATCATTGATGCAACTATGGAAGAGCT
AGCCAAGTTATTTCCTGATGAAATTGCTGCCGACCAGAGTAAAGCAAAGATTCTTAAGTATCATATTGTGAAGACACCGAGATCGGTTTACAAAACTGTT
CCAAACTGTGAACCTTGCCGACCTCTCCAAAGGTCACCGATTGAGGGTTTCTATCTGGCTGGTGATTACACAAAGCAGAAATACTTGGCTTCCATGGAAG
GTGCAGTTTTATCCGGGAAGCTTTGCGCCCAGTCTATAGTGCAGGATTATAGCAGGCTTGCTCTCAGGAGCCAGAAAAGCCTACAATCCGAAGAAGTTCC
CGTCCCATCTTAG

>PDS sugarcane CDS (ScPDS):

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GCACAATAAACTATTTGGAAGCTGGGCAGCTCTCTTCGTTTTTTAGAAACAGCGAACGCCCCAGTAAACCGTTGCAGGTCGTGATTGCTGGTGCAGGATTGGCTGGT
CTATCAACGGCGAAGTATCTGGCAGATGCTGGCCATAAACCCATATTGCTTGAGGCAAGAGATGTTTTGGGTGGAAAGGTAGCTGCTTGGAAGGATGAAGATGGGGA
TTATTACGAGACTGGGCTTCATATCTTTTTTGGAGCTTATCCCAACATACAGAATCTGTTTGGTGAGCTTGGAATTGAGGATCGTTTGCAGTGGAAAGAACACTCCA
TGATATTTGCCATGCCAAACAAGCCAGGAGAATTCAGCCGGTTTGATTTCCCAGAAACTTTGCCAGCCCCTGTAAACGGGATATGGGCCATACTGAGAAACAATGAA
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ACCTTGCCGACCTCTCCAAAGGTCACCGATCGAAGGTTTCTATCTGGCTGGTGATTACACAAAGCAGAAATACTTGGCTTCCATGGAAGGTGCAGTTTTATCCGGGA
AGCTTTGCGCCCAGTCTATAGTGCAGGATTATAGCAGGCTCGCTCTCAGGAGCCAGAAAAGCCTACAATCCGAAGGAGTTCCTGTCCCATCTTAG

>PDS sugarcane protein (ScPDS):

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LSTAKYLADAGHKPILLEARDVLGGKVAAWKDEDGDYYETGLHIFFGAYPNIQNLFGELGIEDRLQWKEHSMIFAMPNKPGEFSRFDFPETLPAPVNGIWAILRNNE
MLTWPEKVKFAIGLLPAMVGGQPYVEAQDGLTVSEWMKKQEKHGSKMAFLDGNPPERLCMPIVDHIRSRGGEVRLNSRIKKIELNPDGTVKHFALSDGTQITGDAYV
CATPVDIFKLLVPQEWSEITYFKKLEKLVGVPVINVHIWFDRKLKNTYDHLLFSRSSLLSVYADMSVTCKEYYDPNRSMLELVFAPADEWIGRSDTEIIDATMEELA
KLFPDEIAADQSKAKILKYHVVKTPRSVYKTVPNCEPCRPLQRSPIEGFYLAGDYTKQKYLASMEGAVLSGKLCAQSIVQDYSRLALRSQKSLQSEGVPVPS*

>BRI1 rice CDS_ LOC_Os01g52050:

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TCGGCGGCTGGCGGCGCGAGGTGCGGGAGCAAGCTGCAGGCGCTCGATTTGTCCGGGAATGCCGCGCTCCGGGGCTCCGTCGCCGACGTGGCGGCCCTGGCCAGCGC
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CCAACAACAAGATCACCGACGATAGCGACCTCCGGTGGATGGTGGATGCCGGAGTCGGGGCAGTACGGTGGTTGGACCTTGCCCTGAACAGGATCTCCGGTGTCCCG
GAGTTCACCAACTGCTCCGGGCTTCAGTACCTTGACCTCTCCGGCAACCTCATCGTCGGTGAGGTGCCCGGCGGGGCACTTTCCGACTGCCGCGGTCTGAAAGTGCT
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CGGCGGCATCCCAGACGCCGTCTCCAACTGCACCAGCCTCGTCTCCCTCGACCTCAGCCTCAACTACATCAATGGGTCCATCCCGGCATCCCTCGGCGACCTTGGCA
ACCTGCAGGACCTCATCCTGTGGCAGAACGAGCTGGAGGGCGAGATACCGGCGTCCCTGTCGCGCATTCAGGGCCTCGAGCATCTCATCCTCGACTACAACGGGCTC
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CTACTTGGCTATCTTGAAGCTCAGCAACAATTCCTTCTCGGGGCCTATACCGCCAGAGCTCGGTGACTGCCAGAGCTTGGTGTGGCTGGACCTGAATAGCAATCAGC
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GAGTGCCGTGGCAAGGGGAGCTTGCTGGAGTTTACCAGCATCCGACCTGATGACCTCAGTCGGATGCCGAGCAAGAAGCTGTGCAACTTCACAAGAATGTATGTGGG
GAGCACGGAGTACACCTTCAACAAGAATGGTTCGATGATATTTCTCGATTTGTCATATAATCAGCTGGACTCGGCGATTCCTGGCGAGCTGGGGGACATGTTCTACC
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GAAGAGCCAGTATGAGAATAACACTGGTTTATGTGGCTTCCCACTGCCACCATGTGACCATAGTTCCCCAAGATCTTCCAATGACCACCAATCCCACCGGAGGCAGG
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GCGAGTACCTCTCGTGATATATATATTGATAGCAGGTCACATTCTGCAACTATGAATTCTGATTGGAGGCAAAATCTCTCCGGTACAAATCTTCTTAGCATCAACCT
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>BRI1 rice protein_ LOC_Os01g52050:

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EFTNCSGLQYLDLSGNLIVGEVPGGALSDCRGLKVLNLSFNHLAGVFPPDIAGLTSLNALNLSNNNFSGELPGEAFAKLQQLTALSLSFNHFNGSIPDTVASLPELQ
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KIKHRNLVPLLGYCKAGEERLLVYDYMKFGSLEDVLHDRKKIGKKLNWEARRKIAVGAARGLAFLHHNCIPHIIHRDMKSSNVLIDEQLEARVSDFGMARLMSVVDT
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>BRI1 sorghum CDS_ Sb03g032990:

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>BRI1 sorghum protein_ Sb03g032990:

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NNCSGLEYLDLSGNLIAGEVAGGILADCRGLRTLNLSGNHLVGPFPPDVAALTSLAALNLSNNNFSSELPADAFTELQQLKALSLSFNHFNGTIPDSLAALPELDVL
DLSSNSFSGTIPSSICQGPNSSLRMLYLQNNYLSGAIPESISNCTRLQSLDLSLNNINGTLPASLGKLGELRDLILWQNLLVGEIPASLESLDKLEHLILDYNGLTG
GIPPELSKCKDLNWISLASNQLSGPIPAWLGQLSNLAILKLSNNSFSGPIPAELGNCQSLVWLDLNSNQLNGSIPAELAKQSGKMNVGLVIGRPYVYLRNDELSSEC
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ANTSRDIYIDSRSHSGTMNSNNWRLSGTNALSVNLAAFEKPLQKLTFNDLIVATNGFHNDSLIGSGGFGDVYKAQLKDGKVVAIKKLIHVSGQGDREFTAEMETIGR
IKHRNLVPLLGYCKCGEERLLVYDYMSYGSLEDVLHDRKKVGIKLNWATRKKIAIGAARGLAYLHHNCIPHIIHRDMKSSNVLIDEQLEARVSDFGMARMMSVVDTH
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>GSK3 rice CDS_ LOC_ Os05g11730:

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GTTCTTTCGGTATCGTCTTTCAGGCTAAATGCTTGGAGACAGGAGAGACTGTTGCCATTAAGAAGGTATTGCAGGACCGACGGTACAAGAACCGTGAGCTTCAGCTT
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GACCTCGCTTCACGCCTTCTTCAATATTCACCGAGTCTCCGCTGCACTGCTCTTGATGCATGTGCACATCCTTTCTTTGATGAGCTGCGAGAGCCGAATGCACGCTT
GCCAAACGGACGTCCATTTCCACCACTATTCAACTTCAAACACGAACTAGCAAATTCTTCTCAAGAGCTCATCAGCAGGCTCATACCAGAACATGTTCGACGGCAAG
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>GSK3 rice protein_ LOC_ Os05g11730:

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VPGEPNISYICSRYYRAPELIFGATEYTTSIDIWSAGCVLAELLLGQPLFPGESAVDQLVEIIKVLGTPTREEIRCMNPNYTEFRFPQIKAHPWHKVFHKRMPPEAI
DLASRLLQYSPSLRCTALDACAHPFFDELREPNARLPNGRPFPPLFNFKHELANSSQELISRLIPEHVRRQATHNFFNTGS*

>GSK3 sorghum CDS_ Sb04g008580:

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AAAGGACTATCAGCTACATGGCAGAGAGAGTTGTGGGAACTGGATCATTTGGAATCGTCTTCCAGGCAAAATGTCTGGAGACTGGTGAGACTGTTGCCATTAAGAAG
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TGGCACAAGATTTTCCACAAGCGGATGCCTCCAGAAGCTATAGATCTCGCTTCACGTTTGCTCCAGTATTCACCAAATCTACGATGCACTGCTCTTGAAGCGTGTGC
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>GSK3 sorghum protein_ Sb04g008580:

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HQVKICDFGSAKMLVKGEANISYICSRYYRAPELIFGATEYTTSIDIWSAGCVLAELLLGQPLFPGESAVDQLVEIIKVLGTPTREEIRCMNPNYTEFRFPQIKAHP
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>DLT rice CDS_ LOC_ Os06g03710:

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LELVRALTACADSLSAGNHEAANYYLARLGEMASPAGPTPMHRVAAYFTEALALRVVRMWPHMFDIGPPRELTDDAFGGGDDDAMALRILNAITPIPRFLHFTLNER
LLREFEGHERVHVIDFDIKQGLQWPGLLQSLAARAVPPAHVRITGVGESRQELQETGARLARVAAALGLAFEFHAVVDRLEDVRLWMLHVKRGECVAVNCVLAMHRL

LRDDAALTDFLGLARSTGATILLLGEHEGGGLNSGRWEARFARALRYYAAAFDAVDAAGLPEASPARAKAEEMFAREIRNAVAFEGPERFERHESFAGWRRRMEDGG
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>DLT rice protein_ LOC_ Os06g03710:

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AAVPRPEQLHAHNGAPAQRVEAMELVVALTACADSLAACNHDAANYYLARLGEMASPAGPTPMHRVAAYFAEALALRVVRMWPHVFDVAPPRELTDGAVADDDDATA
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WMLHVKRGECVAVNCVLAAHRLLRDETGAAIADFLGLARSTGAAILLLGEHEDALNSGRWEARFARALRYYAAAFDAVDAAGLADTSPARAKAEEMFAREIRNAVAF
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>DLT sorghum CDS_ Sb10g001690:

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>DLT sorghum protein_ Sb10g001690:

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AAVPRPEQLHAHNGAPAQRVEAMELVVALTACADSLAACNHDAANYYLARLGEMASPAGPTPMHRVAAYFAEALALRVVRMWPHVFDVAPPRELTDGAVADDDDATA
LRVLNAVTPIPRFLHFTLNERVLRAFDGHDRVHVIDFDIKQGLQWPGLLQSLATRASGPPAHVRITGVGESRQELQETGARLGRVAAALGLAFEFHAVVDRLEDVRL
WMLHVKRGECVAVNCVLAAHRLLRDETGAAIADFLGLARSTGAAILLLGEHEDALNSGRWEARFARALRYYAAAFDAVDAAGLADTSPARAKAEEMFAREIRNAVAF
EAGDRFERHETFAGWRRRMQEGGFQNAGIGEREAMQGRMIARMFAPGNYSVQAQGDGEGLTLRWMDQAMYTVSAWTPISDGGGGGSTVSASVSTTASHSQQS*

>IBH1 rice CDS_ LOC_ Os04g0660100:

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CCTACTCCTCCATGGCGCGGGCGGCGAGCCCGCGCCGCGCGTGGAGCCGGGCGCTGCTCGGCCAGGTCCGCGCGCGGAGGTCCAGGACGCTGATGAGGCGCGCCGCC
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TCCGCCGCCTCCGCGGCAGCAGGGCGAGCCGCCGAGGGCCGAAGCGCTCCGGCGCCTGGTCCCCGGCGGCGCCGGCATGGAGTACTCCAGCCTCCTGGAGGAGACCG
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>IBH1 rice protein_ LOC_ Os04g0660100:

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>IBH1 sorghum CDS_ Sb06g031560:

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>IBH1 sorghum protein_ Sb06g031560:

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AVLLRRRVSAAAPPLMLRASAGETSAPTPPPPALAAVAPRGPPPRQAGEPARADALRQLVPGGAEMEYCSLLDETADYVRCLRAQVQLMQSLVDLFSAQQ*

>ILI1 rice CDS_ LOC_ Os04g54900:

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ACCAGGAGGTGGACAACCTCAGCGAGACGCTCGCTCAGCTGCTCGCCTCCCCCGACGTCACCAGCGACCAGGCGGCCGTCATCAGGAGCCTCCTCATGTGA

>ILI1 rice protein_ LOC_ Os04g54900:

MSSSRRSRSRRAGSSVPSSSSSSRTSISEDQIAELLSKLQALLPESQARNGAHRGSAARVLQETCSYIRSLHQEVDNLSETLAQLLASPDVTSDQAAVIRSLLM*

>ILI1 sorghum CDS_ Sb06g030340:

ATGTCCAGCAGCCGGAGGTCGTCATCACGCACACGGCGTGCGGGGAGCGGCTCGCTGTCGTCGTCGACGTCGAGGTCCATCTCGGAGGACCAGATCTCCGAGCTCCT
GTCCAAGCTTCAGGCGCTGCTCCCGGAGTCTCAAACTCGCAATGGCGCACATAGGGGCTCGGCGGCGAGGGTGCTGCAGGACACGTGCAGCTACATCAGGAGCCTGC
ACCAGGAGGTGGACAACCTCAGCGAGACGCTCGCCGAGCTGCTGGCCTCCGCCGATGTCACCAGCGACCAGGCCGCCGTCATCAGGAGCCTGCTCATGTGA

>ILI1 sorghum protein_ Sb06g030340:

MSSSRRSSSRTRRAGSGSLSSSTSRSISEDQISELLSKLQALLPESQTRNGAHRGSAARVLQDTCSYIRSLHQEVDNLSETLAELLASADVTSDQAAVIRSLLM*

>DWF4 rice CDS_ LOC_ Os03g40540:

ATGGTGTTGGTGGCGATTGGGGTGGTTGTGGCGGCGGCGGTGGTGGTGAGCAGCCTGCTGCTGCGGTGGAACGAGGTGCGGTACAGCCGGAAGCGCGGCCTGCCGCC
GGGGACAATGGGGTGGCCGCTCTTCGGCGAGACCACCGAGTTCCTCAAGCAGGGACCCAGTTTCATGAAGGCCCGGAGGCTCAGGTACGGGAGCGTGTTCAGGACGC
ACATCCTGGGGTGCCCGACGGTGGTGTGTATGGAGGCGGAGCTGAACCGGCGGGCGCTGGCCAGCGAAGGGCGCGGGTTCGTCCCGGGCTACCCGCAGTCGATGCTG
GACATCCTGGGGCGGAACAACATCGCCGCCGTGCAGGGCCCCCTCCACCGCGCCATGCGCGGCGCCATGCTCTCCCTCGTCCGCCCCGCCATGATCCGCTCCTCCCT
CCTCCCCAAGATCGACGCCTTCATGCGCTCCCACCTCGCCGCCTGGTCCTCCTCCTCCTCCTCCGCCGTCGTCGACATCCAGGCCAAGACCAAGGAGATGGCCTTGC

TATCTGCACTCAGGCAGATTGCCGGCGTCTCCGCTGGCCCACTCTCTGACGCTCTCAAGGCAGAGCTCTACACCCTTGTGCTTGGCACCATCTCCCTGCCCATCAAC
CTTCCTGGAACCAACTACTACCAAGGCTTCAAGGCAAGGAAGAAGCTTGTTGCAATGCTAGAGCAGATGATCGCGGAACGGCGATCCTCCGGTCAGGTACACGACGA
CATGCTGGATGCGCTCTTGACCGGTGTCGAGGGCACCAGGGAGAAGCTCACAGATGAGCAGATCATTGACCTGATCATCACCCTTATATACTCTGGATATGAAACCA
TGTCGACGACCTCGATGATGGCTGTCAAGTACCTGTCAGACCATCCCAAAGCTCTTGAGCAACTCAGGAAAGAACATTTTGATATCAGGAAAGGTAAAGCGCCCGAA
GATGCCATCGACTGGAATGATTTCAAGTCCATGACCTTCACTCGAGCTGTTATCTTCGAGACATTAAGATTAGCTACAGTTGTGAATGGGCTGCTGAGGAAAACTAC
CCAAGATGTTGAAATGAATGGGTATGTTATCCCAAAAGGTTGGAGAATATATGTTTACACAAGGGAAATAAATTATGATCCATTCCTGTACCCTGATCCCATGACAT
TCAATCCATGGAGGTGGCTGGAGAAGAACATGGAATCACATCCACACTTCATGCTGTTTGGAGGAGGTAGTCGAATGTGCCCGGGGAAGGAAGTAGGCACCGTAGAA
ATTGCAACATTCCTTCACTATTTCGTGACTCAATACAGATGGGAGGAAGAAGGTAACAACACAATATTGAAGTTCCCCCGAGTTGAAGCTCCCAACGGGTTACATAT
CCGCGTTCAAGATTACTGA

>DWF4 rice protein_ LOC_ Os03g40540:

MVLVAIGVVVAAAVVVSSLLLRWNEVRYSRKRGLPPGTMGWPLFGETTEFLKQGPSFMKARRLRYGSVFRTHILGCPTVVCMEAELNRRALASEGRGFVPGYPQSML
DILGRNNIAAVQGPLHRAMRGAMLSLVRPAMIRSSLLPKIDAFMRSHLAAWSSSSSSAVVDIQAKTKEMALLSALRQIAGVSAGPLSDALKAELYTLVLGTISLPIN
LPGTNYYQGFKARKKLVAMLEQMIAERRSSGQVHDDMLDALLTGVEGTREKLTDEQIIDLIITLIYSGYETMSTTSMMAVKYLSDHPKALEQLRKEHFDIRKGKAPE
DAIDWNDFKSMTFTRAVIFETLRLATVVNGLLRKTTQDVEMNGYVIPKGWRIYVYTREINYDPFLYPDPMTFNPWRWLEKNMESHPHFMLFGGGSRMCPGKEVGTVE
IATFLHYFVTQYRWEEEGNNTILKFPRVEAPNGLHIRVQDY*

>DWF4 sorghum CDS_ Sb01g015040:

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GGGCACAATGGGGTGGCCGCTCTTCGGCGAGACCACCGAGTTCCTCAAGCAGGGCCCCAGCTTCATGAAGCAGAGAAGGCTCAGGTACGGGAGCCTGTTCCGGACGC
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CGATGATGGCGGTGAAGTATCTGTCGGACAATCCGAAAGCTCTTGAACAAATCAGGAAAGAGCATCTTGACATAAGGAAGGCTAAATCACCAGACGATGCCCTTGAC
TGGAATGATTATAAGTCAATGACCTTCACCAAAGCTGTCATTTATGAGACTCTAAGACTAGCTACAGTTGTCAATGGGCTGCTGAGGAAAACTACCCAGGATGTAGA
AATGAATGGGTATGTTATTCCAAAGGGCTGGAGAATTTATGTCTACACAAGGGAGATAAATTATGATCCATTCCTGTACCCGGAACCGATGGTTTTCAACCCATGGA
GATGGCTGGAGACGAACCTCGAATCACATCCACACTTCATGTTGTTTGGAGGAGGTGCCCGCATGTGCCCAGGGAAGGAAGTGGGAACAGTTGAAATTGCAACGTTC
CTTCACTATTTCATTACGCGTTACAGATGGGAGGAGGAGGGAAACA

>DWF4 sorghum protein_ Sb01g015040:

MAVLLLLVAVLGVVLASSLLLRWNELRYSRRRGLPPGTMGWPLFGETTEFLKQGPSFMKQRRLRYGSLFRTHILGCPTVVCMEPELNRRTLASDGAGFVPGYPQSML
DILGPNNIAAVHGPLHRAMRGAMLALTRAHMIRAALLPKIDAFMRAHLHGWAGRRVDIQEMTKEMALLSALRQIAGISAGPLSDALKAELYTLVLGTFSLPINIPGT
NYSKGLQARKKLVAMLRQMIADRRSSGCAQDDMLDALLSGNEGTRAKLTDDQIIDLLITLIYSGYETVSTTSMMAVKYLSDNPKALEQIRKEHLDIRKAKSPDDALD
WNDYKSMTFTKAVIYETLRLATVVNGLLRKTTQDVEMNGYVIPKGWRIYVYTREINYDPFLYPEPMVFNPWRWLETNLESHPHFMLFGGGARMCPGKEVGTVEIATF
LHYFITRYRWEEEGNNTISKFPRVAAPNGLHIRVQDY*

8.2 Construction sequences for synthesis by GenOne Biotechnilogies:

A) ScBZR1_OE:

CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT
ATGACGTCGGGGGCGGCCGCGGCGGCGGCGGCGGCCGGAGCGCTGGGGCGGACGCCGACGTGGAAGGAGCGGGAGAACAACAAGCGCCGGGAGCGCCGGC
GGAGGGCCATCGCCGCCAAGATCTTCACGGGCCTCCGCGCGCTCGGCAACTACAAGCTGCCCAAGCACTGCGACAACAACGAGGTGCTCAAGGCGCTGTG
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CTGAGCGCGCCGTCCTCGAGCTTCCCGAGCCCGGTGCCGTCCTACCACGCCAGCCCGGCGTCGTCGAGCTTCCCGAGCCCGACGCGCCTCGACCACAGCA
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CCGCGTCTCCAGCAGCGCGCCCGTCACGCCGCCGCTCTCCTCGCCCACGGCGGCGTCGCGGCCGCCCACCAAGGTCCGCAAGCCGGACTGGGACAACGCC
GTCGCCGACCCGTTCCGCCACCCCTTCTTCGCGGTCTCCGCCCCCGCCAGCCCCACCCGGGCCCGCCGGCGCGAGCACCCGGACACCATCCCCGAGTGCG
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CAAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTA
TTTG

AttL1
AttL2

B) ScLIC_OE:

CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT
ATGAGCCGGCGGCAGGAGCTCTGCAGGAACTTCCAGCGCGGCAGCTGCAAGTACGGAGCGCAGTGTAGGTTCGTGCACGCGTCCTCTCAGCAGCAGCAGC
AGGCGAAGCCCAACCCGTTTGGGTTCGGCTCTCAGCAGCAGCAGCAGGCGAAGCCCAACCCGTTTGGGTTCGGCTCTGGGAGCAGGCAGCAGCAGCAGCA
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GTACAGATCCTGAGTCATGCAGGCACCAGATTGCTGAGGATTTTAAGAATGAAACTCCACTCTGGAAGCTTACGTGTTATGCTCATCTCAGGAGTGGTCC
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AGAAATCTGCAAAATGCAAAGCTGATGGAGTTTAATAATTTTCTGAACACTCCACGTGTATCAGTATCACAAAGTCCAAACTTTCCAACTGTCACTTCCT
TCCCTGAAGTGAAAACTAATTCATCATTTGGGGTTTCTCAAACTAATGGACCACCAGTGTTACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAATA
ATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG

C) ScBZR1_HP:

CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT
GGCTTGCATCCCTTTCGGTAGGTGGTGCCGTCGTCCTCGACGACCCAGCCCGCTTCGCGGCACAGCGCCTTGAGCACCTCGTTGTTGTCGCAGTGCTTGG
GCAGCTTGTAGTTGCCGAGCGCGCGGAGGCCCGTGAAGATCTTGGCGGCGATGGCCCTCCGCCGGCGCTCCCGGCGCTTGTTGTTCTCCCGCTCCTTCCA
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GGAGCGGGAGAACAACAAGCGCCGGGAGCGCCGGCGGAGGGCCATCGCCGCCAAGATCTTCACGGGCCTCCGCGCGCTCGGCAACTACAAGCTGCCCAAG
CACTGCGACAACAACGAGGTGCTCAAGGCGCTGTGCCGCGAAGCGGGCTGGGTCGTCGAGGACGACGGCACCACCTACCGAAAGGGATGCAAGCCACCCA
GCTTTCTTGTACAAAGTTGGCATTATAAAAAATAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG

D) ScPDS_amiRNA:

CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT
GGATCCCAGCAGCAGCCACAGCAAAATTTGGTTTGGGATAGGTAGGTGTTATGTTAGGTCTGGTTTTTTGGCTGTAtacgtaGCAGCAGCAGTTAATCCT
GCACTATACACTGCAGGAGATTCAGTTTGAAGCTGGACTTCACTTTTGCCTCTCTCAGTGAATACTGCAGGATTAATTCCTGCTGCgacgtcTAGGCTGT
TCTGTGGAAGTTTGCAGAGTTTATATTATGGGTTTAATCGTCCATGGCATCAGCATCAGCAGCGGTACCCTGCAGACCCAGCTTTCTTGTACAAAGTTGG
CATTATAAAAAATAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG

E) ScLIC 1_amiRNA:

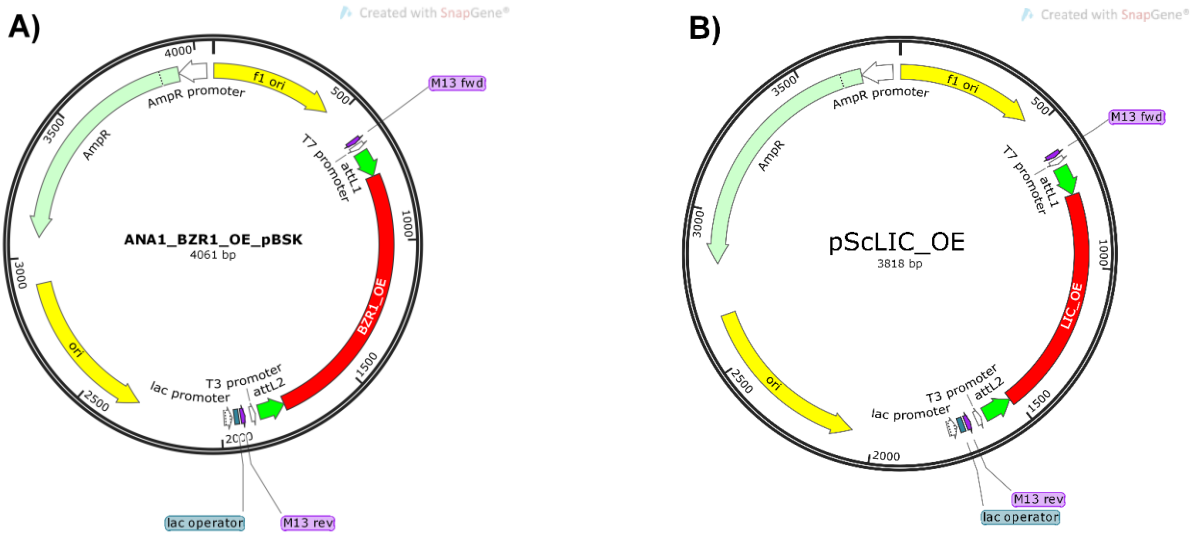
CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT
GGATCCCAGCAGCAGCCACAGCAAAATTTGGTTTGGGATAGGTAGGTGTTATGTTAGGTCTGGTTTTTTGGCTGTAGCAGCAGCAGTTACCCAAATTCGA
GGTACTGCAGGAGATTCAGTTTGAAGCTGGACTTCACTTTTGCCTCTCTCAGTAGCTCCAATTTGGGTAATTCCTGCTGCTAGGCTGTTCTGTGGAAGTT
TGCAGAGTTTATATTATGGGTTTAATCGTCCATGGCATCAGCATCAGCAGCGGTACCCTGCAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAA
TAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG

F) ScLIC 2_amiRNA:

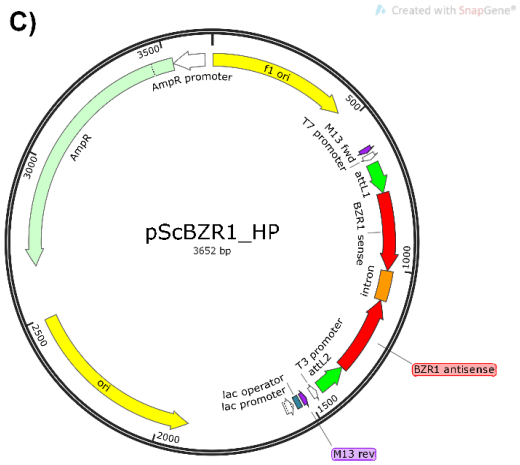
CAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTTGCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAAAAGCAGGCT
GGATCCCAGCAGCAGCCACAGCAAAATTTGGTTTGGGATAGGTAGGTGTTATGTTAGGTCTGGTTTTTTGGCTGTAGCAGCAGCAGTATTTCATATCAGA
ACGGCCACAGGAGATTCAGTTTGAAGCTGGACTTCACTTTTGCCTCTCTTGGCCGTTCTGATATGAAATATTCCTGCTGCTAGGCTGTTCTGTGGAAGTT
TGCAGAGTTTATATTATGGGTTTAATCGTCCATGGCATCAGCATCAGCAGCGGTACCCTGCAGACCCAGCTTTCTTGTACAAAGTTGGCATTATAAAAAA
TAATTGCTCATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAAATAAAATCATTATTTG

Legend: Sequences designed and sent to GenOne Biotechnologies for vectors synthesis. A) ScBZR1_OE vector. B) ScLIC_OE vector. C) ScBZR1_HP vector. D) ScPDS_amiRNA. E) ScLIC1_amiRNA. F) ScLIC2_amiRNA. Sequences in yellow correspond to attL1. Sequences in green correspond to attL2. Sequences in orange correspond to antisense sequence of 250bp flanking BZR1 domain. Sequences in gray correspond to sense sequence of 250bp flanking BZR1 domain. Sequences in light blue correspond to intron of hairpin structure. Sequence in dark blue correspond to amiRNAPDS. Sequence in light purple correspond to amiRNAPDS*. Sequence in red and lowercase letters correspond to SnaB cut site. Sequence in black and lowercase letters correspond to AattII cut site. Sequences in pink correspond to amiRNALIC1 and amiRNALIC2, respectively. Sequences in dark purple correspond to amiRNALIC1* and amiRNALIC2*, respectively.

8.3 Maps of synthesized constructions by GenOne Biotechnilogies in entry vector pBSK:



C)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pScBZR1_HP
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f1 ori
M13 fwd
T7 promoter
attL1
BZR1 sense
intron
BZR1 antisense
T3 promoter
attL2
lac operator
lac promoter
M13 rev
ori
AmpR
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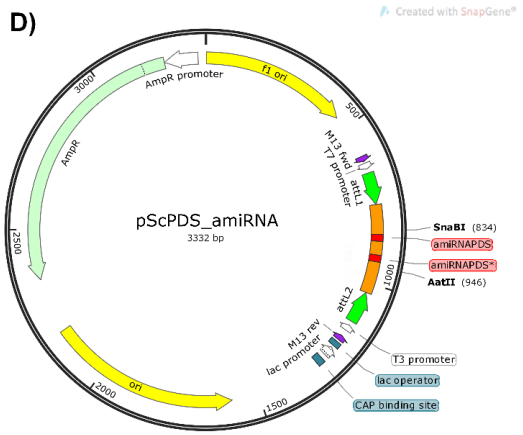
1500
2000
2500
3000

D)


Created with SnapGene®
pScPDS_amiRNA
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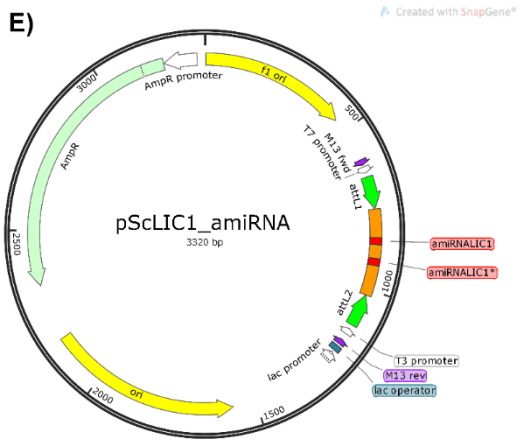
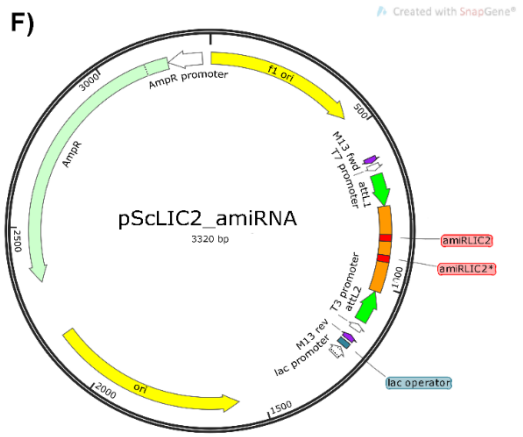
AmpR promoter
f1 ori
M13 fwd
T7 promoter
attL1
SnaBI (834)
amiRNAPDS
amiRNAPDS*
AatII (946)
attL2
M13 rev
lac promoter
T3 promoter
lac operator
CAP binding site
ori
AmpR
500
1000
1500
2000
2500
3000

E)


Created with SnapGene®
pScLIC1_amiRNA
3320 bp
AmpR promoter
f1 ori
M13 fwd
T7 promoter
attL1
amiRNALIC1
amiRNALIC1*
attL2
lac promoter
T3 promoter
M13 rev
lac operator
ori
AmpR
500
1000
1500
2000
2500
3000

F)


Created with SnapGene®
pScLIC2_amiRNA
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AmpR promoter
f1 ori
M13 fwd
T7 promoter
attL1
amiRLIC2
amiRLIC2*
T3 promoter
attL2
M13 rev
lac promoter
lac operator
ori
AmpR
500
1000
1500
2000
2500
3000

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I N F O R M A Ç Ã O

INFORMAMOS que o projeto **CIBio/IB No. 2007/03 - Genômica funcional de plantas**, cujo pesquisador responsável é o Prof. Dr. Marcelo Menossi Teixeira, sub-projeto "*O papel da via de sinalização de brassinosteróide no desenvolvimento e fisiologia da cana-de-açúcar*", do pós-graduando Ana Laura Garcia Leme Peres, encontra-se devidamente aprovado e regularizado junto a CIBio/IB-UNICAMP e a CTNBio, conforme legislação vigente.

Cidade Universitária "Zeferino Vaz",
21 de maio de 2019.

Prof. Dr. JOSÉ LUIZ PROENÇA MÓDENA
Presidente da CIBio
Instituto de Biologia - UNICAMP

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Comissão Interna de Biossegurança
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RG n.° 49266885-1

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RG n.° 4231026-3