Differential expression of the brassinosteroid receptor-encoding *BRI1* gene in *Arabidopsis thaliana*

Ph.D. dissertation

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LIST OF ABBREVIATIONS

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BL	brassinolide
BR	brassinosteroid
CS	castasterone
DD	continuous dark
GUS	*Escherichia coli* β-glucuronidase
LD	daily cycles of light and dark photoperiods
LL	continuous light
LRR	leucine-rich repeat
LUC	firefly (*Photinus pyralis*) luciferase
qRT-PCR	quantitative real-time PCR
RLK	receptor-like kinase
WT	wild type

1. INTRODUCTION

Brassinosteroids (BRs) represent a recently recognized class of phytohormones that regulate a wide range of physiological functions throughout the life of plants from germination to seed production. Although intense studies of BR action started only following the middle of the 1990s, as a result of these investigations BR perception and signaling became one of the best known mechanisms of plant hormone regulation. It was established that BRs are perceived by cell surface-localized receptors which, in cooperation with their co-receptors, initiate a phosphorylation-based intracellular signaling process. This alters, via two closely related BR-specific transcription factors, the expression of a set of BR-controlled genes.

Whereas the key components of BR perception and their roles were elucidated in detail, little information was available on their distribution within the plant. Based on early studies it was proposed that the receptor is expressed without spatial or other differential regulation, and that site- or development-specific BR effects depend only on local concentrations of the hormone. But this model was challenged by other results, obtained partly in our laboratory, which suggested organ-specific or time of the day- and light-dependent differences in BR sensitivity.

Therefore, the aim of our project was to find out whether the expression of the BR receptor, a key component in the signaling process that interacts with the hormone and initiates the phosphorylation cascade, is differentially regulated, and if so, whether this control mechanism can influence receptor distribution and alter BR susceptibility. For this work the model plant *Arabidopsis* was ideally suited, because its BR receptor, co-receptors and signaling components were well characterized, and several mutants in these elements were

available. We expected that answering these questions of receptor expression and abundance would contribute to better understanding of the ways how BR sensitivity is modulated, and whether this adjustment is coordinated with local levels of the hormone in order to evoke optimal physiological effects.

2. RESEARCH BACKGROUND

Since plants have sessile (sedentary) lifestyle, being anchored to their substrates, they need to adapt to their challenging conditions by integrating diverse environmental stimuli into their endogenous programs. Plant responses to external cues are coordinated by phytohormones which are often growth-promoting factors, thus being involved in cell division, elongation and differentiation during plant development. Besides classic types of hormones, such as auxins, gibberellins, cytokinins, ethylene and abscisic acid, BRs have recently been recognized as a novel class of phytohormones. These steroidal compounds that occur ubiquitously in vascular plants at nanomolar concentrations are important regulators of growth and development (Clouse and Sasse, 1998; Bajguz and Tretyn, 2003). Although their physiological role is deeply intertwined with those of auxins (Nemhauser *et al*., 2004; Vert *et al*., 2008), BRs *per se* orchestrate the fine-tuning of numerous physiological and morphogenic functions.

2.1. Brassinosteroids: steroidal plant hormones

2.1.1. Discovery and early research history

BR research dates back to the early studies of Mitchell *et al*. (1970) who screened pollen extracts from roughly sixty plant species for biologically active regulatory substances. They found that about half of the samples exhibited growth-promoting effect when tested on bean seedlings. The regulatory substances of the pollen, eliciting responses distinct from those of auxins, were termed 'brassins' (Mitchell *et al*., 1970).

Brassinolide (BL), the main bioactive compound of brassins, was first isolated and characterized by Grove *et al.* in 1979. This was soon followed by the identification of castasterone (CS; Yokota *et al.*, 1982), another regulatory steroid, which was extracted from insect galls of Japanese chestnut (*Castanea crenata*). Soon thereafter the term 'brassinosteroid' was coined (Arteca *et al.*, 1983) to designate the group of structurally related endogenous plant steroids possessing growth-regulatory activity. Up to now more than 60 BR forms have been identified, but only few of these proved to be ubiquitous (Bajguz and Tretyn, 2003). The isolation of the first hormone-deficient and insensitive mutants and their characterization were instrumental for identifying the regulatory functions of BRs and understanding their essential role in plant development (Clouse *et al.*, 1996; Li JM *et al.*, 1996; Szekeres *et al.*, 1996). These studies resulted in the acceptance of BRs as a novel class of *bona fide* plant hormones (Clouse, 1996).

2.1.2. Chemical structure

BRs are polyhydroxylated steroids showing similarity to ecdysone, the molting hormone of insects. BRs have been defined as compounds sharing 5α-cholestane skeleton, carrying oxygen moiety at C-3, and further ones at one or more of the C-2, C-6, C-22 and C-23 carbon atoms (Bishop and Yokota, 2001). In addition to the physiologically important BRs of C_{28} steroid structure, occasionally their C_{27} and C_{29} congeners have also been detected. These steroids, differing from the common C_{28} BRs only in their C-24 substitution, are functional, but their low levels exclude their regulatory role (Yokota, 1997).

Structural-functional modeling studies were carried out in order to elucidate the physiological effects of different BRs (Brosa *et al.*, 1996). These provided valuable information about the structural requirements of hormone action but, due to the

metabolization in the bioassay systems, could not identify compounds of inherent bioactivity. It was only after the characterization of biosynthetic mutants that BL and CS were found to be the only BR forms possessing biological activity (Nomura *et al*., 2005). Their structural features show that hormonal activity of BRs requires oxigen-containing substituents at all potential substitution sites of the 5α-cholestane skeleton, namely at the C-2, C-3, C-6, C-22 and C-23 positions (Figure 1). Uniquely among natural steroids, BL features a seven-member steroid B ring, which is formed in CS by oxa-lactonization at the C-6 carbon. This extra oxidation was found to increase the biological activity of BL roughly tenfold compared to that of CS (Sung *et al*., 2000).

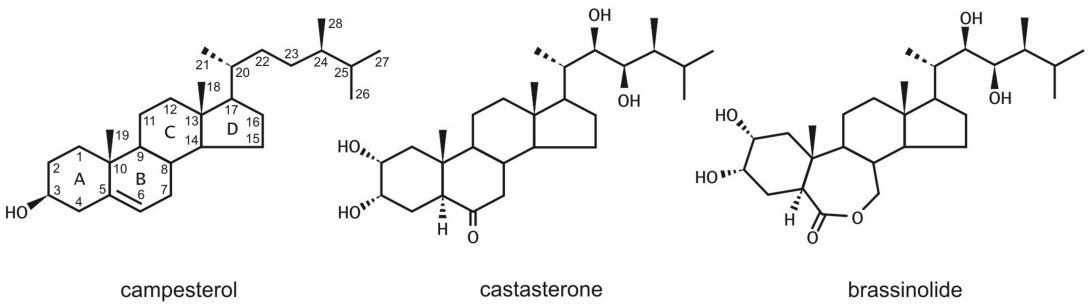

Figure 1. The chemical structures of campesterol, CS and BL

Campesterol, an abundant phytosterol, is the source of BR biosynthesis that leads to the formation of the biologically active forms CS and BL. Numbering of the carbon atoms and designations of the steroid rings are shown in the structural formula of campesterol.

2.1.3. Physiological roles

Early studies of the biological responses to BRs relied on exogenous application of the hormone, followed by recording the various responses. These experiments clearly indicated the pivotal role of BRs in growth promotion, by facilitating the elongation and division of cells (Mandava, 1988). It has been shown that BRs enhance cell elongation by promoting transverse orientation of cortical microtubules (Mayumi and Shibaoka, 1995), and that during

xylogenesis BRs stimulates the differentiation of tracheary elements (Iwasaki and Shibaoka, 1991; Clouse *et al.*, 1992). Furthermore, BRs were also reported to alleviate the effects of multiple types of abiotic (salinity, drought, temperature) stress effects in various crop plants (reviewed by Cutler, 1991).

The full spectrum of physiological BR effects was revealed by the phenotypic and functional characterization of BR-deficient and -insensitive *Arabidopsis*, tomato (*Solanum lycopersicum*) and pea (*Pisum sativum*) mutants. In the *det2* (*de-etiolated 2*) mutant of *Arabidopsis* constitutive photomorphogenesis, short hypocotyl, diminished growth and reduced male fertility were accompanied by late flowering and delayed senescence (Chory *et al.*, 1991). Subsequently it was shown that the phenotypic features of *det2* result from BR deficiency caused by the defect of a steroid 5α-reductase (Li JM *et al.*, 1996). In the case of another *Arabidopsis* mutant, *cpd* (*constitutive photomorphogenesis and dwarfism*) featuring similar developmental defects, it was shown that BR deficiency interferes with normal photomorphogenesis and cell elongation, causes aberrant vascularization, and induces multiple pathogenesis-related genes (Szekeres *et al.*, 1996). Later studies provided evidence that BRs also control important functions related to seed germination (Steber and McCourt, 2001), and the gravitropic (Kim SK *et al.*, 2000), shade avoiding (Kozuka *et al.*, 2010) and various stress responses (Nakashita *et al.*, 2003) of the plants. Recent results that elucidate connections between BR and light signaling are reviewed by Wang ZY *et al.* (2012).

Physiological responses to changing environmental conditions are often determined by crosstalks between interdependent hormonal signaling pathways. This type of regulation can ensure that plants respond only to stimuli that are confirmed by independent signaling mechanisms. During growth induction, BRs act synergistically with auxins to increase the expansion and proliferation of the cells (Hardtke, 2007), and additively with cytokinins to enhance cell division (Mandava, 1988). Several genes, such as those influencing senescence,

are regulated antagonistically by BRs and abscisic acid (Goda *et al.*, 2002). Recent studies revealed complex interactive control of flowering transition by BRs, gibberellins and ABA (Domagalska *et al*., 2010), and shade avoidance by BRs, auxins and gibberellins (Sorin *et al*., 2009).

2.2. Adjustments of endogenous BR levels

Phytohormones are important coordinators of cellular functions, exerting their effects via concentration gradients that both elicit and fine tune a defined set of specific responses. Actual levels of the hormones depend on the balance between their local biosynthesis and deactivation, as well as on the transport processes allocating them within the plant.

2.2.1. BR metabolism

In the various organs and tissues the accumulation or depletion of bioactive BRs is determined by well coordinated metabolic events. Additionally, because too high or too low levels of the hormone would be harmful for the plant, BR concentrations are kept within a physiological range by BR-dependent homeostatic regulatory mechanisms that can attenuate biosynthesis or deactivation at extreme hormone levels (Hategan *et al.*, 2011; Zhao and Li, 2012).

2.2.1.1. Biosynthesis

The main pathway of BR biosynthesis has been deduced from the conversion of isotope-labeled BR intermediates in suspension cultures of Madagascar periwinkle

(*Catharanthus roseus*) (Suzuki *et al.*, 1993a; Fujioka *et al.*, 1995). Later studies, also involving cultured *Arabidopsis* seedlings, revealed that the biosynthetic reactions do not follow a single route, but they can take place in parallel sub-pathways, which form a complex biosynthetic network (Choi *et al.*, 1997; Fujioka *et al.*, 2002; Ohnishi *et al.*, 2006). The main reactions of this network functioning in vegetative tissues are shown in Figure 2.

The physiologically important C_{28} BRs are synthesized from campesterol via mostly oxidative reactions, which are carried out by cytochrome P450 monooxygenases belonging to the CYP90 and CYP85 families (Figure 2). The characterization of BR biosynthetic mutants has been instrumental in clarifying the functions of these enzymes in *Arabidopsis*, pea, tomato and rice, as well as in demonstrating that these and their regulatory mechanisms are well conserved in both dicot and monocot species (reviewed in: Szekeres and Bishop, 2006; Vriet *et al.*, 2013). The enzymological properties of heterologously expressed *Arabidopsis* CYP90 and CYP85 monooxigenases were investigated in detail (Fujita *et al.*, 2006; Ohnishi *et al.*, 2006; Ohnishi *et al.*, 2012). These studies revealed that all these P450s are multisubstrate enzymes that catalyze, albeit by markedly different efficiencies, conversions of several, structurally related BR intermediates (Figure 2). CYP85A2, one of the C-6 oxidases that produce bioactive BRs, was also shown to be multifunctional. In addition to forming CS by introducing a keto-group at C-6, this enzyme also catalyze the synthesis of BL by subsequent, Bayer-Villiger-type lactonization of the steroid B ring (Nomura *et al.*, 2005). Differential substrate preferences of the CYP90 and CYP85 enzymes are indicative of preferred routes in BR biosynthesis (Figure 2), and modulate the efficiency of BR production through the availability of more or less preferred substrate pools.

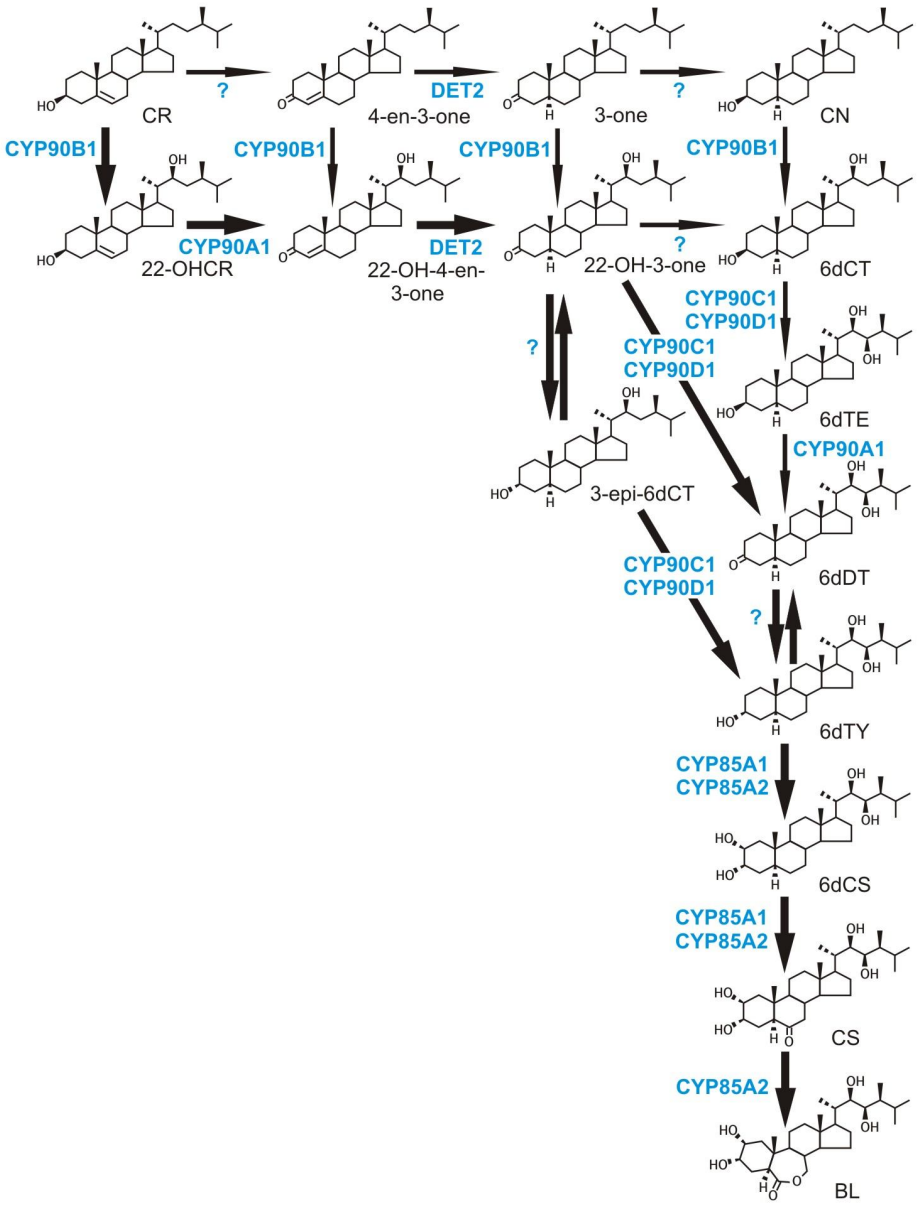

Figure 2. The pathways of BR biosynthesis

BRs are synthesized from campesterol (CR) via a network of biosynthetic routes. Biosynthetic enzymes are indicated by their conventional symbols (in blue) at the arrows corresponding to conversion steps. Bold arrows highlight enzymologically preferred reactions. Intermediates are abbreviated as 4-en-3-one: (*24R*)-ergost-4-en-3-one; 3-one: (*24R*)-ergost-3-one; CN: campestanol; 22-OHCR: (22S-)22-hydroxycampesterol; 22-OH-4-en-3-one: (*22S,24R*)-ergost-4-en-3-one; 22-OH-4-en-3-one: (*22S,24R*)-ergost-3-one; 6dCT: 6-deoxocathasterone; 3-epi-6dCT: 3-*epi*-6-deoxocathasterone; 6dTE: 6-deoxoteasterone; 6dDT: 3-dehydro-6-deoxoteasterone; 6dTY: 6-deoxotyphasterol; 6dCS: 6-deoxocastasterone.

2.2.1.2. Deactivation and catabolism

In addition to biosynthesis, proper homeostasis and physiological adjustment of BR levels also requires removal of the bioactive hormone. One way of achieving this is reversible glycosylation or acylation of the C-3 or C-23 hydroxy groups, which allow strong accumulation ('storage') of inactive but accessible BRs in seeds and pollen. Alternatively, BRs can be deactivated irreversibly via hydroxylation and subsequent modifications at the C-26 position, which render them to enzymatic degradation (Fujioka and Yokota, 2003; Bajguz, 2007). Recent studies have revealed that not only bioactive BRs, but their precursors can also be targets to irreversible deactivation (Sakamoto *et al.*, 2011), and that deactivating substitutions can enhance cellular retention of such covalently modified BRs (Husar *et al.*, 2011).

2.2.1.3. BR metabolism and its physiological control

To ensure optimal BR levels, plants utilize enzymatic functions capable of deactivating excess amounts of the hormone temporarily or permanently. Reversible deactivation is achieved primarily via glycosyl or acyl conjugation at the C-23 or C-3 hydroxy groups. In addition to its homeostatic effect, temporal BR deactivation can also produce storage forms of the hormone during seed development (Suzuki *et al.*, 1993b; Asakawa *et al.*, 1996). In *Arabidopsis* the main pathway of permanent BR deactivation is initiated by C-26 hydroxylation and subsequent glycosylation of BL and CS by the P450-type enzyme BAS1/CYP734A1 (Turk *et al.*, 2003). Other functions of less importance also contribute to permanent BR deactivation, but their molecular mechanisms are largely unknown. The

emerging view is that in some catabolic reactions the preferred substrates are BR intermediates, rather than active end products (Fujioka and Yokota, 2003; Bajguz, 2007).

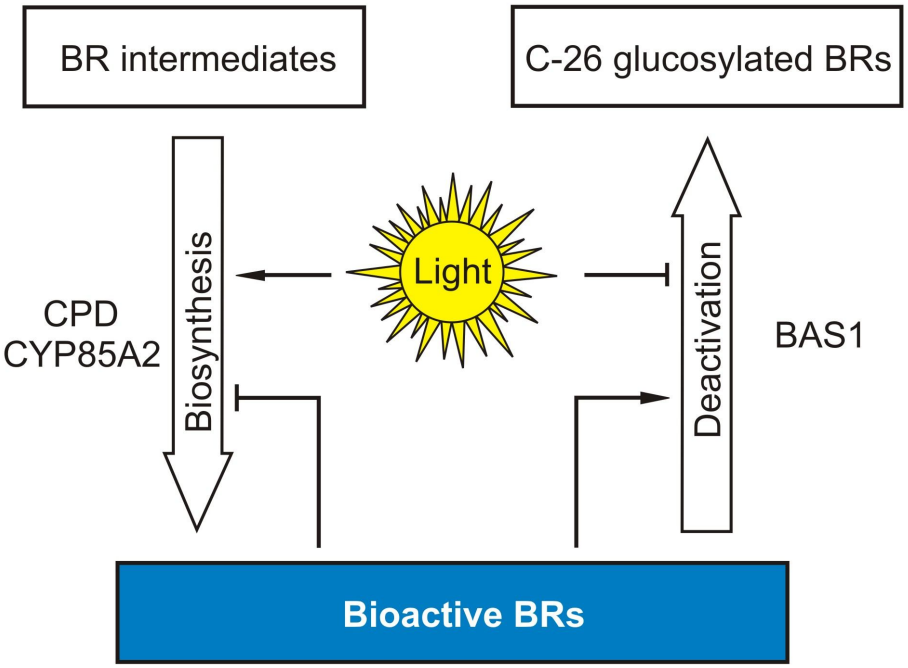

Figure 3. Coordinated transcriptional regulation of BR biosynthetic and deactivating functions
The expression of key BR biosynthetic (CPD and CYP85A2) and deactivating (BAS1) enzymes is oppositely regulated by light and end-product feedback at the transcriptional level.

Plants need to maintain phytohormone levels within a narrow, physiologically optimal range. This results from a dynamic balance between biosynthesis and turnover, therefore precise adjustments of effective hormone concentrations require proper coordination between these two processes (Bishop and Yokota, 2001; Hategan *et al.*, 2011). In *Arabidopsis* the expression levels of BR-deactivating genes, just as those of the BR-biosynthetic ones, are stringently controlled at the transcriptional level, but mostly by contrasting mechanisms (Figure 3). Whereas biosynthetic P450 genes are feedback-regulated by active BRs (Bancos *et al.*, 2002; Tanaka *et al.*, 2005), *BAS1* that encodes the main BR deactivating enzyme shows feedforward response to the hormone (Choe *et al.*, 2001, Tanaka *et al.*, 2005). And while light induces the expression of *CPD* and *CYP85A2* (Bancos *et al.*, 2006), it represses *BAS1* (Turk

et al., 2003). This opposite regulation can maintain proper coordination between biosynthesis and deactivation, thereby contributing to efficient BR homeostasis (Hategan *et al.*, 2011).

2.2.2. BR transport

Several lines of evidence indicate that BRs, unlike the other phytohormones, do not undergo long-distance translocation. This was first indicated by the variegated phenotype of tomato plants comprised of BR-deficient (*dwarf*) mutant and revertant sectors (Bishop *et al.*, 1996). The lack of both basipetal and acropetal transport was convincingly demonstrated by reciprocal grafting in pea and tomato, where BR-deficiency could not be rescued by either stocks or scions of the corresponding wild types (WTs) (Symons and Reid, 2004; Montoya, *et al.*, 2005). These studies revealed that BRs act primarily in a paracrine manner, at or near the sites of their synthesis. The lack of BR transport highlights the importance of precise local control and coordination of metabolic functions in order to optimize the level of the hormone. This regulation seems to act primarily through the transcriptional control of the biosynthetic and deactivating genes (Hategan *et al.*, 2011).

2.3. BR signaling

Over the past decade, a combination of genetic, genomic and proteomic approaches have elucidated the pathway of BR signaling from the perception of the hormone to the nuclear events controlling the activities of BR-responsive genes (Kim TW and Wang ZY, 2010; Tang *et al.*, 2010). Due to ambitious research efforts and thanks to the limited genetic redundancy of the signaling components, during this short time period the signaling pathway

of BRs became one of the best known ones among phytohormones (Kim TW and Wang ZY, 2010).

2.3.1. BR perception

2.3.1.1. BRI1, the receptor of BRs

The first BR signaling mutant was isolated from a population of ethyl methanesulfonate-mutagenized *Arabidopsis* seeds by screening for seedlings that showed uninhibited root growth in the presence of 1 μM 24-epibrassinolide, a synthetic analog of BL. The mutant, designated *bri1* (*brassinosteroid insensitive 1*), had severe dwarf phenotype very similar to those of BR-deficient plants, indicating that its genetic lesion inactivates a positive signaling element (Clouse *et al*., 1996).

Molecular genetic characterization of the *BRI1* gene (At4g39400) revealed that it encodes a plasma membrane-localized leucine-rich repeat (LRR) receptor-like kinase (RLK), which was proposed to be a putative BR receptor (Li JM and Chory, 1997). It was not clear, however, if BRs directly interact with BRI1, because LRRs typically bind peptide ligands (Bishop and Koncz, 2002). Direct binding of radiolabeled CS was demonstrated by Kinoshita *et al*. (2005), who evidenced interaction between the hormone and a specific 'island' region within the extracellular part of BRI1.

As steroid hormones in fungi and animals are perceived by evolutionarily conserved nuclear receptors that directly participate in transcriptional regulatory complexes (Beato *et al*., 1995), the localization of BRI1 in the plasma membrane signifies a unique, plant-specific mechanisms of steroid perception. It is also worth noting, that a genome-wide search for

genes of nuclear-type steroid receptors failed to uncover such sequences in *Arabidopsis*, suggesting that plants lack this type of steroid perception.

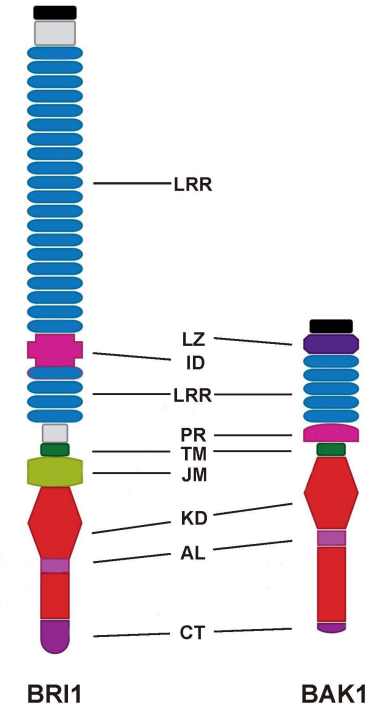

Figure 4. Structures of the BRI1 and BAK1 proteins

The BR receptor BRI1 and its co-receptor BAK1 are plasma membrane-localized LRR-RLK proteins comprising multiple domains of diverse functions. AL: activation loop of the kinase domain, CT: C-terminal region, ID: island domain, JM: juxtamembrane region, KD: kinase domain, LRR: leucine-rich repeat (numbered), LZ: four leucine zippers, PR: proline-rich region, TM: transmembrane region. (Based on the scheme of Kim TW and Wang ZY, 2010).

Structurally, BRI1 is a membrane-spanning protein of 1196 amino acid residues, which is composed of extracellular and intracellular domains that are connected by a transmembrane segment. The extracellular part, responsible for hormone binding, consists of an N-terminal signal peptide and 24 LRR segments. These are interrupted between segments 21 and 22 by a 70-amino-acid 'island', which is responsible for binding the BR ligand (Kinoshita *el al*., 2005). The intracellular part contains a serine-threonine kinase domain and a short C-terminal tail (Kim TW and Wang ZY, 2010). The highly conserved kinase domain

shows more than 80% amino acid identity in both mono- and dicotyledonous plant species, whereas sequence identity between the island regions is only around 60%. LRR units are even less conserved (<50%), but the LRRs next to the island retain higher (62-72%) identity (Szekeres, 2003).

The genome of *Arabidopsis* contains three close homologs of the *BRI1* gene, of which two, *BRL1* (*BRI1-LIKE 1*) and *BRL3*, encode functional BR receptors that are specifically expressed in vascular tissues (Caño-Delgado *et al*., 2004; Zhou *et al*., 2004). But while the loss of both the BRL1 and BRL3 functions in BRI1-deficient background result in more severe dwarfness and complete male sterility, *brl1* and *brl3* null mutations alone or in combination do not have visible effects on growth and fertility (Caño-Delgado *et al*., 2004). These data clearly indicate a primary role for BRI1 in mediating BR responses during plant development (Vert *et al*., 2005).

2.3.1.2. BAK1, the co-receptor of BRI1

Screens for *bri1* suppressor mutants (Li J *et al*., 2002) and yeast two-hybrid screen for BRI1 interactors (Nam and Li JM, 2002) identified BAK1 (BRI1-ASSOCIATED RECEPTOR KINASE 1), which also belongs to the family of LRR-RLK proteins. In addition to the yeast two-hybrid results, direct interaction between BRI1 and BAK1 could also be confirmed by co-immunoprecipitation (Li J *et al*., 2002; Nam and Li JM, 2002) and Förster resonance energy transfer (FRET) assays (Russinova *et al*., 2004).

BAK1 (At4g33430) belongs to the five-member family of *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) proteins, and has also been designated SERK3 (Hecht *et al*., 2001). Accordingly, this relatively small, 615-amino-acid protein (Figure 4) appears to be a functional partner in various LRR-RLK receptor complexes,

such as those involved in the perception of pathogenesis-related elicitors (Chinchilla *et al*., 2007; Roux *et al*., 2011). Due to a redundancy between SERKs, insertional *bak1* mutations result only in weak bri1-like phenotypes (Li J *et al*., 2002; Nam and Li JM, 2002), but the inactivation of all but one *SERK* gene causes severe BR insensitivity (Guo *et al*., 2012), indicating an indispensable role of the co-receptors in the perception process.

2.3.1.3. Receptor-associated regulators of BR signaling

In addition to association with BAK1, BR-elicited receptor signaling also requires homodimerization of BRI1. Activation of the kinase domain requires multiple steps of transphosphorylation between the BRI1 partners that affect up to four phosphorylation sites (Wang X *et al*., 2005).

The C-terminal domain of BRI1 is an important regulator of downstream signaling. Its interaction with BRI1 KINASE INHIBITOR 1 (BKI1; At5g42750) efficiently blocks intracellular BR responses. Transphosphorylational activation of the BRI1 partners, however, prevent further interaction with BKI1, opening the way for undisturbed BRI1 kinase function (Wang X and Chory, 2006).

2.3.2. Intracellular signaling

2.3.2.1. BSKs, the substrates of BRI1 kinase

Proteomic studies of BR-induced changes in the plasma membrane led to the identification of BSKs (BR-SIGNALING KINASE 1 to 3; At4g35230, At5g46570 and At4g00710, respectively), a small kinase family comprising the direct phosphorylation

substrates of BRI1 (Tang *et al*., 2008). It was shown that BRI1-activated BSKs dissociate from the receptor complex and phosphorylate downstream kinase and/or phosphatase elements of the signal route which, in turn, regulate the stability of BR-dependent transcription factors (Tang *et al*., 2008).

2.3.2.2. BIN2 kinase and BSU1 phosphatase

Most of the intracellular components of BR signaling have been identified by gain-of-function mutations suppressing weak bri1 phenotypes. One of these was BRASSINOSTEROID INSENSITIVE 2 (BIN2; At4g18710), an *Arabidopsis* homolog of glycogen synthase kinases (GSKs) functioning as a negative regulator of BR signaling (Li JM *et al.*, 2001). BIN2, a likely target of BSKs (Tang *et al.*, 2008), phosphorylates BR response-mediating transcription factors, decreasing their stability and DNA-binding affinity (He *et al*., 2002). Though primarily a cytosolic protein, BIN2 can move into and function inside the nucleus (Belkhadir and Chory, 2006). In *Arabidopsis* BIN2 is only one of the GSK kinases, and has redundant role with at least some of the other kinases of this group (Yan *et al*., 2009).

BSU1 (BRI1 SUPPRESOR 1; At1g03445), another essential intracellular signaling element, was also identified by suppressor screening (Mora-Garcia *et al*., 2004). This mainly nuclear tyrosine phosphatase with an N-terminal kelch-repeat dephosphorylates BIN2 in a BR-dependent manner, rendering it to degradation via the 26S proteosome (Kim TW *et al*., 2009). Apparently, this mechanism is responsible for abolishing the inhibitory effect of BIN2 on BR signaling (Kim TW and Wang ZY, 2010).

2.3.3. Signaling in the nucleus

BR-elicited cellular responses rely on differential activation or repression of gene activities by the actions of BR-specific transcription factors inside the nucleus.

2.3.3.1. The BZR1 and BZR2 transcription factors

Activation-based genetic screens for mutants unaffected by the BR synthesis inhibitor brassinazole were instrumental in identifying BZR1 (BRASSINAZOLE RESISTANT 1; At1g75080) and BZR2 (At1g19350; also designated as BES1: BRI1 EMS SUPPRESSOR), two closely related transcription factors of BR signaling (Wang ZY *et al*., 2002; Yin *et al*., 2002). These two proteins, sharing 88% amino acid sequence identity, constitute a novel class of transcription factors that contain a destabilizing PEST motif (Yin *et al*., 2005).

BZR1 was shown to bind specifically to a CGTG(T/C)G DNA sequence, termed BR response element (BRRE), which is present in upstream regulatory regions of all P450-encoding BR biosynthetic genes and ensure their strong downregulation in response to BRs (He *et al*., 2005). Although originally BZR2 was proposed to bind to a different type of regulatory sequence, the so-called E-box motif, and act oppositely to BZR1 by activating, rather than repressing genes (Yin *et al*., 2005), later studies clarified that BZR1 and BZR2 bind to the same BRRE sequence motif and function redundantly (Kim TW *et al*., 2009). The way how BZR1 and BZR2 can participate in both inductive and repressive regulatory mechanisms still needs to be elucidated, but it seems possible that this functional versatility results from specific interactions with regulatory proteins, such as BIM1 that confers activating role to the complex (Yin *et al*., 2005).

2.3.3.2. BR-dependent gene expression

BR effects are exerted primarily by the up- and downregulation of BR-responsive genes which, in addition to phosphorylational and selective proteolytic regulation of the signaling components, are also subject to control by nuclear import mechanisms (Ryu *et al*., 2007) and endocytotic recycling of the receptor complex (Russinova *et al*., 2004). The basic scheme of the BR signaling machinery is shown in Figure 5.

The trancriptional responses to BR treatments were studied by cDNA microarray hybridization analyses (Goda, *et al*., 2002; Müssig *et al*., 2002). These revealed that, unlike most phytohormone-induced shifts in the mRNA levels resulting in substantial increases or decreases of transcripts, the changes elicited by BRs were rarely more than twofold. Of the roughly 27000 Arabidopsis genes these studies consistently identified 424 as upregulated, and 332 as downregulated in response to BRs (Vert *et al*., 2005).

While the roles of most BR-regulated genes are still unknown, among the rest of them some functional profiles are clearly recognizable. The roles of several BR-induced genes relate to cell elongation. These encode cell wall components, cytoskeletal elements and xyloglucan endotransglucosylases (XETs), enzymes that are required for the loosening and restructuring of the cell wall, and often they are also subject to auxin regulation. BR-repressed genes encode several BR biosynthetic enzymes, and most (>80%) of them code for various transcription factors (Vert *et al*., 2005).

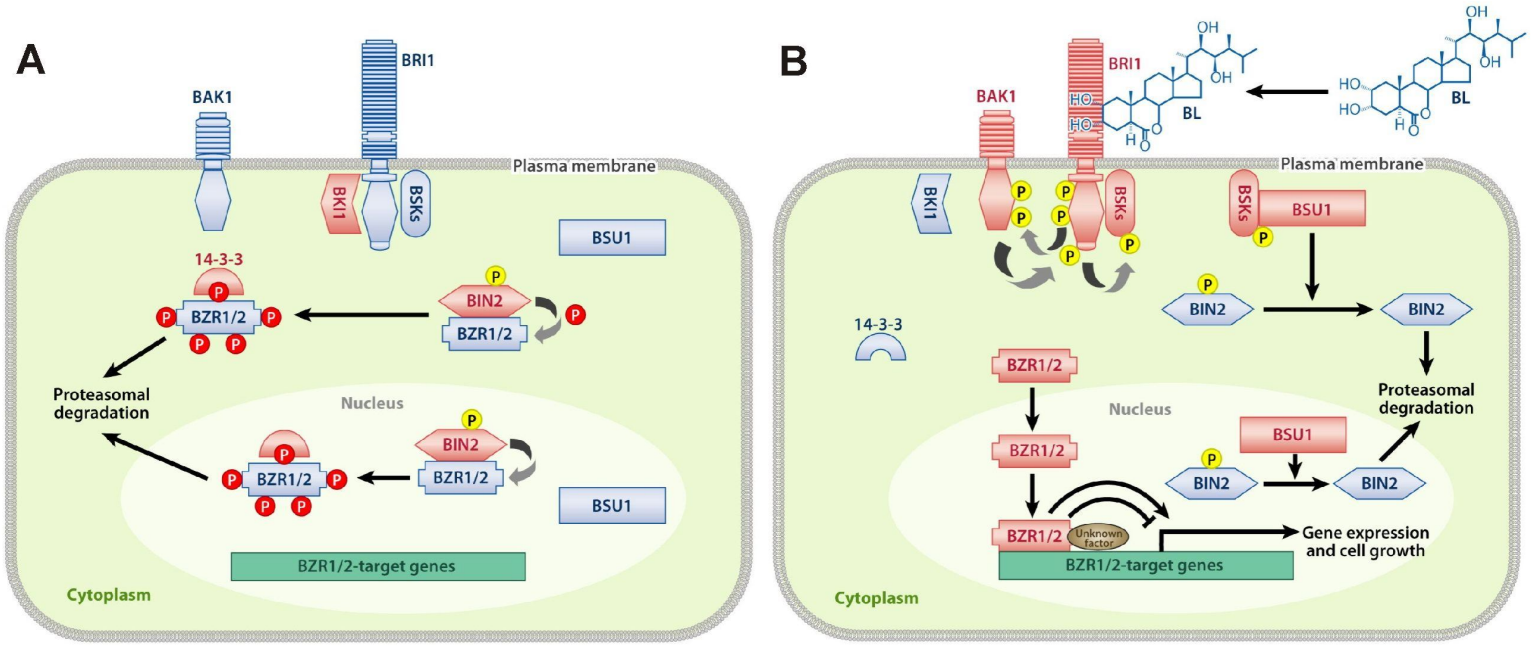

Figure 5. Cellular mechanisms of BR signaling

Active and inactive forms of the signaling components are indicated by pink and blue colors, respectively. Phosphorylation can exert either positive (yellow circles) or negative (red circles) regulatory effects. A: In the absence of BRs, the kinase inhibitor BKI1 prevents interaction of the BRI1 receptor with its co-receptor BAK1. BSK signaling kinases remain associated with the receptor, resulting in inactive (dephosphorylated) BSU1 phospatase. This allows constitutive phosphorylation of the BZR transcription factors by the BIN2 kinase, leading to their nuclear export, cytoplasmic retention by 14-3-3 proteins, loss of DNA-binding activity, and proteosomal degradation. B: In the presence of BRs, hormone-binding by BRI1 induces its dissociation from BKI1 and association with BAK1. Following transphosphorylation steps between BRI1 and BAK1, the receptor becomes activated and phosphorylates BSKs. These are released from the receptor complex and activate (phosphorylate) BSU1. Active BSU1 inhibits (dephosphorylates) BIN2, allowing accumulation of active (non-phosphorylated) BZRs in the nucleus. (The illustration is from the 2010 review of Kim TW and Wang ZY.)

2.3.4. Regulation of BR signaling

2.3.4.1. Control mechanisms at the plasma membrane

BR signaling is initiated when BL binds to the extracellular domain of BRI1. This results in ligand-induced di/oligomerization and subsequent hetero-oligomerization with the BAK1 co-receptor. BRI1 itself has weak kinase activity, but this is strongly enhanced by multiple auto- and transphosphorylation events within the oligomeric complex formed with its co-receptor BAK1 (Li J *et al.*, 2002; Nam *et al.*, 2002). Mass spectrometry revealed that during these not only serine and threonine, but also tyrosine residues become phosphorylated in the intracellular part of BRI1 (Oh *et al.*, 2009). These post-translational modifications lead to full activation of the BRI1 kinase, which can then phosphorylate BKI1, relieving its inhibitory effect (Wang X *et al.*, 2005; Wang X and Chory, 2006). This opens the way for BRI1 to initiate intracellular BR signaling by phosphorylating its BSK substrates (Tang *et al.*, 2008). Like LRR-RLKs of animals and yeast, BRI1 complexes also undergo endocytotic sorting (Russinova *et al.*, 2004), and it was demonstrated by Geldner *et al.* (2007) that this internalization facilitates autocatalytic activation of the receptor and enhances transcriptional BR responses.

2.3.4.2. Cytoplasmic regulation

Several details of the intracellular events in BR signaling still need to be elucidated, but the emerging picture points toward a central role for the BIN2 kinase. This GSK-like kinase can inhibit intracellular signaling by destabilizing the BZR1 and BZR2 transcription

factors via phosphorylation of their central domain, thereby targeting them to 26S proteosome-mediated degradation (He *et al*., 2002; Yin *et al*., 2002). Additionally, phosphorylation by BIN2 was also shown to hinder the entrance of BZR1 and BZR2 in the nucleus and inhibit their binding to BR response elements of the DNA (Vert and Chory, 2006).

Conversely, dephosphorylation of the BR-responsive transcription factors enhances their stability and binding activity (Kim TW *et al*., 2009). This function was first attributed to the BSU1 phosphatase, which was found to bind and dephosphorylate BZR2 *in vitro* (Mora-Garcia *et al*., 2004). More recent studies, however, implicated phosphorylated BIN2 as the main substrate for BSU1, showing that efficient BSU1-mediated dephosphorylation of BZR2 depends on the presence of BIN2 (Kim TW *et al*., 2009). It was found that the substrate of BSU1 in BIN2 is a phosphotyrosine residue, and that its dephosphorylation not only inhibits kinase activity, but also decreases the stability of BIN2 (Kim TW *et al*., 2009).

The details of the regulation of BIN2 by BRI1 are not yet clear. This is likely achieved through the BRI1 → BSK → BSU1 phosphorylation cascade, which enables BSU1 to inactivate BIN2 by dephosphorylation. Protein interactions between the kinase domain of BRI1 and BSKs, as well as between BSKs and BSU1 have been demonstrated both *in vitro* and *in vivo*. Furthermore, BSKs were shown to be efficiently phosphorylated by BRI1 *in vitro* (Tang *et al*., 2008).

2.3.4.3. Regulatory events in the nucleus

Immunoblotting assays carried out with different cellular fractions revealed that BZR1 and BZR2, as well as BIN2 and BSU1, are nucleocytoplasmic of dual localization. The transcription factors BZR1 and BZR2 shuttle between the nucleus and the cytoplasm

depending on their phosphorylation state, which can be modified by the BIN2 kinase and the BSU1 phosphatase in both compartments. Phosphorylation by BIN2 is thought to be particularly important for facilitating the nuclear export of the transcription factors in response to low BR concentrations (Ryu *et al*., 2007; Kim TW and Wang ZY, 2010). Upon exposure to the hormone, BSU dephosphorylates the BIN2 kinase, promoting its export from the nucleus and leading the accumulation of functional (hypophosphorylated) BZR1 and BZR2 (Deng *et al*., 2007; Kim TW and Wang ZY, 2010).

An intriguing question is how BZR1 and BZR2 can function as both positive and negative regulators of distinct gene functions (Li JM, 2010). The likely reason of this versatility is that they act cooperatively with other transcription factors or regulatory cofactors, forming complexes of different specificities and functions. For instance, BZR2 was shown to interact with BIM1, a basic helix-loop-helix (bHLH) protein (Yin *et al*., 2005), and also with the EFL6, REF6 and Myb30 transcription factors (Yu *et al*., 2008; Li L *et al*., 2009). While BZR1 has structural features very close to those of BZR2, in its case similar functional interactions have not yet been demonstrated.

2.3.4.4. Interactions between light and BR signaling

Light is one of the most important environmental factors for plants, which mediates the transition from dark-grown (skotomorphogenic) to light-grown (photomorphogenic) development. The diurnal rhythmicity of multiple gene functions involved in physiological responses results from the combined actions of light and the free-running internal circadian clock (Dunlap, 1999). The ability of plants to perceive light is achieved by photoreceptors. These in *Arabidopsis* include red/far-red-absorbing phytochromes (PHYA to PHYE),

blue/UV-A-absorbing cryptochromes and phototropins and the UV-B-absorbing UVR8 receptor (Sullivan and Deng, 2003; Rizzini *et al*., 2011).

Whereas the perception of light signals is an intracellular process, responses at the tissue and organ levels are mediated and coordinated through the action of phytohormones. De-etiolation phenotypes of BR mutants implied cross-talk between BR and light signaling (Chory *et al*., 1991; Li JM *et al*., 1996; Szekeres *et al*., 1996). As BR levels were seen to influence photomorphogenesis and the expression levels of light-regulated genes (Chory and Li JM, 1997), light was also shown to control BR production via the induction of biosynthetic genes (Bancos *et al*., 2006).

Recent studies elucidated that BRs, together with auxins, play an important role in cryptochrome 1- and phytochrome B-mediated shade avoidance responses (Keller *et al*., 2011; Keuskamp *et al*., 2011). Furthermore, the BRs were shown to interfere with light control of flowering (Domagalska *et al*., 2010), and the regulators of flowering ELF6 and REF6 were detected in transcriptional complexes formed with BR-specific BZR-type transcription factors (Yu *et al*., 2008).

3. OBJECTIVES

Earlier results of our laboratory indicated increased BR sensitivity of *Arabidopsis* seedlings upon prolonged dark treatment (Bancos *et al*., 2006). This suggested that, in addition to BR levels, physiological responses to the hormone are also influenced via differential regulation of susceptibility. A highly specific, non-redundant element of BR signaling is the BRI1 receptor, which interacts directly with the hormone, and affects the activity and/or stability of all downstream signal components (Kim TW and Wang ZY 2010). Therefore our aim was to elucidate how BRI1 expression is regulated, and how it can influence the developmental and morphogenic processes of plant life. Our goals were as follows:

(1) To determine developmental and organ-specific expression patterns of the *BRI1* gene using transgenic plants that carry *BRI1* promoter-reporter fusions.

(2) To find out how light conditions influence *BRI1* activity.

(3) To develop a transgenic system for direct monitoring receptor distribution by replacing BRI1 with a BRI1-LUC fusion.

(4) To characterize the morphogenic effects of targeted ectopic expression of BRI1 under the control of well characterized tissue-specific promoters.

These studies were expected to clarify the role of BRI1 expression and abundance in modulating BR sensitivity, and the extent to which it can alter developmental effects of the hormone.

4. MATERIALS AND METHODS

At the description of laboratory techniques I concentrate on the methods that are specifically adapted to our experimental systems or include details that are necessary for reproducing our results. Widely and uniformly used methods are only referenced or described in less detail.

4.1. General molecular biology techniques

General microbiological and molecular biology methods (such as those used for maintaining and transforming *Escherichia coli*, preparing and screening recombinant DNA, etc.) have been done according to the laboratory manual of Sambrook and Russel (2001). Whenever specific or modified techniques are used their details are provided in the relevant methodical sections.

4.2. Plant material and growth conditions

All plant material used for the experiments was obtained from *Arabidopsis thaliana* (thale cress) ecotype Columbia-0 (Col-0) or mutants and transgenic lines generated in the same background. The BRI1-deficient *bri1-101* missense mutant (originally described as *bin1-1*: Li JM and Chory, 1997), inactivating the BRI1 function by an E1078K amino acid substitution, was received from Jianming Li (University of Michigan, Ann Arbor).

For *in vitro* cultures seeds were surface-sterilized by 10 min treatment with a 5% (w/v) solution of calcium hypochlorite and several subsequent washes with sterile distilled water.

Sterilized seeds were then spread over MS plant medium (Murashige and Skoog, 1962) adjusted to pH 5.7, supplemented with 1% (w/v) sucrose, and supported by 0.2% Phytagel (Sigma, St. Louis, MO, USA). Efficient and synchronized germination was facilitated by overnight refrigeration (4°C) and subsequent illumination (white light, 12 h) of the seeds. Seedlings were grown at 22°C in controlled-environment chambers (SANYO Electronic, Tokyo, Japan) under alternating regimes of 12 h white fluorescent light (50-60 µmol photons/m^2/s) and 12 h dark (LD). Continuous light (LL) as well as continuous dark (DD) were provided using the same conditions as in the corresponding phases of LD.

For maintenance and phenotypic characterization plants were grown in temperature-controlled (20-22°C) greenhouse. Following four-five weeks of vegetative growth under short day conditions (8 h L/16 h D), the plants were brought to flowering and seed production under long day illumination cycles (16 h L/8 h D).

4.3. Generation of transgenic plants

4.3.1. Transgene construction

4.3.1.1. Plasmid vectors

For cloning PCR-amplified DNA segments and assembling gene constructs for transgenic expression the high copy number pBluscript II (SK+) (Stratagene, Santa Clara, CA, USA) plasmid vector was used. The generation of functional chimeric genes and their integrative transfer into the *Arabidopsis* genome were achieved by modified versions of the T-DNA-based pPCV812 binary vector (Koncz *et al.*, 1994). To facilitate selection for transformed plants, in pPCV-GUS the hygromicin resistance-conferring *HPT* gene of

Streptomyces albus was replaced by the 552 bp *BAR* coding sequence of *Streptomyces hygroscopicus* that ensures resistance to glufosinate-type herbicides. In pPCV-LUC, a derivative of pPCV-GUS used for *in vivo* monitoring of gene expression, the β-glucuronidase reporter-encoding *uidA* (GUS) coding sequence of *Escherichia coli* was replaced by the luminescence-optimized coding region of firefly (*Photinus pyralis*) luciferase gene (*LUC*) (Altschmied and Duschl, 1997).

4.3.1.2. Promoter-reporter constructs

Organ-specific and temporal expression patterns of the *BRI1* gene were studied by using promoter-reporter fusions. To this end a 1899 bp segment of the *BRI1* promoter (-1906 to -8, relative to the translational start) was PCR-amplified with the BRI1pr-F and BRI1pr-R oligonucleotide primers (Table 1), and then cloned in *Sma*I-cleaved pBluscript vector. The resulting *Bgl*II-*Sma*I segment was then excised and introduced in *Bam*HI-*Sma*I-cleaved pPCV-GUS and pPCV-LUC. Sequences of the entire BRI1 promoter and the cloning junctions were verified by sequencing.

4.3.1.3. Complementation constructs

For complementation studies the intronless 3590 bp *BRI1* coding sequence without the termination codon was amplified from Col-0 genomic DNA using BRI1cs-F and BRI1cs-R primers (Table 1), which allowed cloning in pBluescript as a *Stu*I-*Bam*HI fragment. To ensure easy detection, the 3' end of the *BRI1* coding sequence was translationally fused to the *LUC* reporter. This was done by first ligating a 30 bp synthetic oligonucleotide linker (Figure 6A), designed to code for a flexible hinge, to the PCR-generated truncated 3' end of the *BRI1*

sequence. Then the linker-extended *BRI1* sequence (3619 bp *Stu*I-*Sma*I fragment) was inserted in *Stu*I-cleaved pPCV-LUC to provide in frame *BRI1-LUC* fusion.

Table 1. Gene-specific oligonucleotide primers

gene	primer	sequence (5' → 3')
Arabidopsis thaliana Col-0		
ATHB8 (At4g32880)	ATHB8pr-F	TTAAAATGGCCTGCAACTGTACGGATAAAC
	ATHB8pr-R	gggTTTGATCCTCTCCGATCTCTC
BRI1 (At4g39400)	BRI1pr-F	agatcTGCTTGATTATGATGACATTATAG
	BRI1pr-R	ggGTTTGTGAGAGAGAAAAGTGTGGG
	BRI1cs-F	GAGAAATGAAGACTTTTTCAAGCTTCTTTCTCTCTG
	BRI1cs-R	ctcatgggatccCATAATTTTCCTTCAGGAACTTC
	BRI1av-F	GATGAGAATTTGGAAGCTCGGGTTTCAG
	BRI1av-R	CCAAGGAAAATCGGACTGACCCTTAG
TUB2-3 (At5g62690, At5g62700)	TUBrt-F	CCAGCTTTGGTGATTTGAAC
	TUBrt-R	CAAGCTTTCGGAGGTCAGAG
Photinus pyralis		
LUC (GenBank AF 027126)	LUCrt-F	GGAGCACGGAAAGACGATGACGG
	LUCrt-R	ACAAACACAACTCCTCCGCGCA

Non-complementary nucleotides are shown in lower-case letters.

To ensure localized expression of the *BRI1-LUC* fusion, well characterized organ- and tissue-specific promoters were used. A 987 bp segment (-988 to -2) of the *CAB3* (*CHLORPPHYLL-A/B-BINDING PROTEIN 3*, At1g29910) promoter, rendering expression in all photosynthetic tissues, was cloned as a *Hin*dIII-*Bam*HI fragment (Mitra *et al*., 1989). A 2128 bp section (-2129 to -2) of the vascular tissues-specific *SUC2* (*SUCROSE TRANSPORTER 2*, At1g22710) promoter was also available as *Hin*dIII-*Bam*HI fragment (Truernit and Sauer, 1995). Of the procambium-specific *ATHB8* (*ARABIDOPSIS THALIANA HOMEOBOX 8*, At4g32880) promoter (Baima *et al*., 1995) a 1720 bp part (-1721 to -2) was

cloned as *Bam*HI-*Sma*I fragment following PCR amplification from Col-0 genomic DNA with ATHB8pr-F and ATHB8pr-R primers (Table 1). For correctly regulated expression of *BRI1-LUC* the above described 1899 bp native *BRI1* promoter was used. Functional gene constructs were generated by inserting the *CAB3*, *SUC2*, *ATHB8* and *BRI1* promoters in the pPCV constuct at the 5' *Stu*I site of the *BRI1-LUC* fusion (Figure 6B). Correct amplification of the promoters and proper joining of restriction sites were verified by sequencing.

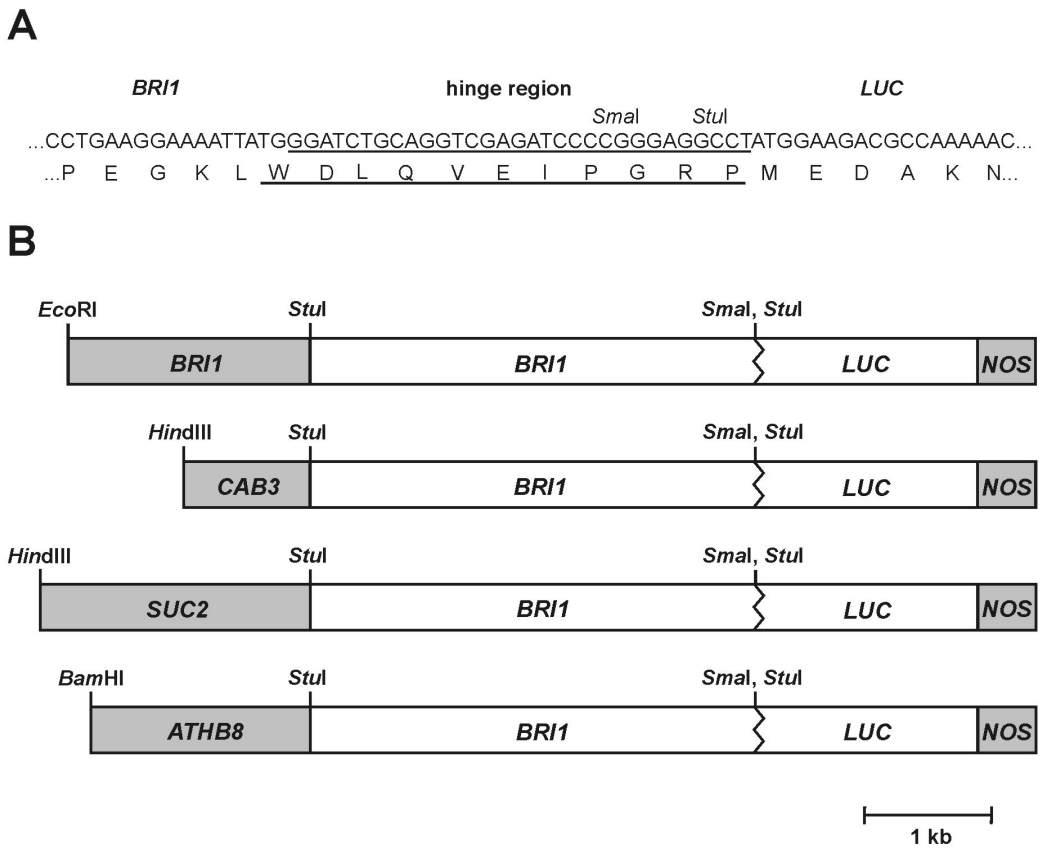

Figure 6. Gene fusion constructs used for *BRI1-LUC* expression

A: Translational junction between the *BRI1* and *LUC* coding regions. The sequence of the oligonucleotide linker and the amino acids of the generated hinge region are underlined. B: Scheme of the chimeric genes constructed using the *BRI1*, *CAB3*, *SUC2* or *ATHB8* promoters and the *BRI1-LUC* translational fusion. White bars represent coding sequences, whereas regulatory regions, promoters and the termination sequence of the *Agrobacterium tumefaciens* nopalin synthase (*NOS*) gene, are shown in gray. Restriction endonuclease cleavage sites at the fusions are indicated.

4.3.2. Generation and phenotypic characterization of transgenic *Arabidopsis* lines

4.3.2.1. *Agrobacterium*-mediated *Arabidopsis* transformation

The chimeric gene-containing pPCV plasmids were transformed in the S17-1 strain of *E. coli* with broad transfer specificity. Transformed cells were then used to conjugate the plasmid constructs in *Agrobacterium tumefaciens* GV3101 (pMP90RK) by biparental mating, as described by Koncz *et al*. (1994). Conjugated *Agrobacterium* cells were selected by their endogenous rifampicin and pPCV-derived carbenicillin resistance. To avoid *E. coli* and non-conjugated *Agrobacterium* background, conjugant bacteria were isolated from single colonies following repeated selection cycles.

The *BRI1pro:GUS* and *BRI1pro:LUC* reporter constructs were introduced in Col-0, whereas the *BRI1-LUC* fusions driven by various promoters in *bri1-101* BR insensitive plants. The transformation was done by the floral dip method of Clough and Bent (1998). *Agrobacteria* containing the pPCV constructs were grown to late exponential phase in 300 ml liquid cultures. Bacterium cells were collected by centrifugation at room temperature (4000x g, 10 min), then they were re-suspended by gentle rotating in 300 ml 5% (w/v) solution of sucrose. Transformation was carried out by brief dipping of *Arabidopsis* inflorescences at the early flowering stage in the *Agrobacterium* suspensions. To promote access to the ovules, the bacterial suspensions were supplemented with 30 µl Silwet L-77 surfactant (Lehle Seeds, Round Rock, TX, USA) just before the transformation procedure. After dipping, the *Agrobacterium*-exposed inflorescence was kept for 12 h in high humidity environment to facilitate gene transfer. In the case of the infertile *bri1-101* mutant bri1/+ heterozygous plants

of wild phenotype were used for the transformations, and the *bri1* background was recovered later in homozygous segregants of the transgenic lines.

4.3.2.2. Isolation of homozygous transgenic lines

Seeds collected from the *Agrobacterium*-transformed plants were subjected to selection with the herbicide Basta (glufosinate; Bayer CropScience, Monheim, Germany). Densely growing one-week-old seedlings were sprayed repeatedly with 1300-fold water-diluted solution of the herbicide. In each case at least 10 Basta-resistant seedlings (primary transformants, T1 generation) were re-planted and raised in individual pots allowing only self fertilization. When required, the presence and identity of the transgenes were verified by PCR or luciferase bioluminescence assays using small leaf samples. Segregation of seed material from the self-fertilized plants was tested to make sure that the isolates contained only single copy of the transgene. Transgenic seedlings from these assays were used for preliminary characterization of the independent transgenic lines. From the samples showing 3:1 segregation of the transgene representative lines were chosen on the basis of their phenotypic traits and transgene expression profiles. These were then used for generating lines that were homozygous for the transgenes and, in the cases of the complementation constructs, also for the *bri1* mutant background.

Homozygocy for the *bri1* allele was assayed by PCR products of genomic DNA with BRI1av allele verification primers (Table 1), relying on the *Xho*I polymorphism caused by the G>A transition in *bri1-101* at nucleotide position 3232 (Li JM and Chory, 1997). DNA samples for these reactions were prepared from 1 cm^2 freshly collected leaf discs using the CTAB (cetyltrimethylammonium bromide) extraction method of Doyle and Doyle (1990).

4.3.2.3. Phenotyping of reproductive organs

Quantitative characterization of inflorescence and silique development in *BRI1-LUC*-complemented *bri1-101* lines was carried out by comparing their inflorescence height, silique length, and the number of seeds per silique. For these measurements we used 10 two-month-old plants of each line, all grown in parallel. Of these batches of complemented plants 50 ripe siliques were collected for size and seed number analyses.

4.4. Transgene-based gene expression analyses

4.4.1. GUS histochemical assays

Histochemical localization of β-glucuronidase activity was carried out according to Jefferson (1987). Immediately after isolation, all plant material was fixed by vacuum infiltration with 2% (w/v) formaldehyde in 50 mM sodium phosphate (pH 7.0). Following two washes in the same buffer, samples were transferred in staining solution containing 0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc; Biosynth A.G., Staad, Switzerland) in 50 mM sodium phosphate (pH 7.0). Following overnight incubation at 37°C in the X-Gluc solution samples were soaked in multiple changes of 50% (v/v) ethanol to remove photosynthetic pigments. GUS-stained seedlings and plant organs were photographed using Nikon SMZ800 microscope with dark background function.

4.4.2. *In vivo* detection of luciferase activity

Measurements of luciferase bioluminescence were carried out at constant 22°C temperature as described in Kay *et al.* (1994), using a liquid nitrogen-cooled backilluminated digital CCD ('charged-couple device') camera (LN-CCD-512-TKB, Princeton Instruments, Trenton, NJ, USA). For detecting diurnal changes of transgene activity, patches of 50 one-week-old seedlings on MS medium were sprayed one day before the onset of the measurements with 2.5 mM sterile solution of D-luciferin (Biosynth A.G.) also containing 5 mM Tris-phosphate buffer (pH 8.0) and 0.01% (v/v) Triton X-100.

For monitoring transgene induction upon germination, seeds were sown over MS medium already containing 5 mM D-luciferin. Germination in DD was facilitated by an illumination period of 12 h following cold treatment. In time-course experiments 10 min exposures were taken in complete darkness with 120 min intervals. During light periods the samples were placed in the dark five minutes before the start of the exposures to avoid background from delayed fluorescence. The luminescence emitted by the seedlings was quantitated using Metamorph imaging software (Meta Series 4.5; Universal Imaging, Ypsilanti, MI, USA), and following background subtraction they were plotted as absolute or normalized values on a time scale. Measurements were carried out in duplicates, with three biological repetitions. For the localization of luciferase expression intact plants or isolated organs were sprayed with luciferin solution, then the sites of activity were determined on the basis of parallelly recorded light and luminescence images.

4.4.3. Quantitation of the BRI1-LUC fusion protein

Batches of 100 DD-grown seedlings, carrying the *BRI1pro:BRI1-LUC* transgene in *bri1-101* background, were harvested on day five following the start of germination. After removal of the cotyledons and roots the hypocotyls were separated to upper (apical) and lower (basal) halves and were frozen in liquid nitrogen. These samples were then used for analyses of the BRI1-LUC protein and *BRI1-LUC* mRNA content.

Relative levels of the BRI1-LUC fusion protein were determined by the luminometric method of Viczián and Kircher (2010). The frozen hypocotyl samples were homogenized, and then mixed thoroughly with 300 µl extraction buffer (100 mM potassium phosphate pH 7.8, 0.05% Tween 20, 1 mM dithiothreitol) in an ice bath. Following centrifugation (15 min, 20000 x g, 4°C) the clear supernatant was collected and kept on ice. In wells of a black microplate 50 µl of each crude extract was mixed with further 100 µl extraction buffer plus 50 µl LUC assay buffer (80 mM glycil-glycine pH 7.8, 40 mM $MgSO_4$, 60 mM ATP). The microplate was placed in the dark, 22°C chamber of a TopCount NXT luminometer (Perkin-Elmer) for 5 min. Then luminescence values were measured for 2 min immediately from the synchronized injection of 100 µl luciferin solution (5 mM in 10 mm Tris-phosphate pH 8.0 and 0.01% Triton X-100). The data obtained were normalized to the total protein levels in the crude extracts, which were determined by amido black-binding assays (Schaffner and Weissmann, 1973). In the same samples the levels of the *BRI1-LUC* mRNA were also quantitated by qRT-PCR using the LUCrt-F and LUCrt-R primers. The measurements were done in triplicate, with two biological replicates.

4.5. Determination of mRNA levels

4.5.1. RNA isolation

Total RNA samples were isolated from batches of 50 one-week-old seedlings using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and RNase-free plasticware. Traces of genomic DNA were removed by treatment with RNase-free DNase (Qiagen) according to the manufacturer's instructions. Each RNA sample was dissolved in 50 µl high purity distilled water and, following OD_{260} measurements and adjustments of the concentrations to 1 µg/µl, they were stored frozen at -20°C until use.

4.5.2. cDNA synthesis

Samples of cDNA were prepared from 1 µg total RNA with the help of RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) using MuLV reverse transcriptase. The syntheses of cDNA were initiated from random hexanucleotide primers.

4.5.3. Quantitative real-time PCR analyses

Transcript levels of the transgenes were compared on the basis of quantitative real-time PCR (qRT-PCR) analyses using 7300 Real Time System and software (Applied Biosystems, Carlsbad, CA, USA). PCR product accumulation was monitored on the basis of increasing SYBR Green fluorescence. Each measurement was carried out in triplicates with 1.5% of the products obtained in the same cDNA synthesis reaction, with two biological

repetitions. PCR programs included denaturation (95°C, 10 min), followed by forty thermo-cycles (95°C, 15 s and 60°C, 1 min). In all measurements tubulin transcripts (*TUB2*, At5g62690 and *TUB3*, At5g62700) were used as constitutive control. The primers used for quantitating the *LUC* (LUCrt-F and LUCrt-R) and *TUB* (TUBrt-F and TUBrt-R) cDNAs are shown in Table 1.

5. RESULTS AND DISCUSSION

In order to maintain coherence and avoid redundancies, in this chapter the presentation of experimental results will immediately be followed by their interpretation and discussion in the context of the related information available in the literature.

5.1. Differential organ-specificity of *BRI1* gene expression

Earlier studies in our laboratory revealed that transfer of *Arabidopsis* seedlings from LD to DD conditions causes strong downregulation of the BR-controlled *CPD* gene, and that this effect resulted from enhanced BR sensitivity, rather than an increase of hormone content (Bancos *et al.*, 2006). This finding was in agreement with the observations of Fujioka *et al.* (1997) and Yang *et al.* (2005), who also reported enhanced BR sensitivity of etiolated seedlings. To clarify whether such modulation of BR sensitivity is correlated with differential expression of the hormone receptor, we generated transgenic plants carrying *BRI1* promoter-driven *GUS* and *LUC* reporters in order to monitor the transcriptional activity of *BRI1*. Although Friedrichsen *et al.*, (2000) suggested that in *Arabidopsis* seedlings *BRI1* expression is not spatially regulated, these reports of differential BR sensitivity implied temporal and/or organ-specific regulation of the BR signaling pathway, likely through the availability of its key components.

5.1.1. *BRI1* gene activity during germination and early seedling development

Developmental processes during and following germination involve the activation of multiple BR-regulated genes that promote the division and elongation of cells, as well as organ differentiation (Goda *et al*., 2002; Müssig *et al*., 2002). These events require enhanced BR signaling, which can be ensured by BR accumulation, more efficient signalization, or both. It has been shown in *Arabidopsis* and pea that germination is accompanied by transient induction of all BR biosynthetic genes, and that this results in an accumulation of bioactive BRs (Bancos *et al*., 2002 and 2006; Nomura *et al*., 2007). To find out if gene expression data also imply changes of BR susceptibility, we examined the spatial and temporal patterns of *BRI1* activity during early development using stable transgenic lines harboring *BRI1* promoter-reporter constructs.

5.1.1.1. GUS reporter-based localization of *BRI1* promoter activity

To localize *BRI1* gene activity at early development, we germinated seeds of a representative transgenic line expressing the *GUS* reporter under the control of the 1899 bp *BRI1* promoter. Seedlings were raised under LD or DD conditions for one week. Samples collected daily were subjected to histochemical analysis.

When seedlings were raised under light/dark cycles, no GUS activity was visible on day one, at the emergence from the seed coat. Subsequently, at days two and three, intense staining could be observed in the straightening hypocotyl, and in the root where it was most prominent around the elongation zone. Later on the GUS activity decreased, but it remained

strong near the root tip and well detectable in the petioles. No staining was seen in the cotyledons (Figure 7A).

Upon dark germination GUS activity was already detectable on day one in the emerging radicle. During days two and three it increased rapidly both in the hypocotyl and the radicle. Subsequent, until day seven, the staining in these organs gradually decreased around their joint region and became centered at distal parts near the root tip and, especially, the hypocotyl hook. Etiolated seedlings, just like those grown in light/dark, lacked GUS staining in their cotyledons (Figure 7B).

Figure 7. Expression of the *BRI1pro:GUS* transgene in seedlings

Histochemical staining of GUS activity during the first week of development in seedlings germinated under LD (A) or DD (B) conditions.

Our GUS reporter-based expression analyses revealed that in young seedlings strong *BRI1* expression is associated with intense growth, that is, organ regions and tissues of rapidly elongating and/or proliferating cells. In green seedlings these are primarily the expanding hypocotyl, the petioles, and the elongation zone of the root, whereas in etiolated plants the

elongation zones of the root and, in particular, the hypocotyl. BRs are essential for proper growth of the hypocotyl and root (Szekeres *et al.*, 1996; Li JM *et al.*, 1996; Müssig *et al.*, 2003), which they control by regulating cell division and elongation in the expanding tissues (Kauschmann *et al*., 1996; Hu *et al*., 2000; Zhiponova *et al*., 2013). Therefore, strong expression of the *BRI1* gene at the sites of intense elongation suggests that BR effect in these tissues can be enhanced by ensuring proper receptor density. Recently van Esse *et al*. (2011) developed a fluorescent method suitable for quantitating the BRI1 receptor in developing roots. They found that uniform receptor abundance in various cell types of the elongation zone is maintained by a substantial, five- to tenfold increase of the number of receptors per cell. This enhancement of the BRI1 content in these cells seems to be in good agreement with the observed transcriptional upregulation of *BRI1* activity.

5.1.1.2. Enhanced *BRI1* expression is accompanied by BR receptor accumulation

The gene expression assays carried out with *BRI1pro:GUS* transgenic seedlings showed that following day 4 in DD the GUS activity was higher in the apical part of the hypocotyl than in its basal region (Figure 7B). To examine how this differential activity of the BRI1 promoter correlates with the accumulation of *BRI1* mRNA and the encoded BR receptor, we determined the relative levels of these products in the apical and basal halves of five-day-old DD seedlings.

To enable detection of both the transcript and receptor protein, we used a line carrying the *BRI1pro:BRI1-LUC* transgene in *bri1-101* background. In this line the severe BR insensitivity caused by the *bri1-101* mutation is fully complemented by the *BRI1*-reporter gene fusion that is expressed under the control of the *BRI1* promoter. As a result, *BRI1pro:BRI1-LUC/bri1-101* plants are morphologically indistinguishable from the Col-0

WT (Figure 8). Our qRT-PCR measurements using the LUC-specific LUCrt-F/LUCrt-R primers (Table 1) revealed that in five-day-old seedlings the upper (apical) half of the hypocotyl contained nearly fivefold higher amount of the *BRI1:LUC* mRNA than the lower (basal) part (Figure 9A). Similar result could be obtained with the *BRI1* transcript in Col-0 seedlings (data not shown). When assaying *BRI1pro:BRI1-LUC/bri1-101* seedlings for BRI1:LUC protein content, crude extracts from the upper halves gave about 12.5-fold stronger luminescence than that of the lower segments (Figure 9B). These data and results of the *BRI1pro:GUS* histochemical assays indicated good agreement between enhanced *BRI1* promoter activity and accumulation of the transcribed mRNA and translated protein products.

Figure 8. Rescue of the *bri1-101* mutant phenotype by the *BRI1pro:BRI1-:LUC* transgene Phenotypes of two-month old Col-0, *bri1-101* and *BRI1pro:BRI1-LUC*/*bri1-101* plants.

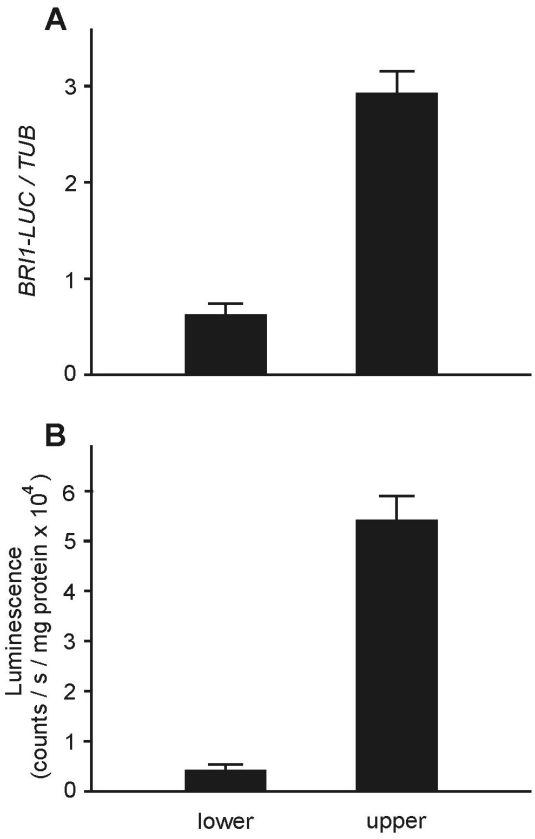

Figure 9. Levels of the *BRI1-LUC* transcript and BRI1-LUC fusion protein in DD-grown *BRI1pro:BRI1:LUC* seedlings

(A) Relative levels of the *BRI1-LUC* mRNA in the lower and upper halves of five-day-old seedlings. qRT-PCR values obtained with *LUC*-specific primers. (B) Luminescence of the BRI1-LUC fusion product in the lower and upper halves of five-day-old seedlings. Data are mean values ± standard deviation.

The similarity of luminescence patterns in two-week-old *BRI1pro:LUC*/Col-0 and *BRI1pro:BRI1-LUC/bri1-101* seedlings also indicate good correlation between *BRI1* transcriptional activity and BRI1 receptor accumulation. In this case of the *BRI1pro:LUC* transgene LUC activity is proportional with the rate of *BRI1* promoter-driven transcription, whereas with *BRI1pro:BRI1-LUC* it corresponds to the level of the receptor-reporter fusion protein. We found, however, that in both transgenic lines LUC activity was highest in the expanding leaves and root tips at this stage of the development (Figure 10).

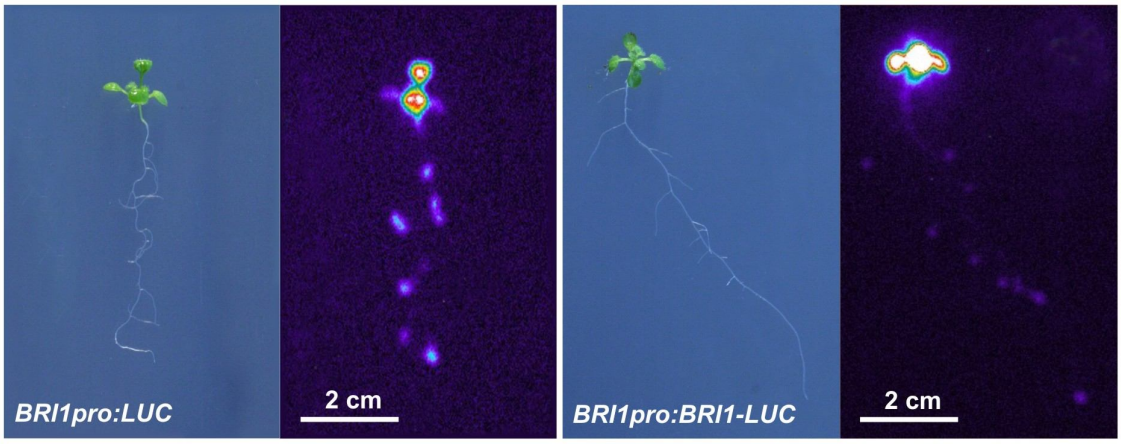

Figure 10. LUC activities in two-week-old *BRI1pro:LUC* and *BRI1pro:BRI1-LUC* seedlings
Morphology and luminescence images of two-week-old *BRI1pro:LUC*/Col-0 and *BRI1pro:BRI1-LUC*/*bri1-101* plants.

These results highlight the importance of transcriptional regulation in determining BRI1 availability, and possibly also BR responsiveness. The BRI1 receptor, which directly interacts with the hormone, is the initiator and a key element in the BR signaling process, even if this is also influenced by the abundance and/or phosphorylation state of downstream signaling components (Li JM and Jin, 2007; Kim TW and Wang ZY, 2010). Accordingly, the morphology of a receptor-overexpressing transgenic line resembles WT plants treated with BL (Wang ZY *et al.*, 2001), and also those that overproduce the hormone (Choe *et al.*, 2001).

Our histochemical studies with the *BRI1pro:GUS* transgenic line indicated enhanced *BRI1* promoter activity in the upper hypocotyl region of DD seedlings following day four (Figure 7B). Quantitative analyses revealed that in five-day-old *BRI1pro:BRI1-LUC/bri1-101* seedlings this increased level of transcription is accompanied by local accumulation of the *BRI1* mRNA, as well as of the encoded BR receptor (Figure 9A-B). At this developmental stage epidermal cell elongation is confined to the apical part of the hypocotyl (Gendreau *et al.*, 1997), and MDP40, a BR-controlled regulator of cell elongation, is preferentially expressed in this hypocotyl region (Wang X *et al.*, 2012). These data strongly suggest that, at

least in the hypocotyl, the abundance of the BRI1 receptor and the efficiency of BR signaling are both influenced by the local transcriptional regulation of the *BRI1* gene.

5.1.2. Organ-specificity of *BRI1* expression in mature plants

Our results evidenced organ-specific regulation of *BRI1* activity in young seedlings, implying that such differential expression may be maintained during later stages of development. Therefore we examined the localization pattern of *BRI1* promoter activity in rosettes and reproductive organs of *BRI1pro:GUS* transgenic plants using GUS histochemical analysis.

In pre-bolting rosettes of four-week-old plants GUS staining was observed in young, expanding leaves, mainly in the petioles and proximal parts of the central veins. Only very weak or no activity could be detected in old leaves and in the roots (Figure 11A). Also low level expression was seen in the flowers, where staining occurred only at the joining of the pedicel and over the stigma (Figure 11B). But strong GUS activity could be detected in the developing seeds of expanding siliques (Figure 11C-D).

We found that the organ specificity of *BRI1* expression is very similar to those of the main *CYP85* genes in *Arabidopsis* (Castle *et al.*, 2005) and tomato (Montoya *et al.*, 2005), which are required for the production of bioactive BRs. Furthermore, the corresponding orthologous genes of pea, *CYP85A1* and the receptor-encoding *LKA*, were also shown to be coordinately regulated upon seed maturation and germination (Nomura *et al.*, 2007). The formation of reproductive organs, especially fruits and seeds, also depends on BR-induced functions. Because plants do not have active BR transport, fruit accumulation of the hormone is achieved by local upregulation of the biosynthetic genes (Montoya *et al.*, 2005; Symons *et al.*, 2006 and 2012). Our results suggest that, in addition to local hormone accumulation,

sensitization of the affected tissues by an increase of receptor abundance may also contribute to the enhancement of BR-responses required during elongation and differentiation.

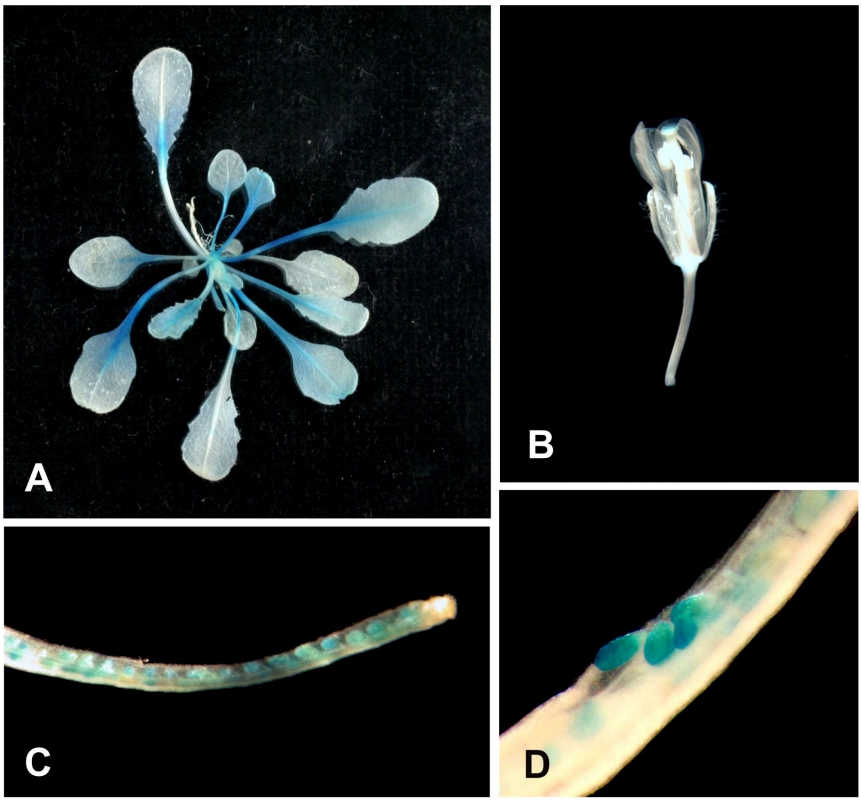

Figure 11. *BRI1* expression in mature plants

GUS staining patterns in four-week-old rosette (A), flower (B), silique (C) and developing seeds (D) of the *BRI1pro:GUS* transgenic line.

5.2. Transient induction of *BRI1* upon germination

BR mediated morphogenic events of germination are accompanied by the activation of BR biosynthetic genes, leading to accumulation of the biologically active CS and BL (Bancos *et al.*, 2002; Nomura *et al.*, 2005). But this increase in steroid hormone content is limited by a transcriptional feedback mechanism that suppresses biosynthesis at high BR concentrations (Mathur *et al.*, 1998; Bancos *et al.*, 2002) and, as it has been evidenced in pea, also by the concomitant induction of BR deactivating functions (Nomura *et al.*, 2005). Therefore, we

wanted to determine the time-course of BRI1 expression upon germination, and to find out if it may indicate a modulating effect on BR signaling during this developmental stage.

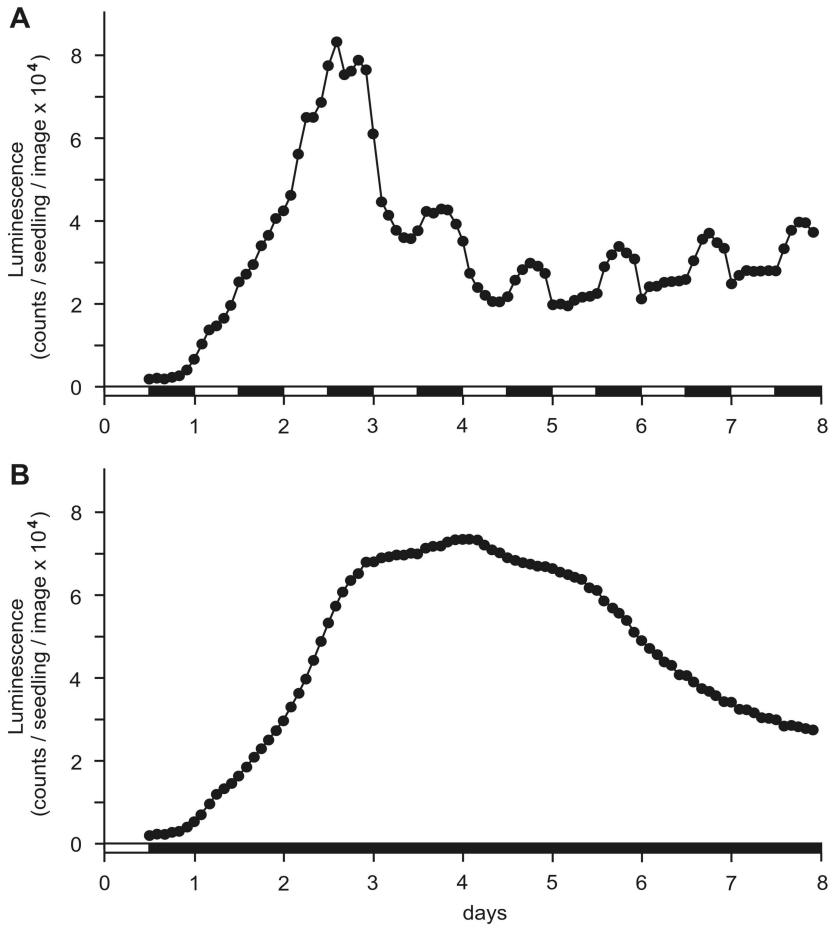

Figure 12. *BRI1* promoter activity during germination and early seedling development

Luminescence of *BRI1pro:LUC* transgenic seedlings from the onset of seed imbibition under LD (A) or DD (B) conditions. White and black bars at the time scale correspond to light and dark conditions, respectively. The graphs show data from single, representative measurements.

Transcriptional activity of *BRI1* was followed by measuring the luminescence emitted by *BRI1pro:LUC* transgenic seedlings. The firefly luciferase version we used (originally designated LUC+) was ideally suited for time-course detection because its short, two to three h half lifetime (Millar *et al.*, 1992), as opposed to 50 h in the case of GUS (Jefferson *et al.*,

1987), allows almost real time monitoring of both increases and decreases of expression activity.

Upon germination we observed strong transgene induction following day one, and maximal or near maximal activities by day three, irrespective of the light conditions. In LD the expression level abruptly decreased after reaching a peak on day three, returning to about 50% of the maximum level by the end of day four. Thereafter it showed gradual decrease and daily fluctuation with maxima in the dark periods (Figure 12A). Compared to the LD profile, DD induction of BRI1 was somewhat delayed, but reached nearly the same level by the end of day three. Unlike in LD grown seedlings, the level of expression remained close to the maximum until day six, and was higher then in the LD plants for further two-three days (Figure 12B).

BRI1 gene activity in young seedlings also well correlates with the timing of BR dependent morphogenic events. Under LD conditions the initial stage of seedling development coincides with a well defined transient induction, which is downregulated as elongation becomes restricted by photomorphogenic control. In DD this control mechanism does not affect hypocotyl elongation, which continues until reaching a growth limit after approximately one week.

5.3. *BRI1* gene activity during the day

When *BRI1* expression was measured in seedlings germinated in LD, periodic daily cycles of the gene activity were observed from day four. In order to characterize this phenomenon, we monitored the changes of luminescence intensity in one-week-old *BRI1pro:LUC* seedlings under varying light regimes.

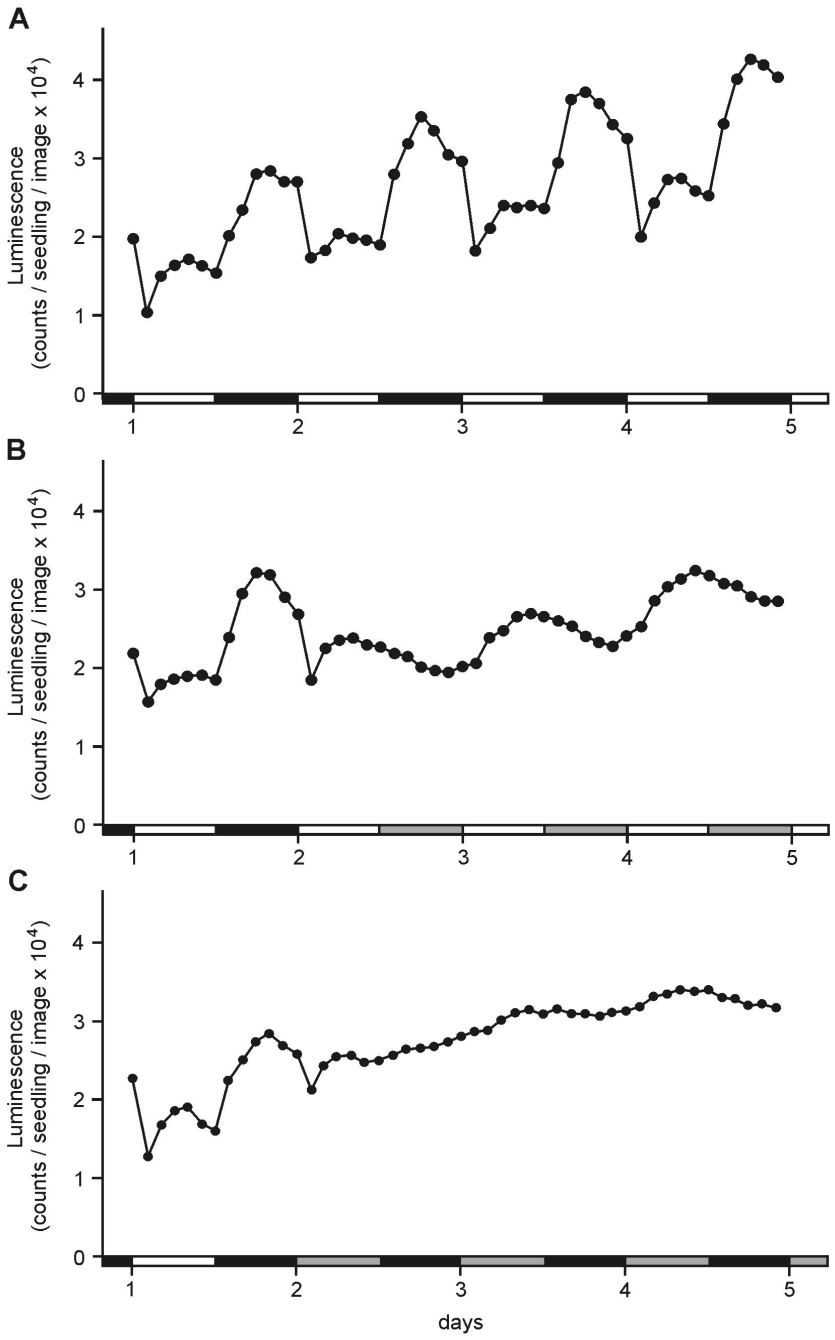

Figure 13. Daily changes of *BRI1* expression under different light regimes

Luminescence of LD-raised one-week-old *BRI1pro:LUC* transgenic seedlings under LD (A), LL (B) or DD (C) conditions. Time scales show days from the penultimate common illumination cycle. White and black bars indicate light and dark periods, gray bars correspond to subjective nights during LL (B) or subjective days during DD (C) treatments. The graphs show data of single, representative experiments.

5.3.1. Diurnal profile of expression

In time-course measurements the *in vivo* luminescence emitted by one-week-old LD-grown *BRI1pro:LUC* seedlings showed characteristic fluctuation, with low levels in the illuminated and about twice higher intensity at the dark periods (Figure 13A). This profile was very similar to that observed after day five in the LD germination experiments (Figure 12A), but *BRI1* activity in these older seedlings also showed small secondary peaks between the night time maxima, around the middle of the light periods.

5.3.2. Light regulation

In the LD measurements we saw a biphasic daily rhythm of *BRI1* expression with elevated activity in the dark. Abrupt changes of luminescence intensity following 'lights on' and 'lights off' suggested an important regulatory role for light. To test this, we carried out similar experiments with LD-raised one-week-old *BRI1pro:LUC* seedlings using constant light conditions during the CCD measurements. In LL the biphasic diurnal expression changed to low amplitude circadian cycling with maxima toward the end of the subjective daylight periods (Figure 13B). In DD a more or less steady increase of the gene activity was observed, while circadian periodicity could barely be detected (Figure 13C). In LL and DD we did not observe sudden up- or downregulation of *BRI1* expression, indicating that in LD these result from changes in the light conditions.

These results implied that *BRI1* expression becomes induced by dark and repressed by light. To test this observation, we carried out an experiment in which gene activities were measured in seedlings that were grown in LD, moved to DD for 60 h, and then returned to LD

(Figure 14A). Compared to the LD control (Figure 14B), in these seedlings *BRI1* activity remained steady and high during the DD period. Then, at the first light period of the resuming LD, the expression level suddenly and strongly decreased before its profile retuned to biphasic, similar to that of the control.

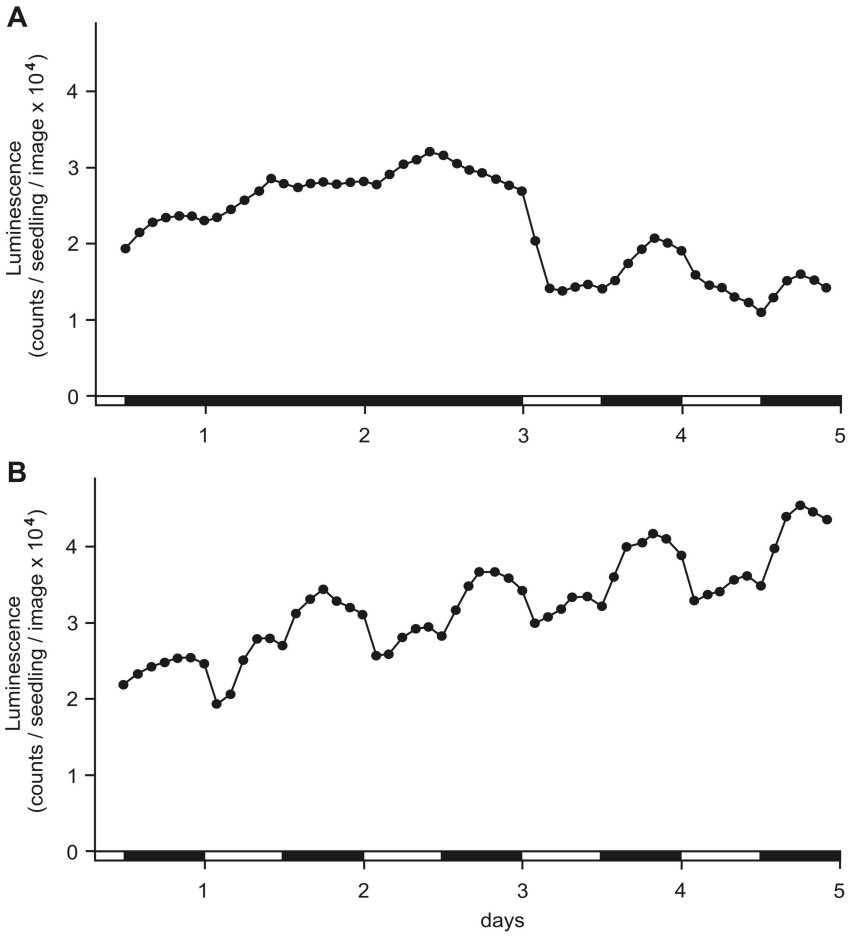

Figure 14. *BRI1* expression under changing light conditions

Luminescence of LD-raised one-week-old *BRI1pro:LUC* transgenic seedlings upon transfer to DD and then return to LD (A), and control seedlings maintained in undisturbed LD (B). Time scales show days from the last 'lights on' before starting the measurements. White and black bars correspond to light and dark periods, respectively. The graphs show data of single, representative experiments.

Our data reveal that the diurnal periodicity of *BRI1* expression is controlled by both light and circadian regulation. Gene activity is enhanced in the dark and suppressed by light.

This fluctuation is modulated by a weak circadian control that generates highest expression during the illumination phase, thereby counteracting, to some extent, the more pronounced repression caused by light. The effects of these two regulatory mechanisms result in the biphasic diurnal profile of *BRI1* activity in expanding leaves. The daily rhythmicity of expression could be observed even in early stage seedlings up to day four after germination, but this was largely obscured by the strong developmental induction occurring between days one and three (Figure 12A).

Michael *et al.* (2008) demonstrated that in LD-grown *Arabidopsis* an artificial shifting of maximal *BRI1* mRNA accumulation from the end to the beginning of the light periods considerably altered BR sensitivity. This result points out the importance of differential *BRI1* expression in determining the susceptibility to BRs, and together with our findings suggests that this is considerably influenced by the light and circadian regulation of transcriptional activity.

5.4. Complementation of the *bri1-101* mutant with the *BRI1-LUC* transgene

Targeted restoration of the BRI1 receptor function in the severely BR insensitive *bri1-101* mutant offered a versatile experimental system for elucidating the role of expressional regulation in proper plant development.

5.4.1. Restoration of the wild phenotype by *BRI1* promoter-driven *BRI1-LUC*

In the *bri1-101* line its BRI1 receptor is fully dysfunctional due to an E1087K missense mutation in the kinase domain. BR insensitivity in these plants causes severe dwarfism and very low fertility (Li JM and Chory, 1997). Our aim was to restore the receptor

function in this background by expressing functional *BRI1* under the control of promoters showing distinct organ specificities.

In order to facilitate detection of the expressed receptor we opted using the *BRI1-LUC* translational gene fusion, rather than WT *BRI1*. In the protein product of this chimeric gene a relatively large (550 aa) reporter is fused to the C-terminal kinase domain of the receptor, which is essential for the initiation of intracellular BR signaling. To avoid steric interference with the kinase activity, the LUC reporter part was joined via a flexible hinge region of 11 aa (Figure 6A), identical with the one used by Friedrichsen *et al*. (2000) for constructing their fully functional BRI1-GFP fusion.

We tested whether our BRI1-LUC construct acts as efficient receptor by expressing its chimeric gene in *bri1-101* under the control of the *BRI1* promoter. In the majority of the independent transgenic isolates the wild phenotype was restored, making them morphologically indistinguishable from Col-0 (Figure 8). This has demonstrated that the BRI1-LUC fusion is a functional receptor that can restore BR perception in severe *bri1* mutant background.

5.4.2. Complementation with ectopically expressed *BRI1-LUC*

Our expression analyses with transgenic plants carrying promoter-reporter fusions revealed well defined developmental and organ-specific regulation of *BRI1* activity. To find out how differential regulation of the receptor gene affects development and morphogenesis, we generated transgenic *Arabidopsis* lines in which it is expressed under the control of well-characterized organ-specific promoters, namely *CAB3*, *SUC2* or *ATHB8*. The expression of *CAB3*, encoding one of the chlorophyll a/b-binding proteins, is confined to photosynthetic tissues, and is most prominent in leaf mesophyll cells (Mitra *et al*., 1989). The sucrose

transporter-encoding *SUC2* and *ATHB8*, which codes for a cell-differentiation-related transcription factor, are both vasculature-specific, but function at distinct developmental stages. Whereas *SUC2* activity localizes primarily to the phloem part of vascular bundles (Truernit and Sauer, 1995), *ATHB8* expression is associated with the differentiation of procambial cells (Baima *et al*., 1995; Kang *et al*., 2003).

5.4.2.1. Mature phenotype

The morphogenic effects of ectopic BRI1 complementation were clearly recognizable when the phenotypes of the transgenic lines were compared to each other and that of *bri1-101* at their fully developed, two-month-old stage (Figure 15). *CAB3* promoter-driven expression of the *BRI1-LUC* transgene gave almost complete complementing effect, resulting in size, leaf shape and inflorescence similar to those of the WT and the *BRI1pro:BRI1-LUC* - complemented plants (Figure 8). By contrast, in the lines with vasculature-specific BRI1 production only partial complementation could be observed. In *SUC2pro:BRI1-LUC/bri1-101* plants inflorescence stems were considerably longer than those of the non-complemented mutant, but much shorter (only about half height) than that of Col-0. No rescuing effect was apparent in the rosette leaves and siliques, which were similar to the ones of the mutant. The *ATHB8pro:BRI1-LUC/bri1-101* line showed a different type of disproportionate organ development. In its case the leaves and siliques were partially expanded, but the inflorescence remained shorter than in *SUC2pro:BRI1-LUC/bri1-101*, and retained the reduced apical dominance characteristic of the mutant (Figure 15).

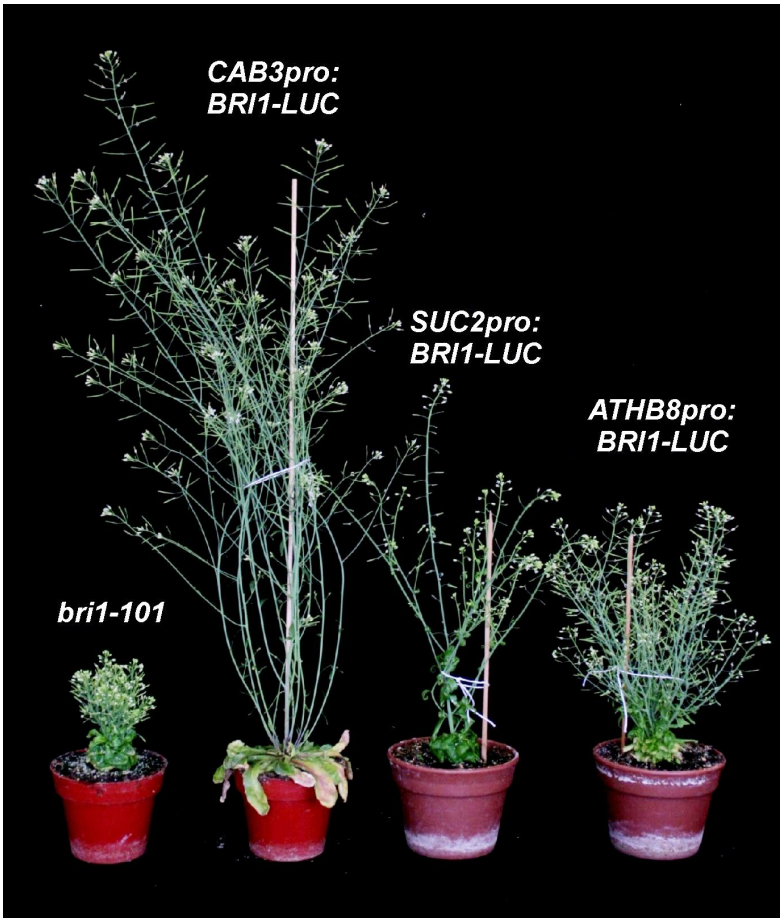

Figure 15. Complementation of the *bri1-101* mutant by ectopically expressed *BRI1-LUC*
Morphology of two-month-old *bri1-101* mutant, as well as *CAB3pro:BRI1-LUC*, *SUC2pro:BRI1-LUC*, and *ATHB8pro:BRI1-LUC* transgenic plants with *bri1-101* background.

To compare approximate basal strengths of the promoters used in the complementation constructs, and find out how these correlate with the corresponding rescue efficiencies, we determined the relative levels of the *BRI1-LUC* transcript in one-week-old LD-grown transgenic seedlings using *LUC*-specific qRT-PCR. In these measurements we found that *CAB3*-specific expression rendered more than twice higher level of the fusion mRNA than that of the control *BRI1*-specific transcription. In the case of *SUC2* promoter-driven activity the mRNA level was nearly the same as seen with the *BRI1* promoter, whereas

ATHB8-specific expression decreased it to nearly 10% of that value (Figure 16). These results reveal marked differences between basal activities of the *CAB3*, *SUC2* and *ATHB8* promoters in seedlings, differences that apparently influence the extent of complementation in the three transgenic lines. On the other hand, the distorted proportions of partially complemented plants show that complete restoration of the wild phenotype also requires proper, *BRI1*-specific developmental and tissue-specific coordination of transgene expression.

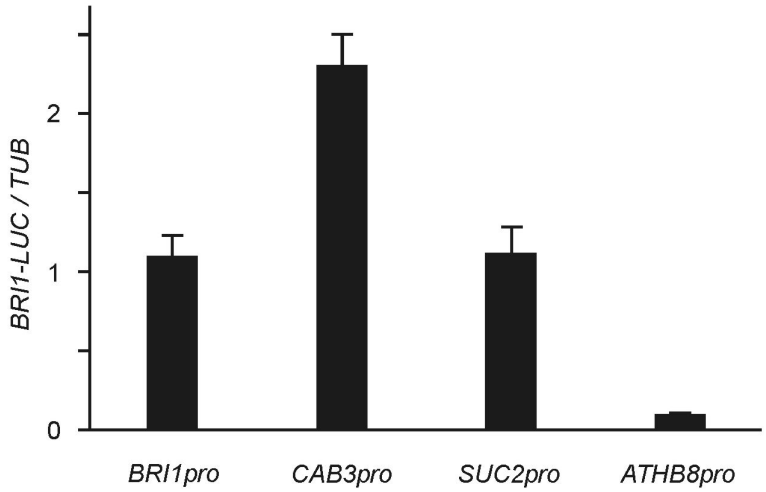

Figure 16. Levels of the *BRI1-LUC* mRNA in the complemented *bri1-101* lines

Relative amounts of the transcript expressed under the control of the *BRI1*, *CAB3*, *SUC2* or *ATHB8* promoters in one-week-old transgenic seedlings with *bri1-101* background. qRT-PCR results obtained with *LUC*-specific primers. The data are mean values ± standard deviation.

5.4.2.2. Leaf development

One of the most conspicuous features of the complemented lines was their strikingly different leaf morphology (Figure 17). The plants carrying the *CAB3pro:BRI1-LUC* fusion developed rosette leaves that were similar to those of the WT control and the *BRI1pro:BRI1-LUC*-complemented mutant, except that in fully expanded form they often became

hyponastic, a shape with upward-bent leaf blade. This was in sharp contrast to the leaves seen in the plants with *SUC2* promoter-driven transgene activity, which produced leaves with very short petioles and short, rounded blades. These epinastic (downward-bent) leaves were very similar to those of the non-complemented *bri1-101* mutant. *ATHB8*-specific expression of *BRI1-LUC* resulted in rosette leaves with normal lateral, but reduced longitudinal expansion. In this case the leaf blades were flat, just as in the WT (Figure 17).

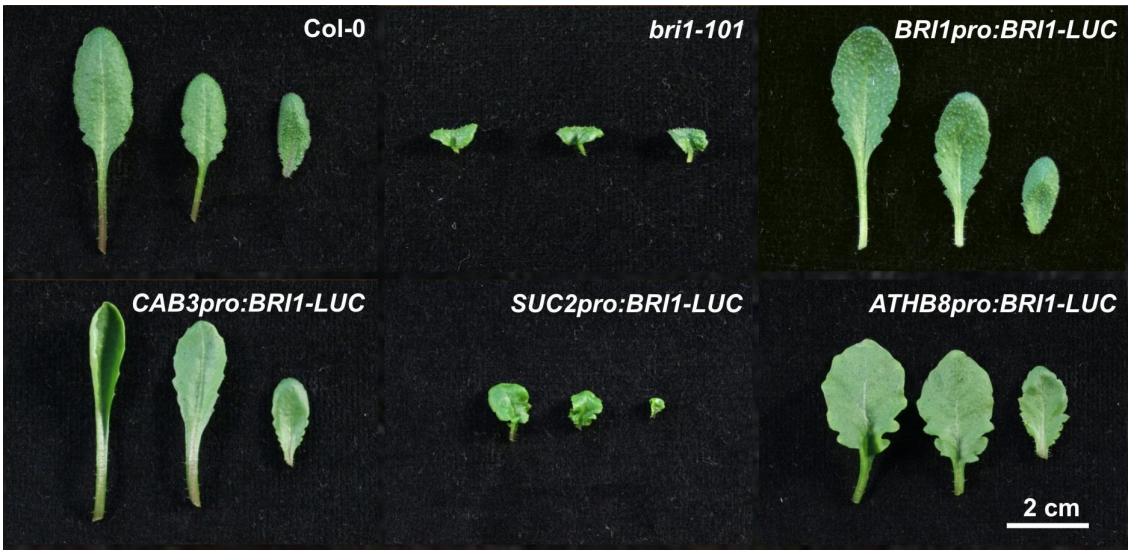

Figure 17. Leaf morphology of the *BRI1-LUC*-complemented *bri1-101* lines
Leaves isolated from six-week-old rosettes.

Luminescence imaging of the rosettes provided information on the distribution of transcriptional activities in the transgenic lines, and confirmed that these corresponded to the known specificities of the promoters used (Figure 18). In the case of the *CAB3pro:BRI1-LUC* transgene strong, more or less uniform expression could be observed over the entire leaf area. Apparently, the level of the produced receptor was not limiting and could ensure proper leaf elongation. As photosynthetically active mesophyll cells are more numerous toward the lower (abaxial) side of the leaves, it seems possible that the hyponastic shape of older leaves is the consequence of receptor overproduction and the resulting overexpansion of this tissue layer.

Alternatively, this may simply be due to an overall increase of receptor abundance, as opposed to receptor deficiency that is known to cause epinasty (Kauschmann *et al.*, 1996; Szekeres *et al.*, 1996).

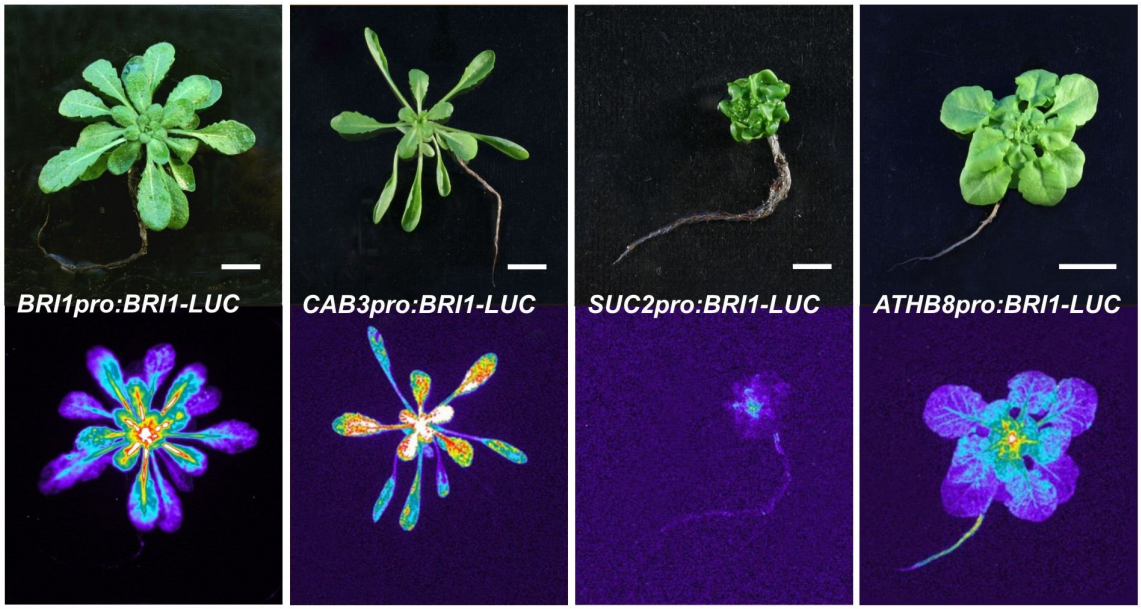

Figure 18. Ectopic expression of the *BRI1-LUC* transgene at the rosette stage

Morphology (upper row) and luminescence images (lower row) of one-month-old transgenic plants with *bri1-101* background. Scale bars correspond to 1 cm.

Vasculature-specific expression of the transgene exhibited only partial rescue effect, which was in agreement with the observation of Savaldi-Goldstein *et al*. (2007) who obtained only very weak complementation in seedlings of another severe *bri1* mutant upon vascular expression of the WT *BRI1* allele. Luminescence images of both *SUC2pro:BRI1-LUC/bri1-101* and *ATHB8pro:BRI1-LUC/bri1-101* rosettes confirmed vascular expression of the transgene but, in accordance with the different phenotypes, revealed distinct patterns of expression. In the respective lines *SUC2*-specific activity was confined mainly to differentiating leaves and roots, whereas *ATHB8* promoter-driven expression was also apparent in the better developed leaf vasculature (Figure 18). These differences in the

transcriptional regulation can be caused by the contrasting functions of the *SUC2* and *ATHB8* genes. The former encodes a sucrose transporter that functions in differentiated vascular bundles (Truernit and Sauer, 1995), the latter itself participates in the differentiation of these structures (Kang *et al*., 2003).

5.4.2.3. Formation of reproductive organs

BRs play an important role in the initiation and development of reproductive organs (Montoya *et al*., 2005; Symons *et al*., 2006 and 2012), as well as in functions required for fertility (Clouse *et al*., 1996; Szekeres *et al*., 1996). Therefore, we determined how *BRI1* expression controlled by the different promoters affected inflorescence and silique formation, and how efficiently was fertility restored in the *bri1-101* background.

Inflorescence height and structure was almost completely restored to WT in *CAB3:BRI1-LUC/bri1-101* plants (Figure 15, Table 2). However, the rescue of transgenic lines with vascular BRI1 expression was less efficient and showed contrasting effects on inflorescence and silique development. Whereas the *SUC2* promoter-regulated transgene substantially increased elongation of the inflorescence, but not of the siliques, *ATHB8* promoter-driven *BRI1-LUC* more efficiently rescued silique development but only moderately inflorescence growth (Figure 15, Table 2). Unlike the inflorescence of *SUC2:BRI1-LUC/bri1-101*, featuring normal WT-like stature, that of the *ATHB8:BRI1-LUC/bri1-101* plants showed the reduced apical dominance (with multiple stems) characteristic of *bri1* mutants (Figure 15, Table 2).

Table 2. Inflorescence and silique development in *BRI1-LUC*-complemented lines (Data are mean values ± standard deviation.)

Arabidopsis line	inflorescence height (mm)	silique length (mm)	seeds per silique
Col-0 control	396 ± 52	13.6 ± 0.9	40 ± 5
bri1-101 control	63 ± 9	6.5 ± 0.6	8 ± 3
BRI1pro:BRI1-LUC	388 ± 68	14.4 ± 0.8	41 ± 7
CAB3pro:BRI1-LUC	412 ± 75	12.9 ± 1.0	37 ± 7
SUC2pro:BRI1-LUC	227 ± 40	6.7 ± 0.8	27 ± 4
ATHB8pro:BRI1-LUC	149 ± 14	10.9 ± 0.8	38 ± 3

In contrast to complete infertility of *bri1* null mutants, the severe BR insensitive *bri1-101* isolate retained weak reproductive capacity (Li JM and Chory, 1997). WT-like seed production was almost fully recovered by *CAB3* and *ATHB8* promoter-driven complementation, but not in *SUC2:BRI1-LUC/bri1-101* that developed siliques with only two-thirds of the seed number seen in Col-0 siliques (Table 2). LUC imaging revealed relatively strong transgene activity in all parts of the *BRI1:BRI1-LUC/bri1-101* and *ATHB8:BRI1-LUC/bri1-101* inflorescences. *CAB3*-specific *BRI1-LUC* expression was associated with the photosynthetically most active uppermost nodes, silique apices and sepals at the inflorescence tip. On images of the *SUC2:BRI1-LUC/bri1-101* line all visible luminescence was confined to vascular elements of the inflorescence stem and silique peduncles (Figure19). Just like in the case of leaf development, the LUC activities detected in green siliques of the complemented lines were roughly proportional with the respective rescue

efficiencies in these organs. In agreement with the GUS histochemical staining that demonstrated intense *BRI1* promoter activity in developing seeds (Figure11C-D), the complementation experiments also highlighted the importance of silique-localized BRI1-LUC production for efficient seed production. These results suggest that local upregulation of BRI1 expression contribute to the onset of BR-dependent functions that were shown to be essential for ovule and seed differentiation (Huang *et al*., 2013).

5.4.2.4. The importance of properly regulated *BRI1* expression

Our experiments, in which BR sensitivity was partially restored in the *bri1-101* mutant by ectopic expression of the BRI1 receptor, provided evidence that proper coordination of plant development requires precise, *BRI1* promoter-specific transcription of the receptor gene. Analyses with reporter fusions revealed complex differential regulation of *BRI1* promoter activity, which is controlled both spatially and developmentally. Coordinated synthesis (and likely: also distribution) of the receptor was disturbed in the transgenic lines that expressed BRI1 ectopically. This resulted in plants with various morphogenic defects, ones that showed disproportionate organ development. This effect was more pronounced in the cases of vasculature-specific expression, when BRI1 availability in other tissues was clearly limiting, and less conspicuous upon *CAB3*-specific expression, which allowed receptor synthesis in all photosynthetically active cells.

In a recent study van Esse *et al*. (2011) found that in *Arabidopsis* roots the surface density of BRI1 can considerably vary between cell types, and that increased receptor abundance coincided with enhanced BR sensitivity. In line with these results, our data indicate precisely coordinated differential expression of the *BRI1* gene, which can influence receptor abundance and, as a consequence, BR sensitivity.

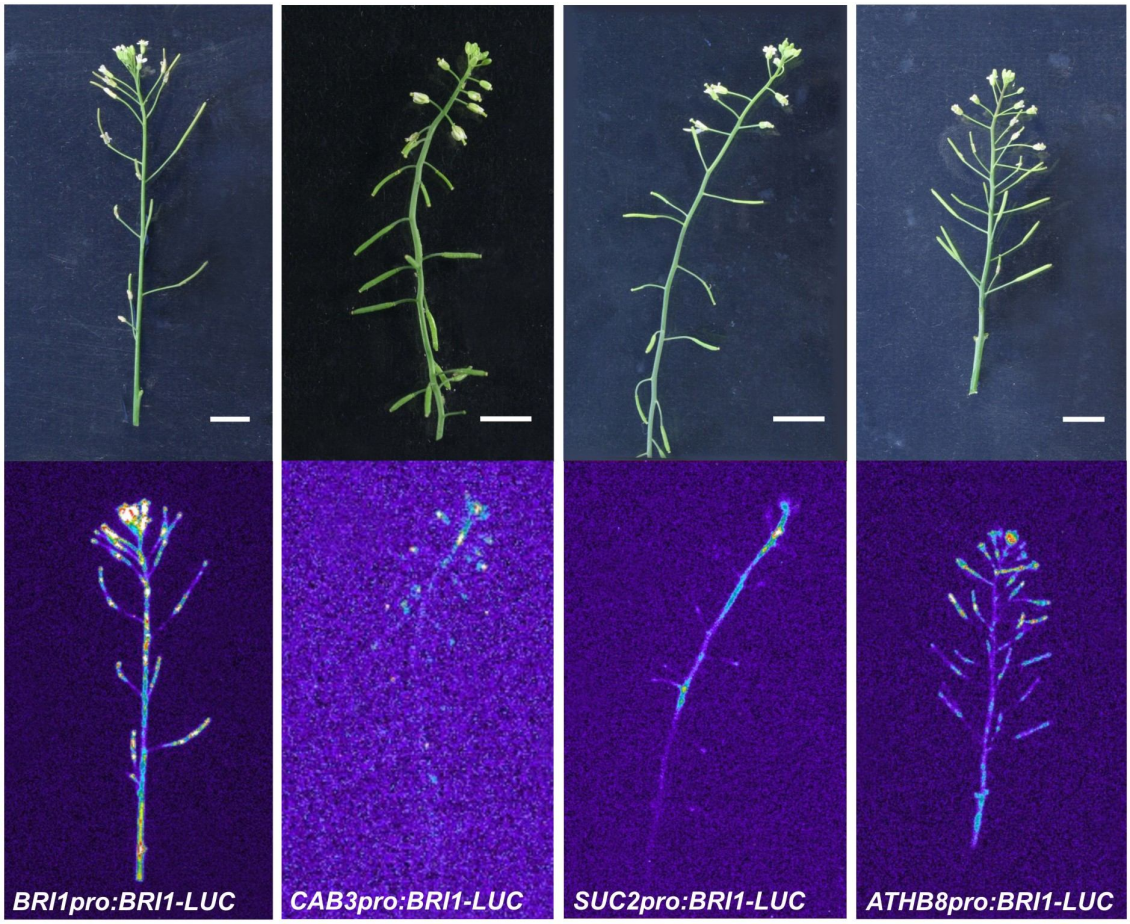

Figure19. Ectopic expression of the *BRI1-LUC* transgene in the inflorescence

Morphology (upper row) and luminescence (lower row) images of inflorescences from six-week-old transgenic plants with *bri1-101* background. Scale bars represent 1 cm.

6. CONCLUSIONS

Our study revealed differentially regulated expression of the *BRI1* gene, and that this can alter the abundance of the encoded receptor and influence BR sensitivity in *Arabidopsis*. The main results of our work can be summarized as follows:

(1) Using transgenic plants that harbored promoter-reporter gene fusions we determined the organ-specific and developmental patterns of *BRI1* gene activity. We demonstrated that in the elongation zone of the hypocotyls the upregulation of transcription results in the accumulation of *BRI1* mRNA, and also of its receptor protein product.

(2) With time-course measurements we detected and characterized a diurnal pattern in *BRI1* promoter activity, identifying light signaling and circadian control as the main determinants of this periodic daily regulation.

(3) We constructed transgenic plants in which BRI1 was replaced by a BRI1-LUC fusion that retained its receptor function, allowing the direct *in vivo* monitoring of receptor distribution.

(4) Ectopic expression of the *BRI1-LUC* transgene resulted in plants with disproportionate organ development. This highlighted the requirement of properly controlled receptor expression for coordinating BR-dependent morphogenic functions.

Our results revealed complex, differential regulation of the *BRI1* gene, and its coordination with BR-controlled physiological and morphogenic events. The *BRI1* promoter ensures the organ- and developmental stage-specificity, as well as the light-dependence of gene expression. It is also under hormonal control, as its activity has been shown to be repressed by high levels of BRs (Goda *et al.*, 2002) and induced by auxin (Nemhauser *et al.*, 2004; Sakamoto *et al.*, 2013). This multi-level transcriptional regulation, resembling those of the key BR biosynthetic genes (Hategan *et al.*, 2011; Zhao and Li J, 2012), can allow

adjustments between BR accumulation and sensitivity, and suggests an important role for the differential regulation of receptor abundance in enhancing or attenuating physiological effects of the hormone.

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ÖSSZEFOGLALÁS

A növényi hormonokként csupán az 1990-es évek vége felé elismert brasszinoszteroidok (BR-ok) meghatározó szerepet játszanak az egyedfejlődés számos fontos folyamatának szabályozásában. A hormoncsaládot alkotó polihidroxiszteroid-típusú vegyületek befolyásolják a magvak csírázását, a csíranövények fotomorfogenezisét, a szervek és szövetek kialakulása során a sejtek megnyúlását és osztódását, a reproduktív képletek iniciációját és kifejlődését, továbbá hatással vannak a növény egyedek stressztűrő képességére is.

Intenzív kutatómunka eredményeként ismertté váltak a BR-ok bioszintéziséért felelős anyagcsereutak, és a hormon érzékelésétől a génexpressziós válaszreakciók kialakulásáig tisztázódott a BR szignálátvitel teljes mechanizmusa is. Mindezeknek köszönhetően mára a BR-ok a növényi hormonok egyik legjobban ismert csoportjává váltak.

BR inszenzitív *Arabidopsis* mutánsok karakterizálása során felismerték, hogy a hormon érzékeléséért és szignalizációjának aktiválásáért a sejtfelszíni membránban lokalizált BRI1 (BRASSINOSTEROID INSENSITIVE 1) leucin-gazdag repetitív elemeket tartalmazó receptor kináz a felelős. A receptor extracelluláris doménjéhez kötődő hormon által kiváltott strukturális átrendeződés révén az intracelluláris kináz domén akrívvá válik. Ez foszforilációs és defoszforilációs lépésekből álló szignál láncolatot indít el, melynek végén a génműködés BR-függő szabályozásáért felelős transzkripciós faktorok aktiválódnak és a sejtmagban koncentrálódnak.

Bár egyes korábbi eredmények alapján úgy vélték, hogy a BRI recepor eloszlása a növényben szabályozatlan, tehát az aktuális hormonválasz mértékét jórészt a helyi

hormonkoncentrácó szabja meg, néhány fontos kísérleti adat a BR érzékenység szervenkénti és fényviszonyoktól függő eltéréseire utalt.

Tekintettel arra, hogy egyes korábbi vizsgálatainkból is erre következtethettünk, célul tűztük ki a *BRI1* gén kifejeződésének részletes vizsgálatát *Arabidopsis thaliana* modell növényben. A BR szignálút komponenseit kódoló gének közül azért a receptoréra esett a választásunk, mert a genomban egyetlen kópiában fordul elő, és az általa kódolt receptor a hormon érzékelése és a jelátvitel elindítása szempontjából is a szignalizáció esszenciális eleme. Munkánkhoz a vad típusú (Col-0) *Arabidopsis* mellett felhasználtuk annak receptorhiányos *bri1-101* mutánsát, valamint olyan β-glukuronidáz (GUS) és luciferáz (LUC) riporterekkel létrehozott génfúziókat hordozó transzgenikus növényvonalakat is, melyek segítségével mind a *BRI1* gén transzkripciós aktivitása, mind a keletkező receptor termék felhalmozódása jól nyomon követhető.

Vizsgálataink legfontosabb eredményei a következők:

(1) Promóter-riporter génfúziókat hordozó transzgenikus növények segítségével meghatároztuk a *BRI1* gén szervspecifikus és fejlődési stádiumtól függő kifejeződési mintázatát. Csíranövényekben kimutattuk, hogy a hipokotil megnyúlása során tapasztalt indukció a *BRI1* mRNS és a termék BR receptor felhalmozódásával jár, továbbá hogy mindez egybeesik a BR szignalizációnak az elongációs régióban tapasztalt felerősödésével.

(2) A *BRI1* aktivitás időbeni változását követve felismertük és jellemeztük annak napszakos (diurnális) szabályozottságát. Kimutattuk, hogy a napi ciklusokban ismétlődő gyengébb kifejeződés a világos, valamint erősebb expresszió egy negatív fényszabályozás és egy kevésbé markáns cirkadián reguláció hatásának együttes eredménye.

(3) BR inszenzitív *bri1-101* mutáns háttérben olyan transzgenikus növényvonalakat állítottunk elő, amelyekben a BR-ok érzékelését a receptor funkcióját megőrzött BRI1-LUC

fúziós protein biztosítja. Ezáltal közvetlenül lehetővé vált a receptor növényen belüli eloszlásának *in vivo* nyomonkövetése.

(4) A kimérás receptort kódoló *BRI1-LUC* transzgént ektopikusan kifejező transzgenikus vonalak esetében morfogenikus rendellenességeket, tipikusan az egyes szervek aránytalan növekedését és differenciációját tapasztaltuk. Ezek a vizsgálatok megerősítették, hogy a növény zavartalan fejlődéséhez elengedhetetlen a receptor kifejeződésének a *BRI1* promóter által meghatározott specifikus szabályozása.

Eredményeink alapján ismertté vált a *BRI1* gén expressziójának regulációja, valamint ennek összehangoltsága a BR-ok által kontrollált élettani és morfogenikus folyamatokkal. Kimutattuk, hogy a *BRI1* promóter aktivitása szerv- és fejlődési stádium-specifikusan, valamint a fényviszonyoktól függően is szabályozott. Ezekhez adódnak még azok irodalomból ismert hormonális (auxin, BR) hatások, amelyek a BRI1 transzkripciót szintén befolyásolják. Ez a többszintű regulációs mechanizmus, amely sok tekintetben mutat párhuzamosságot a BR bioszintézis kulcsenzimeit kódoló gének működésének szabályozásával, lehetőséget biztosíthat a hormon felhalmozódásának és a vele szembeni érzékenység kialakításának precíz összehangolására. Mindezek azt valószínűsítik, hogy a receptorszint és hormonérzékenység finomhangolásával a *BRI1* gén differenciált expressziójának fontos szerepe lehet ebben a szabályozási rendszerben.

SUMMARY

Brassinosteroids (BRs), which were recognized as plant hormones only toward the end of the 1990s, are essential regulators of several important functions during plant development. This hormone group of polyhydroxylated steroids can control seed germination, seedling photomorphogenesis, the elongation and division of cells during tissue differentiation, the initiation and formation of reproductive organs, as well as resistance to various environmental stress factors.

Intense studies of several leading laboratories uncovered the pathways and regulatory mechanisms of BR biosynthesis, and also clarified in detail the signaling route that leads from hormone perception of the to specific responses at the level of gene expression. Due to these results, by now BRs became one of the best characterized groups among plant hormones.

Functional analyses of BR insensitive *Arabidopsis* mutants revealed that BRs are perceived by BRI1 (BRASSINOSTEROID INSENSITIVE 1), a leucine-rich repeat receptor-like kinase. This cytoplasmic membrane-localized receptor interacts with the hormone ligand and initiates the intracellular signalization that leads to the hormone response. BR binding by the extracellular domain of the receptor results in conformational changes that activate the intracellular kinase domain. This kinase activity then generates phosphorylation/dephosphorylation-based signaling steps, which ultimately cause the activation and nuclear accumulation of the transcription factors that mediate BR-responsive gene expression.

Based on initial studies it was proposed that plants do not regulate the spatial or temporal expression and distribution of BRI1, thus differential hormone responses were attributed mainly to changes in the BR levels. This simple model, however, was challenged

by contrasting data of other research teams, which implied that BR sensitivity varies between organs and can be altered by light conditions.

As some earlier results of our group led to similar conclusion, we set out to investigate in detail the expression properties of the *BRI1* gene in the model plant *Arabidopsis thaliana*. We chose this single-copy gene from among those that encode BR signaling components because its receptor product interacts with the hormone and initiates intracellular phosphorylation, making BRI1 is a key element in the signalization process. In addition to wild type *Arabidopsis* (ecotype Col-0), we also used its BR receptor-deficient *bri1-101* mutant, as well as our newly generated transgenic lines expressing fusion products with the bacterial β-glucuronidase (GUS) and firefly luciferase (LUC) reporters, which allowed easy monitoring of *BRI1* transcription and of receptor accumulation.

The main results of our studies can be assessed as follows:

(1) Using transgenic plants that harbored promoter-reporter gene fusions we determined the organ-specific and developmental patterns of *BRI1* gene activity. We demonstrated that in the elongation zone of the hypocotyls the upregulation of transcription results in the accumulation of *BRI1* mRNA, and also of its receptor protein product.

(2) With time-course measurements we detected and characterized a diurnal pattern in *BRI1* promoter activity, identifying light signaling and circadian control as the main determinants of this periodic daily regulation.

(3) We constructed transgenic plants in which BRI1 was replaced by a BRI1-LUC fusion that retained its receptor function, allowing the direct *in vivo* monitoring of receptor distribution.

(4) Ectopic expression of the *BRI1-LUC* transgene resulted in plants with disproportionate organ development. This highlighted the requirement of properly controlled receptor expression for coordinating BR-dependent morphogenic functions.

Our data provided evidence for the differential spatial and temporal control of *BRI1* gene expression, which well coincides with the localization and timing of BR-requiring physiological processes. We found that, in addition to the organ- and developmental stage-specific regulation, *BRI1* promoter activity also depends on light conditions. As a further layer of expressional modulation, literature data also indicate hormonal adjustment by auxin and BRs. This complex, multi-level control of *BRI1* is quite similar to that seen in the case of those genes that code for the key enzymes of BR biosynthesis, indicating the possibility of fine coordination between hormone levels and susceptibility. Accordingly, our data suggest that differential expression and distribution of the BRI1 receptor can be an important means of fine-tuning BR sensitivity and, as a result, ensuring proper plant development.

PUBLICATIONS

The dissertation is based on the following publication:

Hategan L, Godza B, Kozma-Bognar L, Bishop GJ, Szekeres M (2014) Differential expression of the brassinosteroid receptor-encoding *BRI1* gene in *Arabidopsis*. Planta (DOI 10.1007/s00425-014-2031-4; in press)

Other publications:

Hategan L, Bindics J, Szekeres M (2008) Developmentally regulated expression of the BRI1 brassinosteroid receptor in *Arabidopsis thaliana*. Acta Biol Szegediensis 52: 57-58

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