Theses of the Ph.D. dissertation

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

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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 seemed to indicate organ-specific or time of the day- and light-dependent differences in BR sensitivity. In particular, root development was known to be inhibited by BR concentrations that were enhancing the growth of aerial organs. Light-grown seedlings were found less susceptible to treatment by externally applied BRs

than those grown in the dark. This could be evidenced by their different elongation responses, but also by the enhanced transcriptional reactions of BR-responsive genes upon dark treatment.

Therefore, the aim of our project was to find out whether the expression of the BR receptor BRI1, 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. Among these particularly useful was the severely BR insensitive *bri1-101* mutant that is devoid of functional BR receptor. We expected that by finding answers to the questions related to receptor expression and abundance we can contribute to the better understanding of the ways how BR sensitivity is modulated. Additionally, our results might also be useful for elucidating whether such adjustments are coordinated with the local levels of the hormone in order to evoke optimal physiological effects.

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.

METHODS

Growth conditions

For *in vitro* cultures surface-sterilized seeds of wild type *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) or its BRI1-deficient *bri1-101* missense mutant were germinated and grown on solidified Murashige-Skoog plant medium, at constant 22°C temperature. Seedlings were raised under light/dark conditions (12 h 50-60 µmol photons/m^2/s fluorescent white light, 12 h dark), or received treatments of continuous light or dark. Maintenance and phenotypic characterization of the plant lines were done in temperature-controlled (20-22°C) greenhouse. Following four-five weeks of vegetative growth under short day conditions (8 h light/16 h dark), the plants were brought to flowering and seed production under long day illumination cycles (16 h light/8 h dark).

Generation of transgenic plants

Fusion constructs containing either the *BRI1* promoter or one of three tissue-specific *Arabidopsis* promoters, plus coding sequences of the *BRI1* gene and/or the bacterial β-glucuronidase (*GUS*) or firefly luciferase (*LUC*) reporter genes were assembled in T-DNA-based binary plant vectors. Transgenic plants carrying the fusion genes were generated by *Agrobacterium*-mediated flower transformation of Col-0 or heterozygous *bri1-101* plants. Transgenic isolates were propagated by self-pollination to produce lines that became homozygous for both transgene and genetic background. Segregation and gene expression properties of these plants were

characterized, and for each transgene construct representative lines were chosen for detailed expression or phenotype analyses.

Reporter-based assaying of BRI1 promoter activity

BRI1 gene activity was detected via *in vitro* assaying of GUS enzyme activity or *in vivo* measurement of LUC-generated luminescence in *BRI1pro:GUS*- or *BRI1pro:LUC*-expressing transgenic plants, respectively. The results of GUS histochemical staining with X-gluc substrate were evaluated following the removal of photosynthetic pigments by ethanol. For measuring LUC activity the plants were pre-treated by spraying with luciferin substrate, and then the generated luminescence was detected using a high sensitivity CCD camera.

Complementation of BRI1-deficiency

For rescuing BR-insensitivity of the *bri1-101* mutant a fused transgene was used, which expresses an easily detectable BRI1-LUC chimeric protein with fully retained receptor function. The effects of complementation by this coding sequence was studied in transgenic plant lines that expressed it under the control of the native *BRI1*, the photosynthetic tissue-specific *CAB3*, or the vasculature-specific *SUC2* and *ATHB8* promoters. The morphology of the *BRI1-LUC*-expressing lines and the distribution of the chimeric product in their organs were analyzed.

Transcript analyses

Relative levels of the BRI1 and LUC mRNAs were determined by real-time RT-PCR analyses. Samples of cDNA were prepared from

total RNA isolates using MLV reverse transcriptase and random hexanucleotide primers. The RT-PCR reactions were carried out with gene-specific oligonucleotide primers, and product accumulation was monitored on the basis of increasing SYBR Green fluorescence. The measured levels of the transcripts were normalized to those of the constitutively expressed *TUB2* and *TUB3* genes.

Quantitation of the BRI1-LUC fusion protein

The relative levels of BR receptor accumulation were determined by measuring the luminescence produced by the BRI1-LUC chimeric protein expressed in transgenic lines with *bri1-101* background. Soluble proteins were extracted from different regions of etiolated hypocotyls and their LUC activities were measured by luminometer upon addition of the luciferin substrate. The detected values were normalized to the protein contents of the extracts.

RESULTS

The results of our work can be summarized as follows:

(1) We generated transgenic *Arabidopsis* lines expressing the *BRI1pro:GUS* or *BRI1pro:LUC* reporter genes and used them for determining the specificity of *BRI1* expression. Our GUS staining results revealed differential spatial regulation of the receptor gene, and suggested that the sites upregulated *BRI1* activity overlap with those undergoing BR-dependent morphogenic changes.

To clarify if there is indeed correlation between *BRI1* expression, receptor accumulation and BR sensitivity, we measured the levels of the mRNA and receptor protein expressed from the *BRI1pro:BRI1-LUC* fusion in a transgenic line where this gene provided the only functional BR receptor. Both the transcript and its receptor product were seen to accumulate preferentially in the apical half of five-day-old etiolated hypocotyls. At this developmental stage of dark-grown seedlings this part of the hypocotyl is actively elongating. Furthermore, this region also shows enhanced expression of multiple BR-inducible genes.

Our transgenic line carrying the *BRI1pro:LUC* also allowed the monitoring of *BRI1* promoter activity in time-course experiments. We observed that upon germination *BRI1* is strongly induced in both light/dark- and dark-grown seedlings, but under light/dark conditions its expression level quickly declines after three days. By contrast, the activity of the receptor gene remains highly active in continuous dark for about one week. This period coincides with the time of elongation in etiolated seedlings.

(2) As time-course data showed daily periodicity of *BRI1* activity in seedlings toward the end of the light/dark measurements, we verified this phenomenon in one-week-old *BRI1pro:LUC*-expressing plantlets. In these measurements a biphasic diurnal expression pattern was detected, which showed highest activity at the middle of the dark phases, but also a much smaller peak in the light periods. The sharp increases and decreases of *LUC* activity were observed at lights-on and lights-off, therefore we also tested how the expression was affected by light. Under constant light conditions only a circadian oscillation pattern could be detected, whereas prolonged dark or light treatments caused up- or downregulation, respectively. These results showed that the diurnal expression pattern of BRI1 results from negative light regulation and a superimposed circadian effect.

(3) In order to allow *in vivo* monitoring of BR receptor production and accumulation we generated a transgenic line in which the native BRI1 protein is replaced by its LUC-tagged version. To this end the promoter and coding sequence of the *BRI1* gene was translationally fused with the coding region of the *LUC* reporter, and then this chimeric construct was transformed into the *bri1-101* mutant background. The resulting transgenic lines re-gained their BR sensitivity, as could be evidenced by restoration of the wild phenotype. The *BRI1pro:BRI1-LUC*-expressing plants exhibited a luminescence pattern that was similar to that of the *BRI1pro:LUC* lines. These results verified that the BRI1-LUC fusion retains its receptor function, and confirmed the correlation between the induction of the BRI1 gene and the accumulation of its protein product.

(4) Our analyses indicated that the *BRI1* promoter confers spatial and developmental, as well as light-dependent expression to the receptor gene. Therefore, we wanted to find out how alterations of the optimal expression pattern might affect *Arabidopsis* growth and morphogenesis. With this purpose we prepared gene fusions in which the *BRI1-LUC* coding region was placed under the control of well-characterized tissue-specific promoters, and these constructs were used as transgenes for ectopic complementation of the *bri1-101* mutant. *CAB3* promoter-driven expression, rendering activity in all photosynthetic tissues, resulted in almost complete restoration of the wild phenotype, with only minor leaf morphology defect that is likely caused by local BR hypersensitivity. By contrast, *BRI1* expression from the vascular tissue-specific *SUC2* or *ATHB8* promoters had only partial rescuing effect. The *SUC2* promoter, which is active in the phloem part of the differentiated vasculature, produced sufficient *BRI1-LUC* transcript level for restoring the height of the inflorescence, but failed rescuing leaf and silique morphology. *ATHB8* promoter activity, associated with procambial cells of the differentiating vascular bundles, ensured better leaf and silique growth, but had weaker effect in the inflorescence stem. These experiments clearly demonstrated that proportional organ morphogenesis depends on proper, *BRI1*-promoter-controlled expression of the receptor gene.

Altogether, our results reveal that *BRI1* expression is subject to differential regulation at the transcriptional level, and it can influence BR sensitivity. This complex control mechanism allows coordination between the production of and susceptibility toward BRs, and can be instrumental in ensuring optimal effects of the hormone.

LIST OF PUBLICATIONS

(MTMT ID: 10037230)

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) [IF: 3.661]

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

Hategan L, Godza B, Szekeres M (2011) Regulation of brassinosteroid metabolism. In: Brassinosteroids: a plant hormone (S Hayat, A Ahmad, eds), pp. 57-81, Springer, Dordrecht, Heidelberg, London, New York

Ohnishi T, Godza B, Watanabe B, Fujioka S, **Hategan L**, Ide K, Shibata K, Yokota T, Szekeres M, Mizutani M (2012) CYP90A1/CPD, a brassinosteroid biosynthetic cytochrome P450 of *Arabidopsis*, catalyzes C-3 oxidation. J Biol Chem 287: 31551-31560 [IF: 4.651]

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Statement

As corresponding author I declare that Lidia Hațegan made substantial contribution to the research paper "CYP90A1/CPD, a brassinosteroid biosynthetic P450 of *Arabidopsis*, catalyzes C-3 oxidation", which was published during 2012 in the Journal of Biological Chemistry.

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Miklós Szekeres
res. advisor