Statements of the Ph.D. thesis

Examination of brassinosteroid-regulated gene expression in *Arabidopsis thaliana*

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INTRODUCTION

Intercellular communication plays a crucial role throughout the life cycle of higher plants. This feature allows coordinated responses of cells, tissues and organs, according to their genetic program and in response to various environmental stimuli. Natural signalling compounds with the potential to regulate tissue-specific and systemic processes at a distance from the site of their synthesis, are called plant hormones. For a long period of time, the five "classical" phytohormones (auxins, gibberellins, cytokinins, abscisic acid and ethylene) were thought to be responsible for all physiological and developmental processes controlled by hormones.

Brassinosteroids (BRs) are ubiquitous growth-promoting substances throughout the plat kingdom. Based on their physiological effects, these steroids have been proposed to act as a novel family of hormones, although without providing evidence of their indispensability. The recent findings that the phenotype of several dwarf and photomorphogenic *Arabidopsis* mutants can be rescued only by BRs (e.g. cbb1 and cbb3) supported this view. The first genetic data confirming the role of BRs as plant hormones have been provided by showing that several of these mutant genes encode proteins similar to steroidogenic factors (*cpd*, *det2*) and that the corresponding phenotypes are due to the deficiency of brassinolide (BL), the biologically most active BR.

The putative BR receptor (or a member of the BR receptor complex) has also been identified. Interestingly, the *BRI1* gene encodes a leucine-rich repeat receptor-like kinase with proven serine/threonine kinase activity, and the protein is localized in the plasma membrane. This suggests that BRs can only be perceived at the cell surface. Thus, it is very likely that the mechanism of BR action is basically different from steroid hormone perception and signal transduction in animals.

Plant hormones act on cellular processes, often in cooperation with other hormones, in a concentration-dependent manner. The regulation of the amount of these substances is therefore of crucial importance. The control of hormone concentration may take place at the level of synthesis, degradation, and/or conjugation.

The majority of the hormone-regulated physiological effects result from the changes in the expression of several hormone-regulated genes. Since the action spectrum

of BRs is largely overlapping with those of the auxins, gibberellins and partly of cytokinins, it is possible that these hormones may also induce similar changes in gene expression patterns. However, it is also likely that there is at least a subset of genes with primarily BR-regulated expression. The identification of such genes could lead to a better understanding of BR-specific regulatory mechanisms at the molecular level.

OUR KNOWLEDGE OF BR-REGULATED GENE EXPRESSION

The physiological effects of BRs are well documented. These hormones play a role in numerous processes, including cell and stem elongation, gravitropic responses, cell division, pollen development and senescence. BRs can stimulate cell elongation when applied alone, and can also act synergistically with auxin, but the molecular mechanisms of action of these two hormones are different. It is likely that in most cases BRs use pathways different from those used by other substances, which leads to altered gene expression.

Although BRs have pleiotropic effects on the growth and development of higher plants, which involve numerous changes in gene expression, so far only a few BR-regulated genes have been isolated. At the start of our project, only three BL-induced genes were known. All of these encode members of the xyloglucane endotransglycosylase family which plays an important role in determining the rigidity, flexibility and loosening of plant cell walls. Loosening of cell walls is a necessary, but not sufficient condition for elongation, therefore the participation of other BL-responsive genes was also expected. Indeed, the last few years lead to the identification of several other BL-regulated genes, which may be involved in different subprocesses of elongation or cell division, but the central coordinating factors have remained unidentified.

Arabidopsis mutants deficient in BL biosynthesis are optimal subjects for studying BR-regulated gene expression. Since these plants do not contain biologically active BRs, it is easier to detect changes in the abundance of mRNAs upon exogenous hormone treatment, without the background effect of endogenous substances. The

availability of the cpd (for *constitutive photomorphogenesis and dwarfism*) mutant provided a promising tool for isolating novel hormone-regulated genes.

Because of the concentration- and ratio-dependence of hormone action, the level of these biologically active compounds must be strictly regulated. It is possible that the amount of available BL is (partly) determined via the controlled expression of genes involved in its synthesis, degradation, conjugation or perception. At the time when this work was started, of the many possible targets of regulation only a few genes had been isolated. The regulation of their expression had been studied to an even lesser extent. Preliminary data obtained from the analysis of transgenic plants containing a *CPD* promoter-*GUS* reporter fusion showed that the expression of the steroidogenic *CPD* can be repressed by exogenously added BL.

OBJECTIVES OF OUR WORK

The general objective of our work was to gain further information on the molecular basis of BR action. For this purpose, we addressed the following questions:

- **How specific is the response of the *Arabidopsis CPD* gene to BRs and at which level is the expression regulated?**

- **What is the temporal and spatial expression pattern of the *CPD* gene?**

- **Which other BR-responsive genes may participate in steroid hormone action?**

METHODS USED

-Growing of *Arabidopsis thaliana* plants under sterile conditions and in the greenhouse
-Treatment of plants with hormones and other substances
-Isolation of plant RNA
-Northern hybridisation
-GUS histochemical analysis
-Differential display (DDRT-PCR) analysis
-Molecular cloning techniques
-Isolation of full-length cDNA clone
-RT-PCR analysis
-Flower-transformation of *Arabidopsis*
-Generation and examination of transgenic plants

RESULTS AND DISCUSSION

1. We studied the effect of BL on the expression the *Arabidopsis CPD* gene by using stable transformant plants containing a *CPD* promoter-*GUS* reporter transgene construction. The action of BL was monitored by Northern hybridizations, using in parallel the coding regions of *GUS* and *CPD* as probes. The expression of both genes tested was repressed upon treatment with exogenous BL. The expression pattern was independent of the light conditions. In the presence of cycloheximide, a protein synthesis inhibitor, the repression of the genes by BL could not be detected, suggesting the requirement of a *de novo* synthetized protein for the regulation. Since the only common feature of the two genes analysed is their identical promoter sequence, the data indicate that BL-dependent *CPD* expression is regulated mainly at the transcriptional level. Thus, BL may influence mRNA abundance via at least two separate ways, transcriptionally (*CPD*), and by the modification of mRNA stability (soybean *BRU1*). The end-product

repression of a BR biosynthetic gene suggests an interesting analogy with animal systems, where sterol homeostasis can be maintained by the effect of oxysterols on the genes involved in steroid biosynthesis.

2. For the determination of the temporal and spatial expression of the *CPD* gene, plants of the same genotype as above were used. Plants were grown in the light or in darkness for various periods prior to analysing the expression pattern of the *GUS* transgene. We used the indigo dye forming X-gluc substrate for detection. During early development, GUS staining patterns were similar in both etiolated and light-grown plants. GUS activity was first observed in the cotyledons three days after imbibition, and increased throughout the first week of development. Occasionally, a weak signal could be seen in the apical hook of etiolated seedlings. GUS staining appeared in the first developing leaf primordia ten days after germination, whereas the intensity of the staining showed a decrease in cotyledons. When grown in the presence of BL, the seedlings showed similar, but much weaker activity, especially in the cotyledons: intense staining could be seen only in the leaf primordia. In adult plants grown in the light, GUS activity was seen in the leaf buds and decreased gradually in ageing rosette leaves. GUS activity in leaves was localized mainly to the palisade parenchyma and the stomatal guard cells of the adaxial epidermis. Activity was also detected in cauline leaves and sepals.

Since the staining patterns were similar both in light- and dark-grown plants, it can be assumed that *CPD* expression is not influenced by light. But because *CPD* activity is mainly confined to the tissues rich in plastids, it cannot be excluded that these organelles facilitate *CPD* expression. The high level of hormone precursors (isoprenoids, sterols) in the plastids could be a possible explanation for this phenomenon. The differences between the main sites of action, the site of BR accumulation and the localization of *CPD* activity suggest the requirement of a hormone(or precursor) transport apparatus in the plant, and/or of an efficient BL-inactivating capacity, in order to make *CPD* expression possible.

3. To gain further insight into the molecular processes regulated by BRs, we chose the approach of isolating and characterising novel BL-responsive genes by means of the

differential display method. The effect of BL on gene expression was monitored in homozygous BR deficient cpd mutants. In order to exclude indirect hormone responses, the screening was carried out in the presence of cycloheximide.

With this approach we isolated a previously unknown cDNA which responds negatively to BL treatment. The repressing effect of the hormone on the corresponding gene could also be shown in wild-type (wt) *Arabidopsis*. We showed that the expression level of the gene is much higher in the steroid deficient background. The difference in the abundances of the gene between the mutant and wt plants indicates that the regulation by BL takes place *in vivo* too. We isolated the full size cDNA and determined that the gene encodes a polypeptide of 170 amino acids, and contains two identifiable domains. The N-terminal part of the protein (amino acids 15 to 33) consists of aliphatic, hydrophobic residues, and may form a transmembrane segment. The protein may be anchored to a membrane by this part of the molecule. The amino acid residues between positions 94 and 136 define a RING-H2 domain. The gene was therefore given the name *BRH1* (for *Brassinosteroid Responsive RING-H2*).

In *Arabidopsis*, there are more than 350 different putative RING finger proteins; however, only a few of these has been characterized to some extent. Based on sequence homology and domain composition, the *BRH1* gene is a novel member of the *RHA* gene family. Unfortunately there is no available information about the function of the *RHA* genes. The only homologous gene which has been partially analysed is the *Arabidopsis ATL2*, a gene rapidly and transiently induced by elicitor treatment.

The expression of *BRH1* is quickly down-regulated by BL. The amount of the mRNA drops to the minimum level after one hour of hormone treatment, without the requirement of *de novo* protein synthesis. Whether BL acts on *BRH1* expression at the transcriptional or post-transcriptional level remains to be elucidated. The activity of the gene is unaffected by other phytohormones and by the stress-signalling molecules salicylic acid and jasmonic acid. Because of the elicitor-inducibility of *ATL2* , we tested the response of *BRH1* to chitin. Interestingly, *BRH1* expression shows a transient increase after one hour, and returns to the original level after four hours of treatment.

We generated transgenic *Arabidopsis* plants overexpressing *BRH1* in sense and antisense orientations. In our experiments, overexpression of the gene in either

orientation had moderate developmental effect. Plants containing the antisense construct were more robust, produced more inflorescences and yielded more seeds. By contrast, the phenotype of the plants overexpressing the transgene in sense orientation was less conspicuous. Thus, we can conclude that, at least under the circumstances of our experiments, the presence and amount of BRH1 protein is of no crucial importance.

Meantime, several BR-regulated genes have been isolated from *Arabidopsis* and other higher plants. Most of them are also regulated by other phytohormones (e.g. *BRU1*, *Lin6*), whereas in other cases no evidence for co-regulation was presented. *BRH1* is one of the few known BR-responsive genes not influenced by other hormones. The tomato *Lin6* gene encodes an extracellular invertase and is also responsive to pathogen elicitors. However, the expression of this gene is affected parallelly and not in an antagonistic way by BL and elicitors. The product of this gene is responsible for the hydrolysis of extracellular disacharides, providing cells with metabolizable monosacharides for elongation or defense.

RING finger proteins are thought to function as organizing components of multiprotein complexes involved in many cellular processes, e.g. DNA repair, peroxysome biogenesis or signal transduction. During the past few years many RING proteins have been identified as central parts of ubiquitination complexes. Polipeptides with a RING-H2 domain are over-represented in this group. On the basis of our information about BRH1, its function can be modelled in two different ways. *(a)* BRH1 is a member of a ubiquitination complex and therefore acts as a negative regulator. Several preconditions are required for cell elongation, including the presence of the required proteins, sufficient energy supply and optimal environment. Upon pathogen attack energy must be channelled to defense mechanisms and to strenghtening the cell walls. It is possible that BRH1 plays a role in eliminating one or more protein factors involved in elongation processes, thereby acting as part of a switch between cell elongation and defense mechanisms. The opposite regulation of *BRH1* by BL and chitin may support this hypothesis. *(b)* BRH1 is an organizing part of a non-ubiquitinating multiprotein complex, and therefore acts as a positive regulator. From this point of view, BRH1 may be a member of a complex involved in plant defense mechanisms, supported by the fact that

its gene is induced by elicitor and repressed by BL. However, the transient manner of chitin-inducibility contradicts this theory.

For the ascertainment of the real function of BRH1, further examinations are required. The identification of the interacting proteins would allow us to elucidate its role in BL- and stress-regulated mechanisms.

PUBLICATION LIST

(* the thesis is based on these publications)

*Molnár G, Bancos S, Koncz C, Nagy F, Szekeres M (2001) Characterisation of a brassinosteroid-responsive RING-H2 gene from *Arabidopsis thaliana*. *Planta*, accepted for publication.

*Mathur J, Molnár G, Fujioka S, Takatsuto S, Sakurai A, Yokota T, Adam G, Voigt B, Nagy F, Maas C, Schell J, Koncz C, Szekeres M (1998) Transcription of the *Arabidopsis CPD* gene, encoding a steroidogenic cytochrome P450, is negatively controlled by brassinosteroids. *Plant J* **14**(5):593-602.

Bánfalvi Z, Molnár A, Kostyál Z, Lakatos L, Molnár G (1997) Comparative studies on potato tuber development using an in vitro tuber induction system. *Acta Biol Hung* **48**(1):77-86.

Bánfalvi Z, Molnár A, Molnár G, Lakatos L, Szabó L (1996) Starch synthesis, and tuber storage protein genes are differently expressed in *Solanum tuberosum* and in *Solanum brevidens*. *FEBS Lett* **383**(3):159-64.