Theses of Ph.D. dissertation

Regulation of the genes involved in brassinosteroid biosynthesis

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INTRODUCTION

During their development higher plants need to respond to a variety of external and endogenous stimuli. Such signals are perceived by receptor molecules (e.g. photoreceptors) and determine intracellular responses. The responses at the level of tissues and organs are coordinated by phytohormones, the synthesis of which is directly modulated by environmental cues.

Brassinosteroids (BRs) have recently been recognized as a distinct class of phytohormones. They are polyhydroxy-steroids, possessing a structure similar to that of the insect molting hormone ecdysone. BRs were identified in a wide variety of vascular plants and play important roles in elongation, photomorphogenesis, fertility and stress-resistance. Although the chemical structure of the biologically most active BR brassinolide (BL) was determined chemically characterized as late as 1979, by now its biosynthesis is well understood, and a several components of the BR signal transduction pathway have also been identified.

The identification and characterization of BR-mutants, especially in *Arabidopsis thaliana*, has been instrumental in elucidating the precise biological functions and biosynthetic routes of BRs. Most of these mutants exhibit a dwarf phenotype, and functionally they can be divided into two categories. One can be rescued to wild-type upon treatment with exogenously supplied BL, indicating that the mutation caused lesion of one of the BR-biosynthetic genes. The second group is insensitive to BL, therefore in that the dwarf phenotype cannot be rescued. Accordingly, members of this group carry mutations affecting the BR-receptor or members of the downstream signal transduction pathway.

The *Arabidopsis constitutive photomorphogenesis and dwarfism* (*cpd*) mutant lacks the ability to synthesize active BL. The *CPD* gene has been identified and shown to encode a cytochrome P450 monooxygenase (CYP90A1) responsible for C-23 hydroxylation of in the steroid side-chain. CYP90A1 is a key enzyme in the both branches of the pathway, therefore it has the potential to influence the rate of BR biosynthesis.

Earlier results of our research team demonstrated that the *CPD* gene is expressed in a BR-regulated manner. This end product-dependent feedback control takes place primarily at the transcriptional level. Therefore, *in planta* BR homeostasis can be maintained through the control of *CPD* gene activity. Furthermore, *CPD* also shows characteristic temporal and spatial expression patterns, suggesting that BR-biosynthesis is dependent on developmental stage- and n the tissue-specific regulation.

Several genes involved in BR-biosynthesis have been identified and all of them have been found to encode members of two cytochrome P450 monooxygenase families, namely CYP90 and CYP85. In *Arabidopsis*, in addition to CYP90A1 and the C-22-hydroxylating CYP90B1, two further CYP90s: CYP90C1 and CYP90D1 of unknown enzymatic functions have been identified. C-6 oxidation of the steroid B ring is carried out by CYP85A1. A second *Arabidopsis* CYP85, CYP85A2 has also been identified, but until very recently its function has remained obscure.

Despite the increasing amount of knowledge about BR-biosynthesis, the molecular mechanisms regulating its functioning are still unknown. The main questions about the localization of BR-synthesis and its impact on the hormone level remain to be answered. The relationship between biosynthetic gene activities and the efficiency of steroid hormone synthesis also needs to be elucidated.

The pathway of BR biosynthesis. Black arrows indicate conversion steps which are known, or assumed to be, catalyzed by cytochrome P450 enzymes. The symbols of identified P450s and their genes are indicated next to the arrows.

RESEARCH OBJECTIVES

The main goal of our work was the expressional characterization of BR-biosynthetic genes in order to obtain information about the coordination of synthesis steps and their effect on the endogenous hormone level. In particular, we wanted to answer to the following questions:

1. Are the BR-biosynthetic gene activities influenced (and if yes, to what extent) by hormonal, developmental and organ-specific factors? How uniformly individual genes respond to these conditions?
2. Do endogenous brassinosteroid intermediates show differential organ-specific distribution within the plant? If yes, how do these differences correlate with those of the biosynthetic gene activities?
3. What kind of mechanism(s) can be responsible for the regulation of BR-biosynthetic genes, especially for the feedback control of *CPD*?

MATERIALS AND METHODS

- Plant material and growth conditions
- Phytohormone treatments
- RNA isolation
- mRNA analyses by reverse transcription-PCR (RT-PCR) assays

- mRNA analyses by Northern-hybridization
- Quantitative determination of endogenous BR levels
- Isolation of T-DNA-tagged mutants deficient in CYP90 functions

RESULTS AND DISCUSSION

1. Structural analysis of the *Arabidopsis* CYP90s and CYP85s at the protein and gene level revealed a close phylogenetic relationship between the two P450 families, implying that they probably diverged following their specialization to BR substrates. Their amino acid sequence identity levels show that they are also closely related to the CYP88 which includes enzymes participating in gibberellin biosynthesis. By contrast, CYP90 and CYP85 enzymes show only the minimal, P450-specific amino acid sequence identity with two BR-catabolizing members of the CYP72 family. Comparison of the exon-intron organization patterns of the same cytochrome P450s gave a very similar picture about their relationship.

2. The functions of *Arabidopsis* CYP90A1, CYP90B1 and CYP85A1 have been known, but the roles of other CYP90 and CYP85 family members (CYP90C1, CYP90D1 and CYP85A2) were to be clarified. Using semiquantitative RT-PCR assays we showed that the expression of *CYP90* and *CYP85* genes are, as in the case of *CPD*, feedback regulated by BL. Because the extent and kinetics of BR-regulation was the same as in the case of *CPD*, we proposed that these genes are controlled by the same transcriptional mechanism, ensuring coordinated expression of the BR-biosynthetic genes. In contrast to wild-type plants, the BR-deficient *cpd* and *cbb3* mutants accumulate high levels of the *CYP90* and *CYP85* transcripts. This

shows that the feedback regulation is important for maintaining of BR homeostasis, because at physiological hormone levels it can both increase and decrease biosynthetic gene activities. We also showed that the feedback control is dependent on the BRI1 BR-receptor, because in the BR-insensitive *bri1-2*/*cbb2* mutant CYP90 and CYP85 genes are not down-regulated by BL.

3. RT-PCR assays of the *Arabidopsis CYP90* and *CYP85* transcripts revealed that all members of these gene families show high activity at the early stages of development. *CYP90* and *CYP85* mRNA levels showed rapid increase during the first week of plant life, including germination and early seedling stage. During this period transcript amounts reached a peak, then they receded to about 10% of the maximum value by the end of the 7th day. Thereafter no appreciable changes of *CYP90* and *CYP85* gene activities were detected until the end of the second week. The detected Increase in *CYP90* and *CYP85* expression during the first week of growth is in good agreement with the high demand for BRs during this developmental period. Minor differences between the onset and duration of the transient induction suggest that in this respect *CYP90* and *CYP85* genes are not governed by a simple, uniform regulatory system.

4. The mRNA analysis also uncovered that the expression of *CYP90* and *CYP85* genes (except for *CYP90B1*) follow a tissue-specific pattern. *CYP90A1* and *CYP85A2* are mainly expressed in the photosynthetic portion of the plant, whereas *CYP90C1*, *CYP90D1* and *CYP85A1* transcripts preferentially accumulated in the roots. This distribution pattern was largely independent of developmental stages, but the differences between the shoots and roots transcript levels were more prominent

in young seedlings. It is interesting to note that, in contrast to the BR-biosynthetic genes, the sterol-biosynthetic genes *DIM1* and *DET2* were ubiquitously expressed in *Arabidopsis*. Our data suggest that the spatial regulation pattern of *CYP90* and *CYP85* genes is independent of the feedback control mechanism, because (i) in the case of *CYP90A1* tissue-specific expression is maintained in the *bri1-2* BR-insensitive mutant, (ii) in wild-type plants the low root transcript level can be further decreased by BL, and (iii) the uniformly feedback-regulated *CYP90* and *CYP85* genes show individual differences in their expression patterns.

5. Based on the aforementioned results, we hypothesized that the spatial expression pattern of the BR-biosynthetic genes may influence the in planta distribution of BR intermediates. Therefore we carried out gas chromatography-coupled mass spectrometry (GC-MS) analyses to quantitate the BR intermediates in the shoots and roots of *Arabidopsis*, pea and tomato. We showed that, despite minor variations, the distribution pattern of BRs followed the same trend in each of the three analyzed species. Accordingly, the spatial regulation of BR-biosynthesis seems to be ensured by a control system that is well-conserved among higher plants. Intriguingly, early biosynthetic intermediates accumulated especially in the roots, whereas later intermediates, closer to BL, were more abundant in the shoots. One explanation of this phenomenon can be that, due to their high BR-sensitivity, roots cannot tolerate elevated levels of highly hydroxylated (and presumably biologically active) BR compounds. Remarkably, the substrate of CYP90A1/CPD is present at high amounts in the roots. Because the *CPD* gene is weakly expressed in the roots, the accumulation of its substrate in this tissue suggests that the rate of CPD transcription

is an important factor in determining the activity of CYP90A1, and also of net BR-synthesis, within the plant.

6. Structural features and BR-dependent expression of CYP90C1, CYP90D1 and CYP85A2 of hitherto uncharacterized functions strongly implicate these enzymes in BR biosynthesis. On the other hand, at least two conversion steps of the BR pathway are thought to be catalyzed by yet unidentified cytochrome P450s, for which these enzymes could be good candidates. Earlier this year the catalytic function of CYP85A2 was established and, just as CYP85A1, it was found to be a C-6 oxidase.
In order to clarify the possibly redundant functions of CYP90C1 and CYP90D1, we conducted a PCR screen of Dr. Csaba Koncz's T-DNA-tagged *Arabidopsis* mutant collection at the Max Planck-Institut in Cologne, and isolated mutants carrying insertions in the *CYP90C1* and *CYP90D1* genes. Homozygous lines of these mutants are now being crossed in order to obtain *cyp90c1xcyp90d1* double mutant which are expected to show the characteristic BR-deficient dwarf phenotype. Through characterization of the double mutant we will be able to clarify the enzymatic function of the CYP90C1 and CYP90D1 monooxygenases.

7. We demonstrated that BR-dependent repression of the *CYP90* and *CYP85* genes is different from that of the *Arabidopsis BRH1* gene that encodes a RING-finger regulatory protein. The activity of BR-biosynthetic genes could be down-regulated by BL treatment to one tenth of the untreated control value, and the hormone response required *de novo* protein synthesis. By contrast, after BL treatment the transcript level of *BRH1* decreased only to one third of the initial amount. Because the repression cannot be abolished by protein synthesis inhibitors,

the down-regulation of *BRH1* by BRs can be considered a primary hormone response.

CONCLUSIONS

Our results demonstrated that the cytochrome P450s participating in BR-biosynthesis are regulated by a complex transcriptional control mechanism. Our mRNA analyses revealed that *Arabidopsis CYP90* and *CYP85* genes are subject to an end product-dependent feedback-regulation, and they also show specific temporal and spatial expression patterns. Correlation between the *CYP90* and *CYP85* gene activities and the accumulation of endogenous BR intermediates led to the conclusion that transcriptional regulation plays an essential role in controlling the efficiency of BR-biosynthesis, determining its temporal and tissue-specific organization. Our data can be instrumental for determining the developmental periods and actual sites of intense BR synthesis. In addition, our results suggest that the yet uncharacterized CYP90C1 and CYP90D1 enzymes are also involved in BR-biosynthesis. The isolation of the *cyp90c1* and *cyp90d1* mutants provides a means for the identification of CYP90C1 and CYP90D1 functions in BR-biosynthesis. The identification of BR-biosynthetic genes and mechanisms controlling their activity can open the way for further studies aimed at clarifying the mechanism through which environmental and endogenous factors control steroid hormone biosynthesis.

PUBLICATIONS ON WHICH THE DISSERTATION WAS BASED

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