Diurnal regulation of brassinosteroid biosynthesis and perception

Ph.D. thesis

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INTRODUCTION

Brassinosteroids (BRs) are polyhydroxylated steroids that are ubiquitous in vascular pants. In addition to their strong growth promoting effect, BRs also control important developmental processes, such as photomorphogenesis, germination, fertility, and stress resistance. Due to their essential regulatory role and widespread occurrence, BRs have been recognized as an independent family of plant hormones. Like auxins and gibberellins, they have a major role in growth promotion. But unlike these other hormones, BRs act at, or very close to, the sites of their synthesis.

By now the the pathways of BR synthesis are well known, and most of the genes encoding the biosynthetic enzymes have been identified. BRs are synthesized from abundant phytosterols via a series of oxidative reactions that are catalyzed by cytochrome P450-type monooxigenases, which belong to the CYP85 or CYP90 families. Local BR levels are thought to be dependent on the abundance of the enzymes that catalyze rate-limiting reactions in the synthesis route. In *Arabidopsis thaliana* these are encoded by *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*CPD*) and *CYP85A2*. The characterization of these genes has shown that their expression is controlled primarily at the transcriptional level, and involves developmental, organ-specific and end product-dependent negative feedback regulation.

Plant development is determined by the interaction between endogenous and environmental cues. Among the latter, light is the most important, which is not only the basis of photosynthetic metabolism, but also a coordinator of development from germination to seed production. Regular daily changes in light conditions result in substantial rearrangements of biochemical functions. Preparation of the plant to changes in the light conditions is greatly facilitated by the endogenous circadian clock of the plants. Circadian timing is based on light signals, and allows the organism to adapt its functions properly in anticipation of the upcoming changes of the environment. Following entrainment, circadian regulation can maintain the endogenous rhythm for

several days in constant environment. Direct (or acute) light regulation is mediated by photoreceptors, such as phytochromes and cryptochromes. To the tissue and organ level, light and circadian control is exerted mainly via the action of phytohormones. The roles of some hormone groups (e.g. gibberellins or ethylene) in relaying diurnal regulation are well documented. Our project stemmed from the preliminary results that indicated diurnal expression patterns of the BR-biosynthetic *CPD* and *CYP85A2* genes.

OBJECTIVES

The aim of our work was to identify the main environmental and endogenous cues that contribute to the diurnal regulation of the *CPD* and *CYP85A2* genes. To this end, we set forward to the following goals:

(1) Characterization of the daily expression patterns of these two genes, and identification of the mechanisms ensuring their diurnal regulation.

(2) Clarifying the role of light in the regulatory processes, and defining the signaling route(s) that mediate light effect.

(3) Finding out the relationship between the mechanisms of diurnal regulation and the BR-dependent feedback control of *CPD* and *CYP85A2*.

(4) Determining if diurnal changes in the expression of BR-biosynthetic genes are associated with daily changes in the level of bioactive BRs.

METHODS

- Construction of luciferase-based reporter transgenes
- Generation and maintenance of transgenic *Arabidopsis* lines
- In vivo measurements of bioluminescence
- Isolation of total RNA
- Determination of transcript abundance by reverse transcription-based PCR (RT-PCR)
- Northern-blot analysis
- Isolation and quantitative analysis of BR content

RESULTS

(1) We followed the expression of *CPD* and *CYP85A2* promoter-driven luciferase reporter in transgenic Arabidopsis using in vivo imaging by CCD camera. Under Light/dark regimes (LD), *CPD* expression exhibited a biphasic diurnal profile, with maxima coinciding with the onset and the end of the light periods. A very similar type of activity was observed with *CYP85A2*, providing strong indication for the involvement of common regulatory mechanisms in the diurnal control of the two key BR biosynthetic genes. In order to test the reliability of the reporter-based monitoring system, we also measured the daily activity of the *CPD* gene by directly determining its mRNA levels using RT-PCR assay. Parallel changes of the luciferase activity and the transcript amounts of the endogenous *CPD* confirmed that the promoter:reporter allow precise monitoring of *CPD* promoter activity.

(2) In order to clarify the role of light in the diurnal regulation, we measured the luminescence of the *CPD:LUC*-carrying seedlings under constant light conditions. We found that in continuous light *CPD* activity followed a circadian oscillation with maxima in the subjective night. This expression pattern was maintained for several days. By contrast, in continuous dark the activity of *CPD* rapidly decreased, so that its circadian cycling was lost after two days. Because under LD the expression of *CPD* showed sharp maxima, and these coincided with the changes of light regimes, these indicated a regulatory role for light in controlling *CPD* transcription.

(3) In the diurnal profile of *CPD* activity the minima and maxima of the circadian oscillation could be easily recognized. Therefore we concluded that the diurnal pattern *CPD* expression is under dual control: by a basic circadian rhythmicity, and a superimposed positive light regulation.

(4) To determine the spectral specificity of light regulation, we measured the diurnal profile of *CPD* activity under red/dark and blue/dark photoperiods. The monochromatic light sources used were specific for the absorption of the phytochrome and cryptochrome photoreceptors, respectively. These experiments revealed that red light alone was sufficient to maintain the diurnal expression of *CPD*. By contrast, in blue/dark a quick dampening of the gene activity was observed, which was similar to

the one seen in continuous dark. These data indicated a major role for the phytochrome photoreceptors in the light regulation of *CPD*, therefore we measured *CPD:LUC* expression in phytochrome-deficient *phyAphyB* background. We found that deficiency in the two most abundant phytochromes substantially decreased the activity of the transgene, and that its expression profile in the double mutant was largely determined by the circadian regulation. These results suggest the primary importance of phytochrome signaling in the control of *CPD* activity.

(5) Because daily changes in the BR content can result in differential *CPD* activity via the hormonal feedback regulation, we checked the relationship of this control mechanism with those responsible for the diurnal expression. To this end, we measured *CPD:LUC* activity in the BR-insensitive *bri1* mutant background which lacks the BR receptor. We observed that BR-insensitivity did not abolish the diurnal regulation of *CPD*, but in continuous dark it prevents the repression of the gene activity. This suggests that the repression is mediated by BR hormone action.

(6) We determined the endogenous BR content of *Arabidopsis* seedlings during a 24 h LD cycle to find out whether the levels of bioactive BRs show diurnal changes. Gas chromatography-coupled mass spectrometric analyses revealed a sharp transient accumulation of brassinolide, the biologically most active BR, in the middle of the light period. Remarkably, this increase follows the morning light induction of the BR-biosynthetic genes.

(7) The BR-dependent repression of *CPD* expression in the dark suggested that this might be the result of BR accumulation in the plants. Therefore we measured the BR content of seedlings following a 48 h dark incubation. The results of the GC-MS analysis showed no increase in the BR content compared to the LD control. This indicates that the observed exaggerated BR response is due to the increase of BR-responsiveness, rather than that of the BR content.

(8) Our data revealed that the repression of *CPD* activity in prolonged dark is mediated by the BZR1 transcription factor. In line with this, we found no dark repression in *mCPD:LUC* transgenic plants that carried a mutant version of the *CPD* promoter with inactivated BZR1 binding site. These data confirm that the repression of *CPD* in the dark is controlled by the BR signaling pathway.

CONCLUSIONS

The *CPD* and *CYP85A2* genes, which encode key enzymes of BR synthesis, are under diurnal regulation. This control mechanism acts primarily at the transcriptional level and consists of a basic circadian oscillation, and a superimposed light regulation.

The light induction of *CPD* and *CYP85A2* is mediated by phytochrome-dependent signaling pathways.

The diurnal expression of *CPD* is independent of the hormonal effects of BRs, but the repression of gene activity in extended dark requires BR signaling. The suppression of *CPD* activity in the dark is the result of increased BR sensitivity.

The amount of bioactive BRs also shows diurnal fluctuation, which is in good agreement with the expression patterns of the biosynthetic genes during the day.

LIST OF PUBLICATIONS

Bishop G, Nomura T, Yokota T, Montoya T, Castle J, Harrison K, Kushiro T, Kamiya Y, Yamaguchi S, Bancos S, Szatmári A-M, Szekeres M (2006) Dwarfism and P450-mediated C-6 oxidation of plant steroid hormones. Biochem Soc Trans 34: 1199-1201

Publicatons on which the dissertation is based:

Bancos S, Szatmári A-M,, Castle J, Kozma-Bognár L, Shibata K, Yokota T, Bishop GJ, Nagy F, Szekeres M (2006) Diurnal regulation of the brassinosteroid-biosynthetic *CPD* gene in Arabidopsis. Plant Physiol 141: 299-309 (with shared first authorship)

Ohnishi T, Szatmári A-M, Watanabe B, Fujita S, Bancos S, Koncz C, Lafos M, Shibata K, Yokota T, Sakata K, Szekeres M, Mizutani M (2006) C-23 hydroxylation by *Arabidopsis* CYP90C1 and CYP90D1 reveals a novel shortcut in brassinosteroid biosynthesis. Plant Cell 18: 3275-3288 (with shared first authorship)