Regulation of the genes involved in brassinosteroid biosynthesis

Ph.D. theses

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Abbreviations

aa	amino acid
BL	brassinolide
bp	basepair
BR	brassinosteroid
BRI	"BRASSINOSTEROID INSENSITIVE"
BRH	"BRASSINOSTEROID-RESPONSIVE RING-H2"
CBB	"CABBAGE"
cDNA	complementary DNA
CPD	"CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM"
DWF	"DWARF"
EDTA	ethylene-diamine tetraacetate
GC-MS	gas chromatography-coupled mass spectrometry
GUS	*Escherichia coli* β-glucuronidase
PCR	polymerase chain reaction
ROT	"ROTUNDIFOLIA"
rpm	rotation per minute
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl-sulfate
XET	xyloglucan endotransglycosylase

1 INTRODUCTION

The first member of brassinosteroids, steroidal regulators of plant growth and development, was identified only 24 years ago. Since then these compounds have been intensely studied, and by now they are widely accepted as a distinct class of *bona fide* phytohormones. Together with auxins and gibberellins, they participate in the concerted control of elongation, and are indispensable for the proper photomorphogenesis and fertility of plants.

The hormonal function requires precise adjustment of local brassinosteroid concentrations, and the synthesis of these compounds plays an important role in this process. Earlier work in our laboratory revealed that expression of the *Arabidopsis CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*CPD*) gene, encoding a key enzyme of brassinosteroid biosynthesis, is feedback-regulated by brassinolide, the end product of the pathway. This indicated that *de novo* synthesis and the level of steroid hormones could be influenced by the control of biosynthetic gene activity. By now several other genes encoding enzymes of the brassinosteroid pathway have been identified, but the factors determining their expression have remained unknown.

The main purpose of the present study was to clarify how the genes involved in steroid hormone synthesis are regulated, and whether endogenous brassinosteroid concentrations are affected by their differential expression. In particular, we wanted to answer the following main questions:

(1) Are any of the biosynthetic genes, other than *CPD*, regulated by brassinosteroids?

(2) Do these genes have developmental or spatial expression patterns that could provide information about the timing and sites of synthetic activity?

(3) Do endogenous brassinosteroid intermediates show differential organ-specific distribution within the plant, and if yes, do these variations correlate with those of the biosynthetic gene activities?

(4) What kind of mechanisms can be responsible for the regulation of brassinosteroid-biosynthetic genes, and especially for the feedback control of *CPD*?

We expected that answering these questions will bring us closer to understanding how the expression of biosynthetic genes can influence *in planta* levels of brassinosteroids, and how these genes can be regulated by the steroid hormone environment.

The work described in the theses was done primarily in our laboratory. The screening of Csaba Koncz's T-DNA-tagged *Arabidopsis* mutant collection and the identification of the mutants were carried out at the Max Planck-Institut für Züchtungsforschung, Köln. The isolation and characterization of the *Arabidopsis BRH1* gene, except for the mRNA analyses, were Gergely Molnár's work. Endogenous brassinosteroid contents were analysed by Takahito Nomura at Takao Yokota's laboratory (Department of Biosciences, Teikyo University, Utsunomiya).

2 HISTORICAL BACKGROUND

The sessile nature of plants requires that they make fine, responsive adjustments of their growth in order to adapt to environmental conditions, and to optimize their use of limited resources. At the level of tissues and organs, plant reactions to environmental factors are coordinated by phytohormones. Changing hormone levels can modulate cellular events which determine division, elongation and differentiation. Early studies indicated that, in addition to auxins and gibberellins, brassinosteroids (BRs) also have an important growth-promoting effect during plant development. The turning point in BR research was 1979 when Grove et al. isolated and determined the chemical structure of the first biologically active BR which they named brassinolide (BL). Their discovery stimulated research efforts leading to the identification of more than 40 BRs, the characterization of their effects on plants, and the deciphering of the BR-biosynthetic pathway. The isolation of the first BR-deficient and insensitive mutants (Clouse et al., 1996; Li et al., 1996; Szekeres et al., 1996) resulted in a major breakthrough in defining the biological functions and physiological significance of plant steroid hormones. The fact that BRs, but not any other phytohormone, can restore the wild phenotype of BR-deficient mutants proved the indispensable role of these steroidal regulators in plant growth and development. Today BRs are recognized as a distinct class of phytohormones, just as auxins, cytokinins, gibberellins, ethylene and abscisic acid (Yokota, 1997; Müssig and Altmann, 1999).

2.1 Structural features of BRs

The term BRs collectively refers to the growth-promoting steroids found in plants. They are structurally very similar to the insect molting hormone ecdysone, but BRs also

have unique structural features. It was proposed by Bishop and Yokota (2001) that BRs could be defined as steroids with a 5α-cholestane skeleton, carrying oxygen-containing substituents at C-3, and one or more additional ones at the C-2, C-6, C-22 and C-23 positions. (Carbon numbering of steroid molecules is given in Fig. 1).

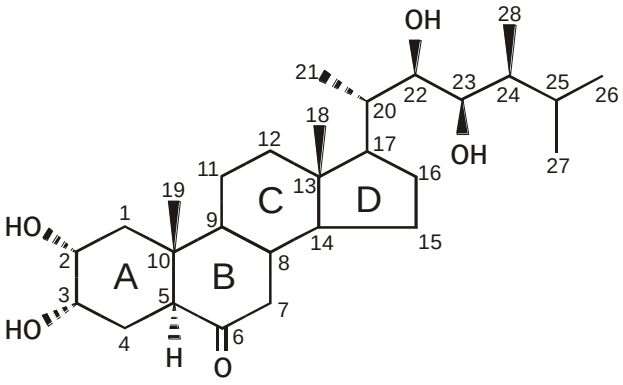


Figure 1. The structural formula of castasterone, a typical BR, with the numbering of carbon atoms and letters designating the steroid rings.

Several bioassays, including the rice lamina inclination test and the bean second-internode assays, have been used to determine the bioactivity of naturally occurring and chemically synthesized BRs (Takatsuto and Yokota, 1999). The most active naturally occurring BR is brassinolide (BL), the end product of the BR-biosynthetic pathway (Mandava, 1988). In contrast to the structure of its precursors BL, by rational nomenclature (2R-23R-24S)-2α-3α-22-23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one, has a lactonized heptameric B ring.

It is worth noting here that feeding and mutant rescue experiments could not reveal if any of the BR-biosynthetic intermediates are biologically active on their own, or they first need to be converted to BL in order to become effective. Therefore their activities measured in bioassays may actually reflect the rate of their uptake and *in planta* conversion to BL. Besides BL, only castasterone is thought to be a bioactive BR (Yokota, 1997).

2.2 Natural occurrence of BRs

On the basis of numerous studies it is now widely believed that BRs are ubiquitous in vascular plants. These steroids have been detected in more then 30 species, including gymnosperms, angiosperms, one green alga (Chlorophiceae) and one fern (Equisetaceae) (Fujioka and Sakurai, 1997). Sterol precursors of BRs, such as campesterol and campestanol, were found at micromolar to millimolar concentrations in plant tissues. By contrast, BRs occur only at nanomolar levels, even in pollen and seeds where they tend to accumulate at the highest level. Castasterone, the direct precursor of BL, was found in a wide variety of plants, while the less abundant BL was not detectable in many of the same species (Fujioka, 1999).

2.3 Physiological effects of BRs

The physiological role of BRs is distinct from, but well coordinated with, those of auxins and gibberellins. Each of these growth-promoting hormones contributes on its own to a complex regulatory system influenced by environmental stimuli, such as light, temperature, stress factors, etc. Overlapping action spectra of these growth regulators presented a major obstacle in delimiting the precise function of BRs in developmental processes.

The effects of naturally occurring and chemically synthesized BRs were tested on various types of explants, as well as on intact plants. BRs have been shown to induce a broad spectrum of responses, such as promotion of cell elongation and division, stem elongation, leaf expansion, xylem differentiation, pollen tube growth, activation of proton

pump and ethylene biosynthesis, and inhibition of root growth (reviewed by Clouse and Sasse, 1998). In field tests useful agricultural applications have been discovered, like increasing crop yield and improving stress resistance (Mandava, 1988). It was proved that BRs act synergistically with auxins in growth promotion, additively with cytokinins in inducing cell division, and antagonistically to abscisic acid in regulating leaf senescence (Mandava et al., 1998). These cross-talks between the phytohormone signaling pathways are necessary for inducing the diversity of responses that are needed for the proper control of plant development under continuously changing environmental conditions.

2.4 **The pathway of BR biosynthesis**

The main routes of BR biosynthesis have been elucidated through extensive step-wise feeding studies, performed with radiolabeled BR intermediates, in suspension cultures of *Catharanthus roseus* (Suzuki et al., 1993; Fujioka et al., 1995). BRs are synthesized from abundant membrane sterols. These plant sterols derive from cycloartenol via a series of methylation, reduction, isomerization and desaturation reactions. Among phytosterols, campesterol is regarded to be the direct precursor of BRs (Yokota, 1997). By now the reactions of BR synthesis are well known in *Arabidopsis*, pea and tomato. These conversion steps follow two parallel reaction routes, the early and late C-6 oxidation pathways (Fig. 2 and Appendix), depending on whether C-6 oxidation occurs before or after the introduction of vicinal hydroxyls at the C-22 and C-23 positions of the side chain (Sakurai, 1999). These two reaction branches become united before the last conversion step leading to BL.

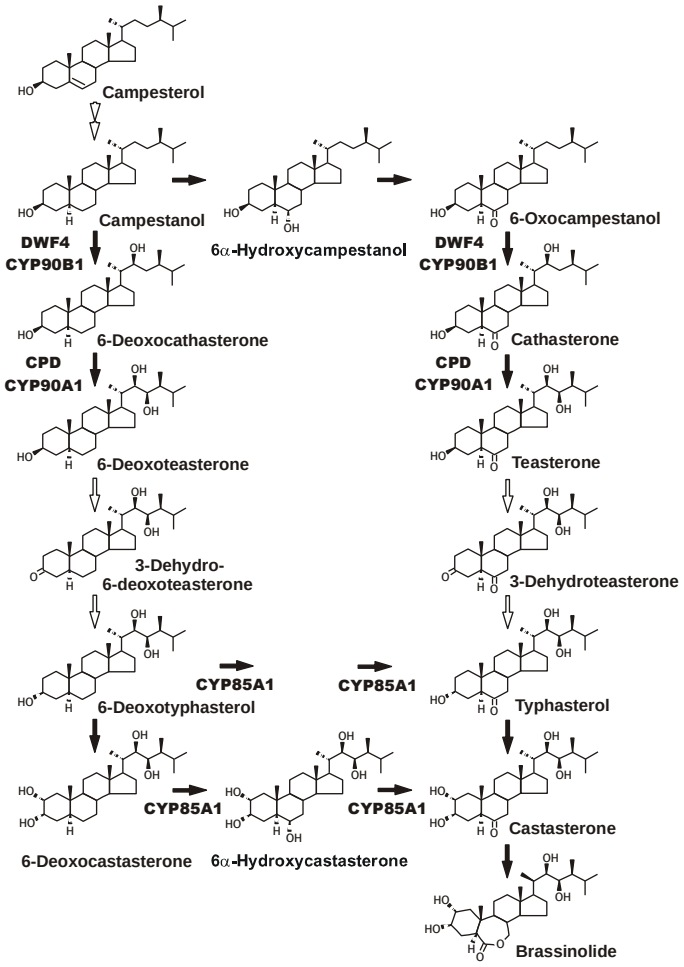


Figure 2. The pathway of BR biosynthesis. Black arrows indicate conversions which are known, or assumed to be, catalyzed by cytochrome P450 enzymes. The symbols of identified P450s and their genes are indicated next to the arrows.

2.5 **BR mutants**

The overall morphology of plants depends on the size, shape and number of their cells. These parameters are modulated by various effects. Environmental signals, such as water, temperature and light, are perceived and transduced to invoke internal hormone signals, including those of the growth-promoting auxins, gibberellins and BRs. These signals then trigger complex responses leading to cell elongation. A block in any of the signal transduction events between the environmental signals and cell elongation can result in dwarfism.

Advances in the studies of BR signaling and characterization of several biosynthetic conversion steps were due to the discovery of BR mutants, primarily in *Arabidopsis*. Most BR-related mutants have severe dwarf phenotype with short, robust stature and dark-green, round, epinastic leaves. (The typical morphology of one such mutant is shown in Fig. 3). In addition, they usually have reduced fertility and prolonged lifespan. When grown in the dark, BR-mutants display abnormal skotomorphogenesis, with a short, straight hypocotyl, open cotyledons, and the emergence of primary leaves. In addition to these morphological features, some of these mutants show increased expression levels of light-regulated nuclear genes, such as *RBCS* (encoding the small subunit of ribulose-1,5-bisphosphate carboxylase) and *CAB* (encoding the chlorophyll a/b-binding protein) (Li and Chory, 1999; Müssig and Altmann, 1999).

Based on their response to exogenously supplied BRs, these mutants can be divided in two distinct groups. In one, having impaired BR biosynthesis, the dwarf phenotype can be rescued to wild-type by treatment with BL or BR intermediates synthesized downstream

of the mutation, but not by any other plant hormones. The second group, with mutants blocked in BR perception or signaling, cannot be rescued by BL, but retain their

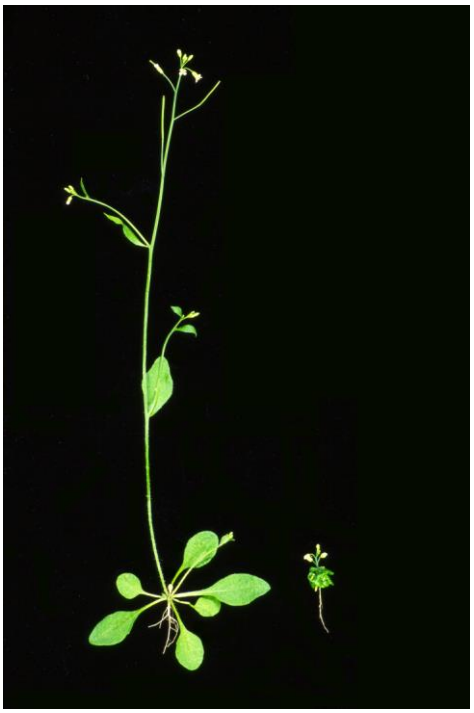


Figure 3. One month old wild-type *Arabidopsis* Columbia-0 plant (left) and the BR-deficient *cpd* mutant.

responsiveness to other types of phytohormones. Biosynthetic mutants have been crucial in uncovering the enzymatic background of BR biosynthesis, whereas the analysis of insensitive mutants was essential for identifying components of the BR signaling pathway (Clouse and Sasse, 1998; Li and Chory, 1999).

2.6 **BR perception and signal transduction**

Structural similarities between BRs and other steroid hormones led to predictions that, like animals and fungi, plants may perceive steroidal regulators by nuclear receptors

(Tsai and O'Malley, 1994). Despite these expectations, thorough analyses of the *Arabidopsis* genome sequence did not uncover genes encoding nuclear steroid receptors (Li and Chory, 1999). When the BR receptor BRI1 was identified with the help of the *bri1* (*brassinosteroid insensitive 1*) mutant (Li and Chory, 1997), it proved to be a plasma membrane-localized leucine-rich repeat (LRR) receptor-like kinase.

Repeated genetic screens for other mutations causing BR-insensitivity uncovered only multiple alleles of *bri1*, each with the characteristic phenotype of BR-deficient mutants (Li and Chory, 1997). In a recent screen, however, a further BR insensitive mutant has been identified and named *bin2* (*brassinosteroid insensitive 2*; Li et al., 2001). *BIN2* has been cloned and shown to encode a cytoplasmic serine/threonine kinase (Li and Nam, 2002). The catalytic domain of BIN2 shows sequence similarities with the *Drosophila* SHAGGY kinase and the mammalian glycogen synthase kinase-3 (GSK-3), both functioning as negative regulators of signal transduction pathways. BIN2 has been shown by biochemical and genetic analyses to be a negative regulator of BR signaling by phosphorylating, and thus inactivating, positive regulatory components (Li and Nam, 2002). More then 10 shaggy-like protein kinases are encoded by the *Arabidopsis* genome, but it is yet unknown if any of them, besides BIN2, are involved in hormone signaling (Clouse, 2002).

The activation-tagged dominant *bes1-D* (*bri1-EMS-suppressor 1-D*; Yin et al., 2002) and *bzr1-D* (*brassinazole resistant 1-D*; Wang et al., 2002) mutants exhibit constitutive BR responses, with a phenotype independent of both BR levels and BRI1 receptor activity. The highly homologous BES1 and BZR1 proteins are localized in both the cytoplasm and the nucleus. It was demonstrated that their translocation to the nucleus is rapidly and significantly enhanced by BRs (He et al., 2002). The present model of BR signal transduction is as follows: after BR perception at the plasma membrane, an

unknown number of signaling steps lead to the inactivation of the negative regulator BIN2, allowing the non-phosphorylated forms of BES1 and BZR1 to enter the nucleus. These proteins then activate the expression of BR-responsive genes by an unknown mechanism. (For a review on BR signaling see: Clouse, 2002).

2.7 **Uptake, transport and metabolism of BRs**

In combination with *de novo* synthesis, uptake, transport and metabolism can substantially influence endogenous BR levels. Therefore studies of these processes greatly contributed to our understanding of the physiological effects and mode of action of these plant hormones. The rescue of BR mutants by exogenously applied BL requires uptake of the hormone and its transport to the site of its action. In experiments using rice seedlings it was shown that BL applied to the root was transported acropetally, whereas when applied to the leaves, it was transported basipetally, but at a much slower rate (Schlagnhaufer and Arteca, 1991; Yokota et al., 1991).

Metabolism of BRs, i.e. their conversion to biologically inactive or degradable compounds, is responsible for the removal of excess amounts of the hormone. Studying the metabolism of low-concentration natural products, such as plant hormones, requires highly sensitive experimental techniques. Most often isotope-labeled hormone is used to facilitate detection of the metabolites by radiodetection methods or gas chromatography-coupled mass spectrometry (GC-MS). Such studies have revealed that, like in the case of most other phytohormones, conjugation (glucosylation or acylation) plays a crucial role in the deactivation of BRs (reviewed by Szekeres and Koncz, 1998).

In tomato cell suspension culture, 24-epicastasterone and 24-epiBL were converted to C-25 and C-26 glucosylated compounds which were subsequently degraded by side-

chain cleavage. The conjugation step was preceded by hydroxylation at the C-25 and C-26 positions by two distinct P450 monooxygenases (Winter et al., 1997). Neff et al. (1999) identified BAS1/CYP72B1, the enzyme responsible for C-26 hydroxylation in *Arabidopsis*. The expression level of this P450 was found to be extremely low under normal physiological conditions, but could be induced by excess amounts of active BRs (Choe et al., 2001). This suggests that CYP72B1 can play an important role in maintaining BR homeostasis.

By contrast, glucosylation or acylation of the C-2 and the epimerized C-3 hydroxyls, also observed in the same cell suspension system, leads to reversible inactivation of the hormone. Because BRs are highly hydrophobic, which restricts their movement across the plasma membrane, reversible glucosylation can make BRs more hydrophilic, thereby facilitating their transport within the plant. On the other hand, temporary inactivation is also advantageous when high amounts of BRs need to be accumulated, without disturbing cell functions, during seed or pollen development (Asakawa et al., 1996).

2.8 **The process of BR biosynthesis**

2.8.1 Cytochrome P450 monooxigenases

All presently known BR-biosynthetic enzymes acting downstream of campestanol in the pathway belong to the superfamily of cytochrome P450 monooxigenases (Fig. 2). Understanding their functioning in the plant steroid hormone pathway requires some background information on these proteins.

2.8.1.1 General features of the P450 superfamily

Cytochrome P450s are heme-containing enzymes catalyzing various types of oxidative reactions, generally involving hydrophobic substrates. P450s were named after their characteristic 450 nm absorption maximum detected upon binding carbon monoxide by the heme prostetic groups. Cytochrome P450s have been found in all forms of living organisms, and they share a well-conserved organization of structural domains. Most eukaryotic P450 proteins are associated with the endoplasmic reticulum, and comprise a proline-rich region immediately after the N-terminal hydrophobic helix, a C-terminal heme-binding region, and a domain involved in oxygen binding and activation. Because of their highly conservative structure, it has been proposed that all known cytochrome P450s evolved from a common ancestral protein (Nelson and Strobel, 1987). Most P450s have no strict substrate specificity, therefore these enzymes have been arbitrarily classified into families according to the degree of their amino acid sequence identity.

In plants P450s play an important role in the synthesis of several regulatory substances (e.g. jasmonic acid, auxins, gibberellins), and various secondary metabolites, including phenylpropanoids, flavonoids, terpenoids, alkaloids and cyanogenic glucosides (Chapple, 1998). The *Arabidopsis* genome encodes 273 different P450s (The *Arabidopsis* Genome Initiative, 2000). Using cDNA microarray hybridization assays, Xu et al. (2001) have shown that many *Arabidopsis* P450 genes, including those involved in BR biosynthesis, are expressed in a tissue-specific manner. Their results also indicate that most P450s are expressed at levels lower than those of housekeeping genes. This may have to do with their function, since many P450 genes are involved in the synthesis of endogenous signal molecules or various secondary metabolites. Many in this latter group of genes are expressed primarily upon induction, for example by stress conditions (Chapple, 1998).

2.8.1.2 The cytochrome P450 enzymes participating in BR biosynthesis

The cytochrome P450s involved in the BR pathway are members of either the CYP90 or CYP85 family. The first CYP90 enzyme was identified with the help of the *Arabidopsis cpd* (*constitutive photomorphogenesis and dwarfism*) mutant (Fig. 3), which is BR-deficient due to a lesion in the *CYP90A1*/*CPD* gene (Szekeres et al., 1996). (According to the nomenclature proposed by Nelson et al. in 1996, in CYP90A1 the letter following the family symbol designates subfamily A, and number 1 indicates the first enzyme within that subfamily.) CYP90A1 is a C-23 steroid side-chain hydroxylase functioning in both the early and late C-6 oxidation pathways (Fig. 2). Another CYP90 side-chain hydroxylase of the same plant, the C-22-specific CYP90B1/DWF4, was described during the characterization of the BR-deficient *dwf4* (*dwarf 4*) mutant (Azpiroz et al., 1998). In addition to CYP90A1 and CYP90B1, the *Arabidopsis* genome encodes two further enzymes of this P450 family, the functionally uncharacterized CYP90C1/ROT3 and CYP90D1. The CYP90C1-deficient *rot3* (*rotundifolia 3*) mutants show only a very mild round-leaf phenotype, therefore the role of CYP90C1 in BR synthesis could not be demonstrated by rescuing it with exogenously applied BL (Kim et al., 1998). As no mutants defective in *CYP90D1* had been isolated, this gene was discovered by a systematic analysis of P450-encoding sequences in the *Arabidopsis* genome (http://drnelson.utmem.edu/CytochromeP450.html), and its role is still unclear.

Members of the CYP85 family have been identified in tomato, *Arabidopsis*, and rice. The BR-deficient *d* (*dwarf*) mutant of tomato was shown to carry a transposon inserted in the *CYP85A1* gene encoding the enzyme responsible for the two-step oxidation of 6-deoxocastasterone to castasterone (Fig. 2; Bishop et al., 1996 and 1999). The *CYP85A1/AtBR6ox* gene of *Arabidopsis*, an ortholog of tomato *CYP85A1/D*, was cloned

and characterized by Shimada et al. (2001). Feeding experiments using radiolabeled intermediates revealed that both the tomato and *Arabidopsis* CYP85A1 enzymes have somewhat relaxed substrate specificities. These P450s could also catalyze, although at much lower rate, the C-6 oxidation of some 6-deoxo BR intermediates upstream of castasterone, but not campestanol, in yeast expression systems (Bishop et al., 1999; Shimada et al. 2001). Until now, of these reactions only the oxidation of 6-deoxotyphasterol to typhasterol could be confirmed by *in planta* conversion experiments (Noguchi et al., 2000). In addition to *CYP85A1*, systematic analyses of the *Arabidopsis* genome identified a second *CYP85* gene encoding CYP85A2, an enzyme with unclarified function (http://drnelson.utmem.edu/CytochromeP450.html).

Some other conversion steps of BR-biosynthesis, such as C-2 hydroxylation and the formation of BL by Baeyer-Villiger lactonization of the steroid B ring, are expected to be catalyzed by other, yet unidentified cytochrome P450s (Asami and Yoshida, 1999). Recently, Kang et al. (2001) have reported that a dark-induced small G protein, Pra2, can mediate light inhibition of a steroid-specific P450 in etiolated pea. This enzyme, designated CYP92A6/DDWF1 (DARK-INDUCED DWARF-LIKE PROTEIN 1), was proposed to catalyze C-2 hydroxylation BRs, converting typhasterol and 6-deoxotyphasterol to castasterone and 6-deoxocastasterone, respectively (Fig. 2; Kang et al., 2001). The significance of this enzymatic step is uncertain, because the *Arabidopsis* genome does not encode any member of the CYP92 family. Therefore, the C-2 hydroxylation reaction in this plant must be performed by a different P450 enzyme.

2.8.2 The regulation of BR biosynthesis

2.8.2.1 Rate-limiting reactions

Maintaining homeostasis of a hormone requires proper balance between its biosynthesis and inactivation. In order to gain insight into the dynamics of BR biosynthesis, all BR intermediates have been quantitated by GC-MS analyses in *Arabidopsis,* tomato and pea (Nomura et al., 2001). The accumulation of particular BR compounds implicated three rate-limiting steps in the biosynthetic pathway (Fig.2). The levels of 6-deoxocathasterone are ca. one to five hundredth of the level of its precursor campestanol in *Arabidopsis,* pea and tomato. This indicates that C-22 hydroxylation may be a rate-limiting step controlling BR biosynthesis. One of the most abundant BR compounds is 6-deoxocathasterone, but the level of the next intermediate 6-deoxoteasterone is lower by one to three orders of magnitude. Thus, C-23 hydroxylation also appears to be a rate-limiting reaction. Finally, the level of 6-deoxocastasterone is at least one order of magnitude higher than that of castasterone, suggesting that the conversion of 6-deoxocastasterone to castasterone is also important in determining the efficiency of BR biosynthesis.

The proportions of individual intermediates in the late C-6 oxidation pathway were quite similar in *Arabidopsis,* pea and tomato (Nomura et al., 2001), suggesting common control mechanisms governing biosynthesis in all these species. Because the analyzed samples were collected from plants of different ages, the data suggest that the control mechanisms of BR biosynthesis function through a considerably long developmental period. Because the flow of intermediates through rate-limiting steps depends on the

availability and activity of the respective catalytic enzymes, the pathway may be efficiently regulated at the level of their expression.

Similar conversion rates and representation of the intermediates indicate that both the early and late C-6 oxidation pathways operate efficiently in cultured cells of *C. roseus* (Fujioka et al., 2000.) However, in most plant species analyzed so far, early oxidation was found to play a subordinate role as compared to late oxidation, and in tomato the early C-6 intermediates could not be detected at all (Nomura et al., 2001). The observation that 6-oxoBRs were more actively rescuing BR-deficient mutants in the dark suggested that the early C-6 oxidation pathway may have a more significant function in the dark (Choe et al., 1998).

2.8.2.2 Regulation of *CPD* gene expression

The expression of the *Arabidopsis CPD* gene, encoding the rate-limiting CYP90A1 C-23 hydroxylase, was investigated by Mathur at al. (1998). Their data showed that *CPD* activity is stringently regulated at the transcriptional level by BL, the end product of the BR pathway. In addition, several BR biosynthesis intermediates and synthetic BR analogs efficiently inhibited the activity of the *CPD* promoter. This inhibition was concentration-dependent, and showed good correlation with the activity of the particular compounds determined by bioassays. Maximum down-regulation was detected in seedlings exposed to BL concentrations higher than 0.1 μM. The BR response was specific, because other hormonal substances, such as auxins, gibberellins, cytokinins, ethylene, jasmonate or salicylic acid, did not have an appreciable effect on *CPD* expression. However, *CPD* activity was not affected by BRs in seedlings pre-treated with the protein synthesis inhibitor cycloheximide. Inhibition of BR-mediated repression suggested that the

transcriptional response of *CPD* requires *de novo* synthesis of a protein with negative regulatory function (Mathur et al., 1998).

In addition to feedback regulation, Mathur et al. (1998) also found developmental and organ-specific variations in *CPD* activity. Histochemical analysis of transgenic *Arabidopsis* carrying *CPD* promoter-driven *GUS* reporter gene revealed highest β-glucuronidase activity in the cotyledons of young seedlings. In mature plants expression could be detected in all green organs, with strongest GUS activity in developing leaves, but no staining was detectable in the roots.

Because all rate-limiting steps of the BR pathway are catalyzed by cytochrome P450s, it seemed possible that the genes encoding these CYP85 and CYP90 enzymes could also be regulated by the same transcriptional mechanism as *CPD*. Apparently, the hormonal feedback regulation is abolished in BR-insensitive mutants, resulting in extremely high levels of active BRs in the pea *lka* and *Arabidopsis bri1* mutants (Nomura et al., 1999; Noguchi et al., 1999). This seems to indicate that intact BR perception and signaling is required for the end-product dependent self-regulation of steroid hormone synthesis.

2.9 **BR-regulated genes**

BRs, like other phytohormones, influence physiological processes by modulating the expression of specific sets of genes via distinct signaling pathways. Local levels of the hormone, concentration gradients within the plant, and different sensitivities of particular organs determine the extent of activation or repression of the target genes, establishing the molecular background of all subsequent developmental or functional effects.

One of the earliest recognized BR-regulated gene was *BRU1* (*BR UPREGULATED 1*) which encodes a soybean XET (xyloglucan endotransglycosylase) enzyme. *BRU1* expression was found to be BR-regulated during the early phase of hypocotyl elongation, at the post-transcriptional level (Zurek and Clouse, 1994). XETs most likely modify cell wall plasticity by incorporating newly synthesized xyloglucan chains during cell growth, rearranging polymer tethers to cause the cell wall loosening required for cell expansion (Fry et al., 1992). Thus, the regulation of XET expression by BRs offers a logical model to explain the molecular basis of BR-induced cell elongation. Another XET gene, the touch-inducible *Arabidopsis TCH4* (*TOUCH4*) was used for studying BR-dependent transcriptional regulation (Xu et al., 1995). *TCH4* is expressed in expanding tissues, particularly in etiolated hypocotyls and differentiating vascular elements (Xu et al., 1995). In addition to mechanical stimuli, *TCH4* also responds to changes in light, temperature, auxins and BRs. Iliev et al. (2002) demonstrated that induction of *TCH4* by the diverse stimuli of touch, darkness, cold, heat and BRs is conferred by the same 102 bp promoter region, indicating that the different signal transduction pathways inducing *TCH4* most likely share at least some common elements.

Most BR-regulated genes have been identified on the basis of altered transcript levels in BR-treated plants or BR-deficient mutants. However, sometimes it was difficult to distinguish between the primary and secondary effects caused by the hormone, or pleiotropic effects in the case of the mutants. Müssig et al. (2002) conducted a large-scale hybridization screen to detect BR-regulated genes using arrays of nearly 5000 *Arabidopsis* cDNAs (representing about 20% of the transcripts). In these assays they analyzed mRNAs isolated from mutant lines showing weak BR-deficient phenotypes. They found that, in addition to a core set of BR-regulated genes, there are several other genes which reveal BR-related activities only under certain environmental conditions (like genes involved in

cell wall modification, phytohormone signaling and response, cold and drought stress, light-signaling, etc.). This work provided the first information on the functions and approximate frequency of genes controlled by plant steroid hormones.

2.10 **Interactions between BR and light signaling**

Light is one of the most important environmental factors for plants, serving both as developmental signal and energy supply. In *Arabidopsis* there are five types of red/far-red-absorbing phytochromes (phyA-phyE), and a few types of blue/UV-A-absorbing photoreceptors (e.g. cryptochromes). The de-etiolated phenotype and de-repression of light-regulated genes in dark-grown BR mutants suggest that light and BR signaling might have common elements. These overlapping effects, and genetic analyses that have demonstrated complex interactions between photoreceptor and phytohormone functions (Chory and Li, 1997; Clouse and Sasse, 1998; Kamiya and Garcia-Martinez, 1999), imply that the growth-controlling effect of light may be achieved, at least partially, through BR signaling.

It is known that the activity or availability of some enzymes in both the biosynthesis or metabolism of BRs are affected by light. The activity of the CYP92A6/ DDWF1 C-2 hydroxylase of pea was shown to be repressed by the light-inducible small G protein Pra2 (Kang et al., 2001). On the other hand, a dominant mutant overexpressing the BR-metabolizing CYP72B1/BAS1 enzyme was isolated in a screen for suppression of phytochrome B-deficiency (Neff et al., 1999).

Thorough knowledge of the regulation of BR biosynthesis, and particularly its rate-limiting steps, would reveal if, or how, *in planta* BR levels can be manipulated. Further components of the signal transduction pathway and their target genes need to be identified

in order to clarify the position of BRs in the whole plant signaling network, and to gain insight into potential cross-talking with other signaling pathways.

3 MATERIALS AND METHODS

3.1 Plant material and growth conditions

For assaying gene expression, *in vitro* cultures of wild-type *Arabidopsis thaliana* (ecotype Columbia-0), BR-deficient *cpd* (Szekeres et al., 1996), *cbb3* and BR-insensitive *cbb2* (Kauschmann et al., 1996) mutants were grown from surface-sterilized seeds. The seeds were treated for 10 minutes with 5% calcium hypochlorite solution, then washed several times with sterile distilled water. The seeds were sown on *Arabidopsis* medium (AM): 2.16 g/l Murashige-Skoog salt-mixture (Sigma Chemical Co.), supplemented with 1% (w/v) sucrose, adjusted to pH 5.7, and supported with 0.2% (w/v) Phytagel (Sigma). In case of antibiotic selection, hygromycin (15 mg/l) was used. The plants were grown at 22˚C, under 14-h-light/10-h-dark cycles. Plants grown in the greenhouse were subjected to 16-h-light/8-h-dark cycles.

3.2 Phytohormone treatments

BR treatments were carried out in liquid AM medium supplemented with 100 nM BL (CIDtech Research Inc., Mississauga, ON, Canada), whereas hormone-free control samples received only the ethanol included in the BL stock solution (0.01%, v/v). The cultures were aerated by horizontal shaking at 50 rpm. The *Arabidopsis* plants used for organ-specific mRNA and BR analyses were grown under similar conditions in Gamborg's B5 liquid medium (Gamborg et al., 1968). Seeds of pea (*Pisum sativum* cv. Torsdag) and tomato (*Lycopersicon esculentum* cv. Sekaiichi) were sown in vermiculite; seedlings were grown in greenhouse under natural light conditions (13-h day, 11-h night). Five-day-old

pea seedlings and 22-day-old tomato plants were then grown hydroponically, in the same greenhouse, in Tadano and Tanaka (1980) liquid medium for further 10 and 14 d, respectively. After harvesting, all plant material was frozen in liquid nitrogen and kept at -80˚C until use.

3.3 **mRNA analyses**

3.3.1 RNA isolation

Total RNA was isolated from 1 g of fresh plant material using the TRI Reagent method (originally described by Chomczynski and Sacchi, 1987). After grinding in liquid nitrogen, the powder was mixed with 10 ml TRI Reagent (Sigma). Following complete lysis, 2 ml chloroform was added, mixed thoroughly, than left for 3 min at room temperature to allow the separation of the organic and aqueous phases. Following centrifugation (12,000 g, 4˚C, 15 min), the aqueous phase, containing the RNA, was transferred to a new centrifuge tube and precipitated with an equal volume of isopropanol. The precipitate was collected by centrifugation (12,000 g, 4˚C, 10 min), washed with 70%, then absolute ethanol. The RNA pellet was dried, and then re-dissolved in RNase-free distilled water. RNA concentration was determined on the basis of absorbance at 260 nm. Traces of contaminating genomic DNA were removed by treatment with RNase-free DNase (Boehringer).

3.3.2 Reverse transcription-PCR assays

Steady-state mRNA levels were analyzed by semiquantitative reverse transcription-PCR (RT-PCR) assays according to Chelly and Kahn (1994), with minor modifications. First, cDNA was prepared from 5 μg of total RNA with “Ready-To-Go" T-primed first-strand kit (Pharmacia Biotech), according to the manufacturer's instructions. One-tenth of the cDNA obtained was PCR-amplified, within the linear range of accuracy, by specific primers spanning 250 to 300 bp regions near the 3’ ends of the coding sequences. Depending on the transcript level, between 15 to 25 amplification cycles were used. For each transcript, the cDNA-specific primers and the number of corresponding cycles are given in Table 1.

Transcript	Nucleotide sequence (from 5’ to 3’; upper forward, lower reverse primer)	Number of PCR cycles
CYP85A1	CGACAGGACGAACCACTCGGT TTCCTTACCAGGACAAAGCCTTGTC	25
CYP85A2	CGACCTGACGAACCGCTCAC CTTTCCAGGGCAAAGCCTAACTC	25
CYP90A1/CPD	GAATGGAGTGATTACAAGTC GTGAACACATTAGAAGGGCCTG	20
CYP90B1/DWF4	AGCATCTTGAGATCGCGAGGGCCAA CCACGTCGAAAAACTACCACTTC	25
CYP90C1/ROT3	GGGAGAAGAATACAAGTGGAC GCAAATACTGCTGTTTGCCGATCC	25
CYP90D1	CTCCTGCTGCCCTCAATCTC TCGAGACCAGGGCACAATCTCTGAC	25
DIM1	CTCGAATGGGTCCACCGCGAAATG CATACAATTCACCATTAAACATTC	20
DET2	AATCTCCTCAATGGTTATATCCAG CGTGTACAGAAAAAATCCAATACC	20
UBQ10	GGACCAGCAGCGTCTCATCTTCGCT CTTATTCATCAGGGATTATACAAGGCC	15

Table 1. Primers used in the RT-PCR analyses and the numbers of reaction cycles.

The PCR conditions were as follows:

94ºC, 2 min;

[94ºC, 30sec; 55ºC, 30sec; 72ºC, 30 sec] x 15-25 cycles;

72ºC, 6 min.

One percent of the RT-PCR product was labeled with [α-^{32}P]-dCTP using a single detection primer that was three nucleotides longer in the 3' direction than one of the amplification primers. The fragments were separated on 2% agarose gel. The gel was dried onto Whatman 3MM filter paper, then the signals were detected by autoradiography, and quantitatively evaluated using PhosphoImiger 445SI (Molecular Dynamics Inc., Sunnyvale, CA).

3.3.3 Northern-hybridization

For the determination of *BRH1* transcript abundance, 20 μg total RNA samples isolated by the TRI reagent method were loaded on formaldehyde-containing 1% agarose gel, then subjected to electrophoresis in 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 10 mM EDTA buffer (pH 7.0). Following separation, RNA was capillarity-blotted onto Nytran N membrane (Schleicher & Schuell). The DNA hybridization probe was prepared by random-primed labeling with radioactive [α-^{32}P]-dCTP. The membrane containing the transferred RNA was first pre-hybridized for four hours in Church buffer (0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, 1% bovine serum albumin) at 65˚C. Then the labeled DNA probe was added to the Church buffer and hybridized with the membrane-bound RNA overnight at 65˚C. The filter was washed twice for 30 minutes at room temperature in 2x SSC solution (SSC: 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0),

supplemented with 0.05% SDS and 0.05% sodium pyrophosphate, then twice for 15 minutes at 65˚C in 0.1x SSC, 0.1% SDS, 0.05% sodium pyrophosphate solution. Hybridization signals were detected and evaluated as described above for RT-PCR analyses.

3.4 **Quantitative determination of endogenous BR levels**

Twenty-day-old *Arabidopsis*, 15-day-old pea and 36-day-old tomato plants were separated into shoots (130, 289 and 241 g fresh weight, respectively) and roots (115, 293 and 78 g fresh weight, respectively). BR extraction and analysis were carried out as described by Nomura et al. (2001). In brief, methanol extracts of the plant material were subjected to solvent partitioning, and purified by several conventional chromatographic steps, followed by HPLC. Quantitative analyses of BRs were conducted by GC-MS/selected ion monitoring, using a JMS AX 505W instrument (JEOL, Tokyo).

3.5 **Identification of T-DNA-tagged *CYP90* mutants**

Screening for mutants with insertion-inactivated *CYP90C1* and *CYP90D1* genes was performed using Csaba Koncz's (Max Planck-Institut für Züchtungsforschung, Köln) T-DNA tagged *Arabidopsis* mutant collection which, at the time of the screening, included 39,700 independent transgenic lines, with an average number of 2 to 3 T-DNA insertions per line. Genomic DNA isolated from individual transgenic lines (of which seed stocks were kept) were combined in basic pools of 100 samples, then these were combined to give 10 super-pools, each containing 4000 samples. For the identification of *cyp90c1/rot3* and *cyp90d1* mutants, we followed the protocol used in the Koncz laboratory (Rios et al.,

2002). In brief: the superpools were first tested for T-DNA insertion in the *CYP90C1/ROT3* and *CYP90D1* genes by long-range PCR assays with combinations of T-DNA and gene-specific primers, using La-Taq DNA polymerase (Takara Shuzo Co).

Due to T-DNA insertion in the target gene, the vicinity of T-DNA- and gene-specific priming sites permits efficient PCR amplification of the DNA segment in between. The T-DNA-specific primers were designed to prime at the left or right border of the T-DNA. These were used in combination with gene-specific primers homologous to the start and end parts of the coding sequences, oriented toward the center of the genes. The primers used were as follows:

CYP90C1	5'	GGAGAATATGGAGATGAAGAGGCGTAAATTG	(forward)
	5'	GAAGCAAATACTGCTGTTTGCCGATC	(reverse)
CYP90D1	5'	GTCATCTTCAACAAGATCAACGGTCTCAG	(forward)
	5'	GTAATTTGTTCTTCATATGCACCGTTGGGAAG	(reverse)

To find the mutant DNA-containing 100-pool within the super-pool identified in the first screen, a second round of PCR screen was performed. The amplified PCR product was isolated and, using the T-DNA-specific primer, it was sequenced to establish the insertion site within the target gene. To isolate single mutant lines, individual seed lines corresponding to the identified 100-pool were sown on hygromycin-containing AM medium. DNA was prepared from two-week-old seedlings using the method described by Rios et al. (2002), and re-assayed by PCR reaction. The primers and reaction conditions were the same as in the first round of PCR assays. Hygromycin-resistant plants from the segregating M2 generation were planted into soil for seed production. Leaf material was collected from 15 such individuals for DNA analyses. Each individual was assayed for phenotype, hygromycin resistance and the presence of T-DNA insertion. Plants carrying the gene-specific insertions were also assayed for the presence of wild-type *CYP90C1* or

CYP90D1 alleles, to identify homozygous individuals. Homozygous lines were maintained by self-pollination.

4 RESULTS

4.1 Phylogenetic relationship between the cytochrome P450 monooxygenases participating in BR biosynthesis

With the help of BR-deficient mutants, several genes involved in biosynthesis have been characterized. All such genes identified to date turned out to encode cytochrome P450 monooxigenases. The *CYP90A1*, *CYP90B1* and *CYP85A1* genes of *Arabidopsis* were functionally well characterized (Szekeres et al., 1996; Choe et al., 1998; Shimada et al., 2001). In addition, three further members of the *CYP90* and *CYP85* gene families have been identified by sequence analysis of the *Arabidopsis* genome and designated *CYP90C1*, *CYP90D1* and *CYP85A2*. (David R. Nelson's cytochrome P450 database, http://drnelson.utmem.edu/CytochromeP450.html). These genes were anticipated to encode BR-biosynthetic enzymes, but their functions were not clarified. Since we expected that their structural conservation may reflect functional similarity, we analyzed the phylogenetic relationship between the CYP90 and CYP85 proteins at the amino acid sequence level, and also between the corresponding genes on the basis of their exon/intron organization.

4.1.1 Sequence similarity analysis between the CYP90 and CYP85 proteins

For comparison of the amino acid sequences, we used the *Arabidopsis* P450 database (http://drnelson.utmem.edu/CytochromeP450.html) generated on the basis of the *Arabidopsis* genome sequence. The deduced amino acid sequences of the CYP90 and

Figure 4. Multiple sequence alignment of the *Arabidopsis* CYP90 and CYP85 proteins. The alignment was created by the PileUp program of the GCG software package. Highlighted letters indicate residues identical with those of CYP90A1. Functional domains are identified above the sequences, asterisks represent intron positions in the corresponding genes. GeneBank accession numbers are given in the brackets.

CYP85 family members showed that they possess all functional domains (i.e. the N-terminal membrane-anchor, the proline-rich sequence and the oxygen- and heme-binding domains) required for monooxigenase activity. An alignment of the CYP90A1, CYP90B1, CYP90C1, CYP90D1, CYP85A1 and CYP85A2 sequences, also indicating their structural domains, is shown in Fig. 4.

Based on the amino acid sequences of *Arabidopsis* P450s belonging to the CYP90, CYP85, and some other arbitrarily selected families, we generated a phylogenetic tree using the ClustalW multiple alignment program (Thompson et al., 1994). This tree (Fig. 5A) shows that members of the CYP90 and CYP85 families are closely related, with aa sequence identity values varying between 35% and 45%. The closest relative of CYP90A1 is CYP90B1, with 45% identity. The highly similar CYP90C1 and CYP90D1 proteins, with 55% identity, represent a distinct branch within the CYP90 group. CYP85A1 and CYP85A2, the two closely related members of the CYP85 family, share 82% sequence identity with each other, and 35% with CYP90A1. As can be seen in Fig. 5A, the CYP85 and CYP90 families cluster together with the CYP88 family (31% to 32% identical to CYP90A1), which comprises enzymes involved in GA biosynthesis (Helliwell et al., 2001). For comparison, we also indicated the position of the CYP72 family including BR deactivating enzymes (CYP72B1 and CYP72C1). These P450s, despite of using similar BR substrates, are only distantly related to the group of CYP90, CYP85 and CYP88 families (15% to 16% identity with CYP90A1).

4.1.2 Exon-intron organization of the BR-biosynthetic cytochrome P450 genes

To see if the observed phylogenetic relationship between the CYP90 and CYP85 proteins is also recognizable at the DNA level, we compared the exon-intron pattern of the

genes encoding the same P450s. The analysis of these sequences revealed that the introns separating the seven to nine exons in each of the *CYP90*, *CYP85* and *CYP88* genes correspond to eight conserved positions (Fig. 5B). By contrast, the distantly related *CYP72B1* and *CYP72C1* genes showed a different pattern of exon-intron organization. The similarities in both protein and gene structure indicate a close relationship between members of the CYP90 and CYP85 families, suggesting that their evolutionary separation occurred, possibly by gene duplication, after their specialization to steroid substrates.

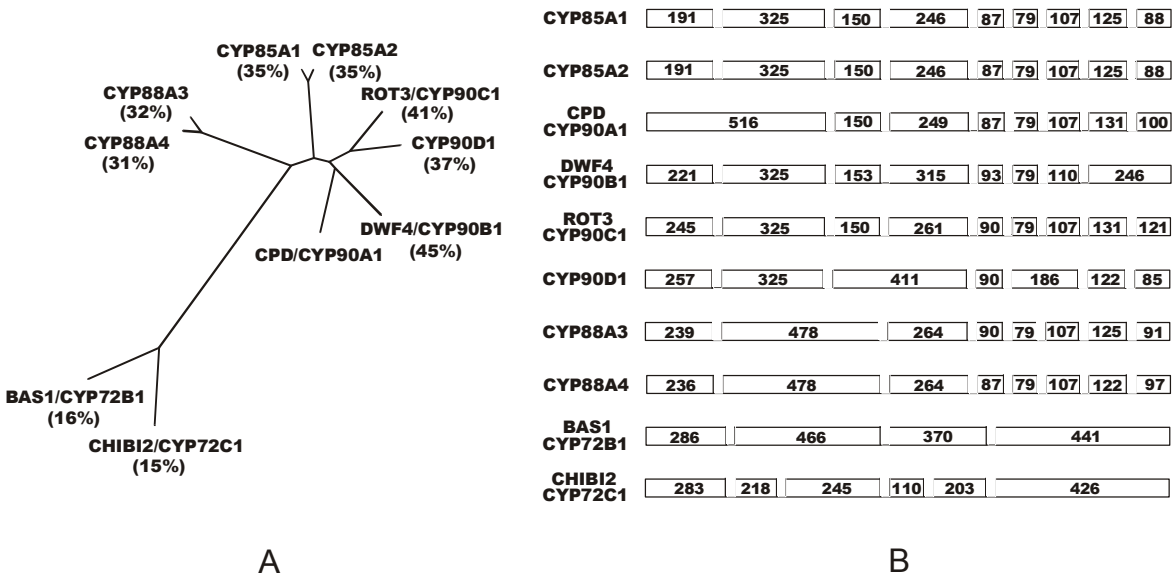


Figure 5. Structural relationship between selected *Arabidopsis* cytochrome P450 proteins and their genes. A: The unrooted cladogram, generated by the ClustalW multiple alignment program, is based on the amino acid sequence identity between P450s involved in BR biosynthesis (CYP90 and CYP85), BR-catabolism (CYP72), and GA biosynthesis (CYP88). The percentage values in brackets indicate the level of sequence identity with the CYP90A1 protein. B: Exon-intron structures of the genes encoding the same cytochrome P450s. Exon sizes are given in bp.

4.2 **Feedback regulation of the *CYP90* and *CYP85* genes**

In order to maintain BR homeostasis, i.e. balanced hormone levels optimal for growth and development, plants evolved an end product-dependent feedback regulation of

the biosynthetic pathway. Mathur et al. (1998) showed that the *CYP90A1*/*CPD* gene is negatively regulated by BL in a concentration-dependent manner. This means that, *CPD* activity can be repressed by BL treatment, but BL deficiency, for example in BR-deficient mutants, up-regulates the expression.

In order to find out if other *CYP90* and *CYP85* genes with similar BR-biosynthetic functions are also feedback-regulated, we analyzed the changes in their transcript levels in intact *Arabidopsis* plants following BL treatment. Because the abundance of these P450 mRNAs is very low, often below the detection level of Northern-hybridization, we determined their steady-state levels using semi-quantitative RT-PCR assays. As constitutive control we used *UBQ10* (*POLYUBIQUITIN 10*) mRNA, which was shown to be expressed at a constant level, from the first day of germination, under a wide variety of environmental conditions (Sun and Callis, 1997). Two hours of BL treatment reduced the amount of *CYP90A1*/*CPD*, *CYP90B1*/*DWF4*, *CYP90C1*/*ROT3*, *CYP90D1*, *CYP85A1* and *CYP85A2* transcripts to approximately 10% or less of the level detected in untreated control seedlings (Fig. 6A). These data show that the activity of all *Arabidopsis CYP90* and *CYP85* genes is down-regulated by BL, consistently with the idea of BR-dependent feedback control.

The levels of the same transcripts have also been analyzed in BR-deficient and -insensitive *Arabidopsis* mutants. We chose *cpd* and the allelic *cbb3* as BR-deficient mutants for our assays. (Because *cpd* is a null mutant lacking the *CYP90A1*/*CPD* transcript, we used the *cbb3* missense mutant to monitor the *CYP90A1*/*CPD* mRNA.) In accordance with the feedback regulation, the transcript levels of *CYP90* and *CYP85* genes were significantly higher in the mutants than in the wild-type plants. But, just as in the case of the wild-type plants, the mRNA levels of all four *CYP90* and both *CYP85* genes were

down-regulated after BL-treatment, but remained somewhat higher than in BL-treated wild-type plants (Fig. 6B).

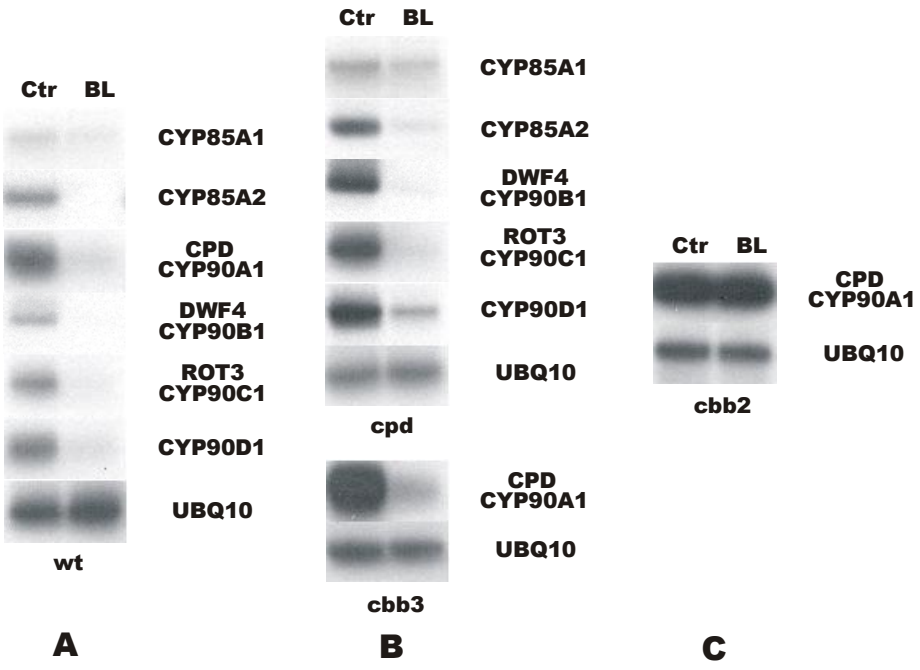


Figure 6. The effect of BL on the steady-state mRNA levels of BR-biosynthetic P450s. RT-PCR products obtained from total RNA of 7-d-old intact *Arabidopsis* seedlings incubated for 4 h in the presence (BL) or absence (Ctr) of 100 nM BL. Relative transcript levels in A: wild-type, B: BR-deficient *cpd* and *cbb3* mutants, C: BR-insensitive *cbb2* mutant. *UBQ10* was used as internal control. The PCR reactions of each panel were made with the same cDNA samples, BL and Ctr signals of each transcript were obtaied from the same gels.

In the BR-insensitive *cbb2/bri1-2* mutant, BL treatment had no effect on the expression of *CPD* (Fig. 6C), or any other *CYP90* and *CYP85* genes (data not shown). The results indicate that the BR-mediated feedback regulation of these genes is dependent on the function of the BRI1 leucine-rich repeat receptor kinase (Li and Chory, 1997), which is inactivated in the *cbb2* mutant.

4.3 **Regulation of *CYP90* and *CYP85* mRNA levels during germination and early seedling development**

Our earlier data indicated that *CPD* is expressed at a much higher level in young seedlings than in adult *Arabidopsis* plants (Mathur et al., 1998). We analyzed the transcript

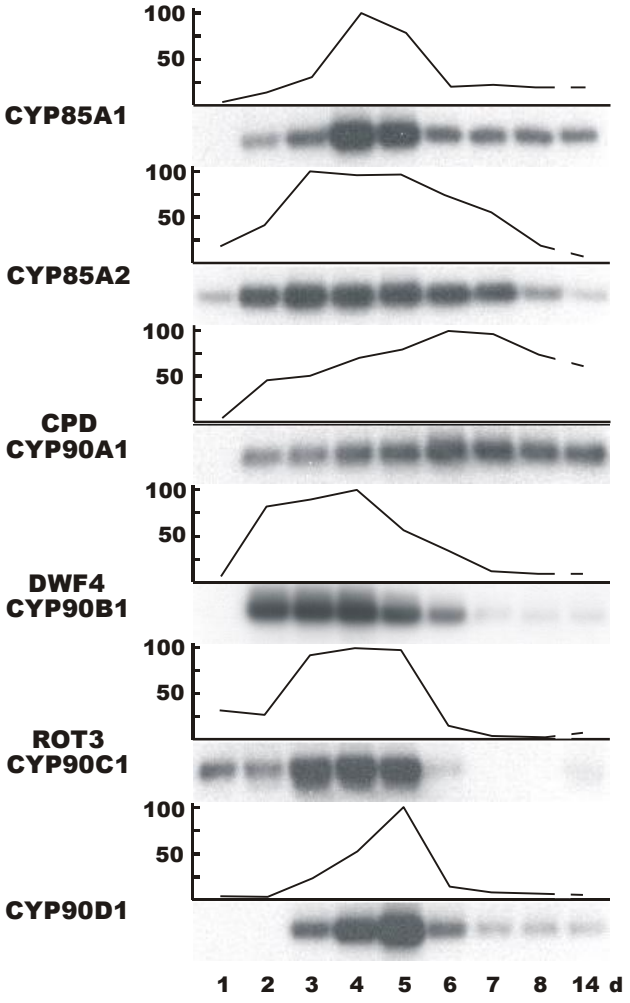


Figure 7. Changes in transcript levels of the *CYP90* and *CYP85* genes during germination and seedling development. RT-PCR products prepared from total RNA of developing wild-type seedlings and young plants (1 through 8 d, and 14 d after imbibition). Quantitative data are plotted as percentage of the highest value measured during the experimental period. The level of each transcript was assayed on the same gel.

levels of all *CYP90* and *CYP85* genes in germinating seeds and young seedlings in order to find out if they also exhibit a developmental pattern of expression.

We determined the abundance of *CYP90* and *CYP85* transcripts by RT-PCR in seedlings and young plants throughout the first 8 days following imbibition, and after two weeks of development (Fig. 7). The abundance curves show that all *CYP90* and *CYP85* genes are strongly induced during the experimental period. Although individual *CYP90* and *CYP85* genes featured some variation in their temporal profiles of expression, each of them reached a maximum of activity during the first week of development. After one week, their abundance (except that of *CPD*) declined to about 10% or less of the maximum values, and thereafter their levels remained unchanged up to the 14th day. The transient induction of *CYP90* and *CYP85* genes suggests that the regulation of BR-biosynthetic genes is well coordinated with the high demand for BRs during early plant development.

4.4 **Differential regulation of *CYP90* and *CYP85* transcripts in shoots and roots**

Using a *CPD* promoter-driven *GUS* reporter construct, Mathur et al. (1998) showed that the *CPD* promoter is more active in the aerial parts than in the roots of *Arabidopsis*. Because BR regulation of plant functions requires fine, localized adjustment of the hormone level, we were interested in the spatial expression pattern of the *CYP90* and *CYP85* genes. Their transcript levels were determined by RT-PCR assays in the shoots (representing combined cotyledon and hypocotyl tissues) and roots of seven-day-old seedlings (Fig. 8A). Our data confirmed the earlier finding that *CYP90A1*/*CPD* is preferentially expressed in the above-ground tissues. *CYP85A2* expression showed similar organ-specific pattern, but its activity was much lower than that of *CYP90A1*. The expression of *CYP90C1*/*ROT3*, *CYP90D1* and *CYP85A1* was stronger in the roots, whereas

CYP90B1 was equally expressed in both the roots and shoots. Activities of the closely related *CYP90C1* and *CYP90D1* displayed similar localization but, surprisingly, the highly homologous *CYP85A1* and *CYP85A2* genes showed opposite preferences for shoot or root expression.

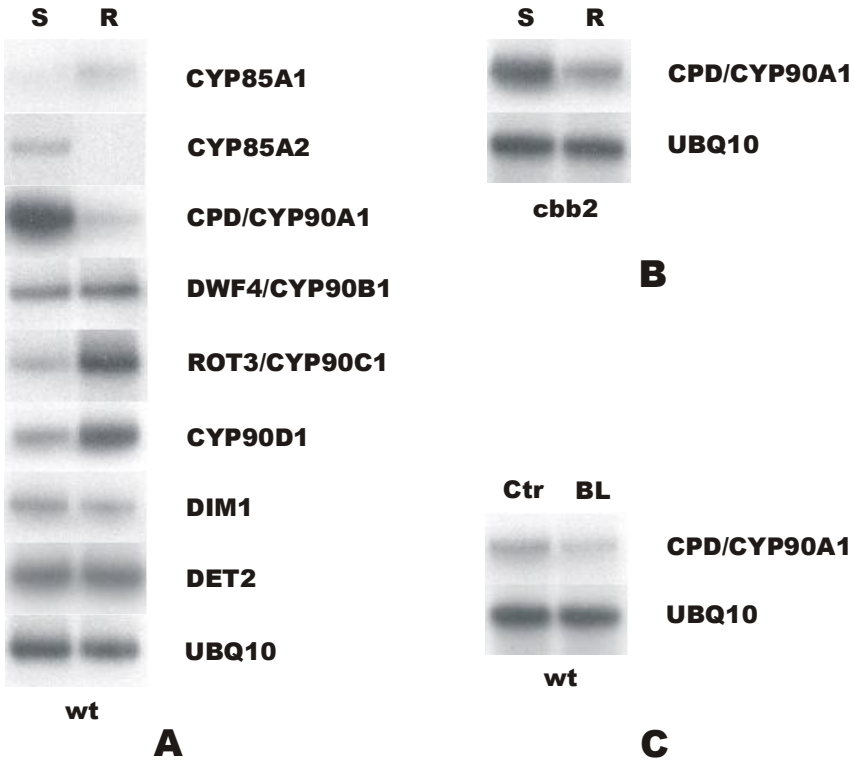


Figure 8. Differential accumulation of BR-biosynthetic P450 mRNAs in shoots and roots. A: Transcript levels in shoots (S) and roots (R) of wild-type seedlings. B: *CYP90A1*/*CPD* transcript levels in shoots (S) and roots (R) of BR-insensitive *cbb2* seedlings. C: *CYP90A1*/*CPD* transcript levels in roots of wild-type seedlings incubated for 4 h in the presence (BL) or absence (Ctr) of 100 nM BL. RT-PCR products were obtained from total RNA of 7-d-old seedlings. *UBQ10* was used as internal control. The PCR reactions of each panel were made with the same cDNA samples, S and R, as well as Ctr and BL signals of each transcript were obtaied from the same gels.

In order to see if genes of non-P450 steroidogenic enzymes, acting immediately upstream of BR biosynthesis, are differentially expressed in roots and shoots, we also determined the levels of the *DIM1* (Klahre et al., 1998) and *DET2* (Li et al., 1996)

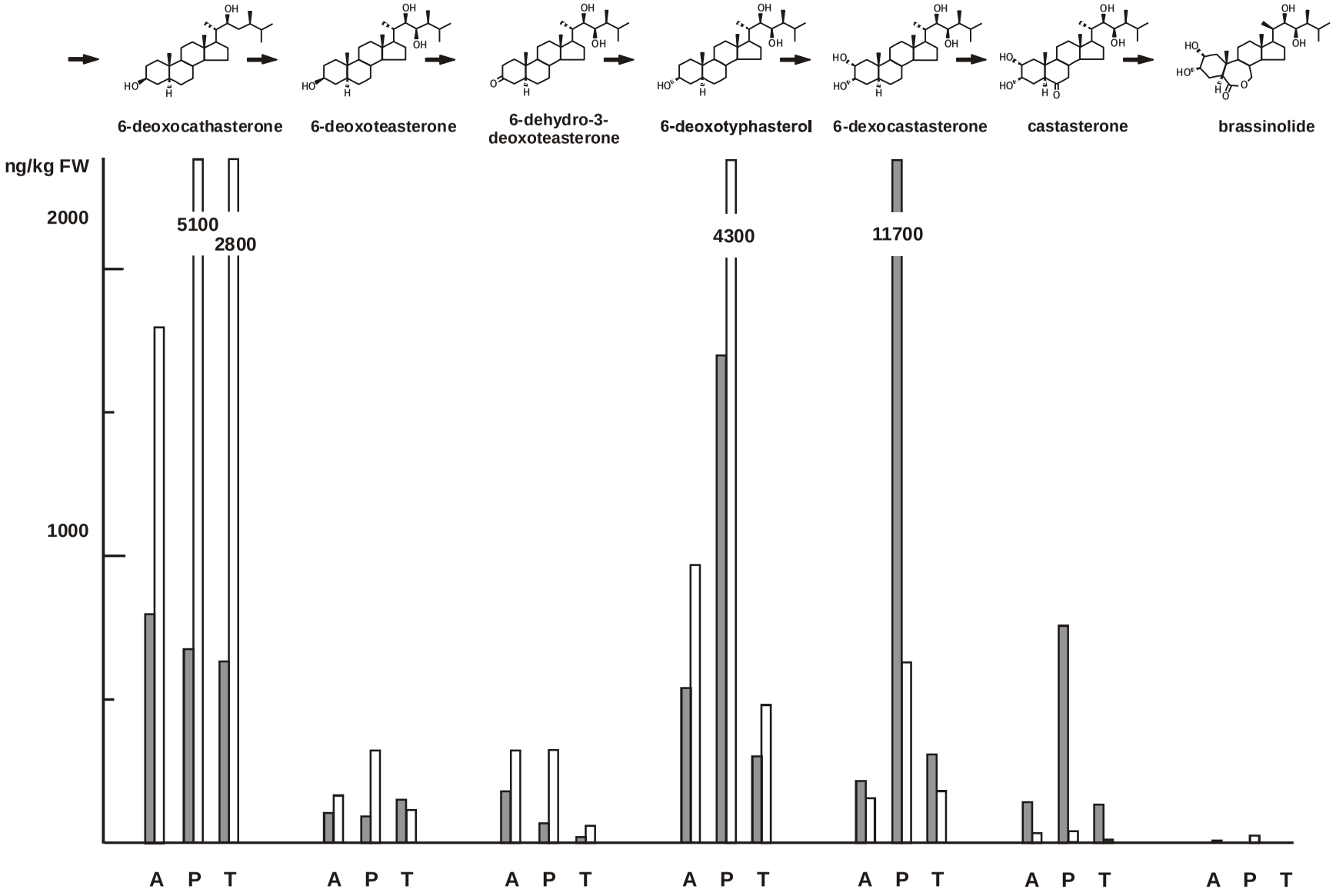
transcripts. In contrast to the *CYP90* and *CYP85* messages, the *DIM1* and *DET2* transcripts were uniformly distributed in the roots and shoots of the seedlings.

To clarify whether the organ-specific expression of BR-biosynthetic genes is dependent on hormonal feedback regulation, we assayed the transcript level of *CYP90A1* in BR-insensitive *cbb2* seedlings (Fig. 8B). Even in this mutant, the mRNA level of *CYP90A1* was lower in the root than in the shoot, in agreement with the organ-specificity found in wild-type plants. On the other hand, in wild-type roots the low level of the *CYP90A1* transcript could be further decreased by BL treatment (Fig. 8C). These data indicate that the observed difference between the shoot and root expression of *CYP90A1* is not the consequence of the hormonal feedback regulation.

4.5 **Endogenous BR levels in shoots and roots of *Arabidopsis*, pea and tomato**

The tissue specificity of *CYP90* and *CYP85* gene expression is expected to influence BR distribution in the plant. To see if this is the case, we determined the levels of endogenous BRs in the shoots and roots of *Arabidopsis* using quantitative GC-MS analysis. The data obtained were compared with those of similar analyses carried out in pea and tomato. As anticipated, these analyses detected differential distribution of the BR biosynthesis intermediates in the shoots and roots of these plants (Fig. 9). Although the levels of individual BRs showed certain variations between the three plant species the distribution patterns of the compounds studied were very similar. Intermediates of the early

Figure 9. (See next page.) Distribution of BR biosynthesis intermediates in shoots and roots of *Arabidopsis* (A), pea (P), and tomato (T). The data were obtained by using quantitative GC-MS analysis. The amounts of endogenous BRs are shown in ng per kg fresh weight of plant material. Dark columns indicate shoot and white ones, root values. Very high values are given numerically on the columns. The corresponding section of the pathway, with the late C-6 oxidation steps, is shown above the graph.


6-deoxocathasterone
6-deoxoteasterone
6-dehydro-3-deoxoteasterone
6-deoxotyphasterol
6-dexocastasterone
castasterone
brassinolide
ng/kg FW
2000
1000
5100
2800
4300
11700
A P T
A P T
A P T
A P T
A P T
A P T
A P T

C-6 oxidation route were at or below detection level in all samples. Within the late C-6 oxidation pathway, earlier intermediates (6-deoxocathasterone, 6-deoxoteasterone, 3-dehydro-6-deoxoteasterone, and 6-deoxotyphasterol) were preferentially represented in the roots, whereas 6-deoxocastasterone and castasterone were more abundant in the shoots. BL was not detectable in tomato at all, and was found only in trace amounts in the roots of pea and *Arabidopsis*.

Intriguingly, as compared to the shoots, a more than twofold higher 6-deoxocathasterone level was detected in the roots which contain low amounts of the *CPD* transcript. Because CYP90A1/CPD catalyzes the conversion of 6-deoxocathasterone to 6-deoxoteasterone in the late C-6 oxidation pathway, the accumulation of its substrate in roots indicates a low conversion rate in this organ and, hence, a good correlation between transcript abundance and the actual enzyme activity.

4.6 **Identification of *Arabidopsis* mutants carrying T-DNA insertion in the *CYP90C1/ROT3* and *CYP90D1* genes**

Our data on the structure and regulation of the yet uncharacterized *CYP90C1*/*ROT3*, *CYP90D1* and *CYP85A2* genes strongly suggested that they encode BR-biosynthetic enzymes. One strategy for determining the function of these P450s is to characterize mutants defective in these genes. To date, no *cyp90d1* or any *cyp85* mutants have been isolated in *Arabidopsis*, and the *cyp90c1/rot3* mutants did not show the phenotypic features of BR-deficiency. Because we knew that the function of CYP85A2 was already investigated at another laboratory, we set out to identify mutants in *CYP90C1* and *CYP90D1* by PCR-based screening of a T-DNA tagged *Arabidopsis* mutant collection.

In two rounds of screening we isolated T-DNA insertion mutants in both the *CYP90C1*/*ROT3* and *CYP90D1* genes. The identity of these mutants was confirmed by PCR assays. The position of T-DNA insertions was determined by sequence analysis of the specific DNA fragments obtained with the combination T-DNA- and gene-specific primers. In the mutants the *CYP90C1* gene was interrupted in the sixth exon, whereas *CYP90D1* in the third intron. Fig. 10 shows the positions of the T-DNA insertion in the isolated mutants, and the DNA sequences around the insertion sites.

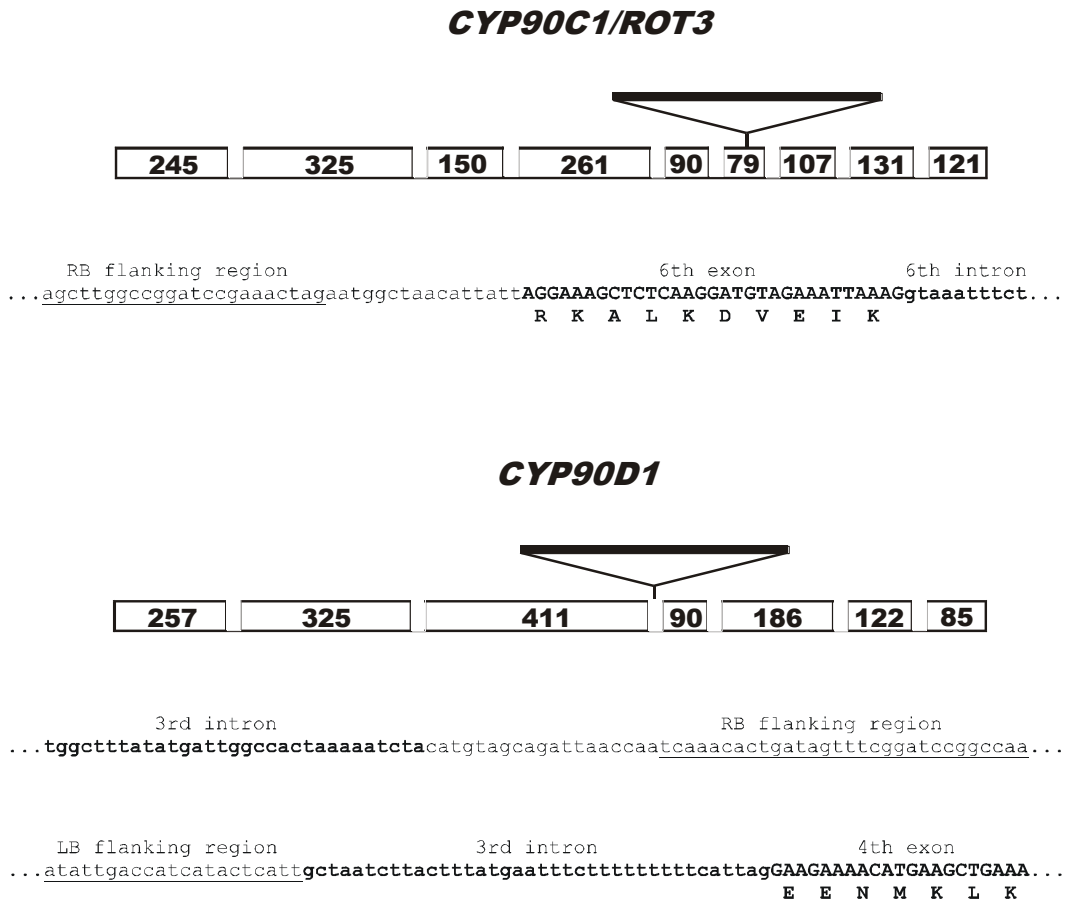


Figure 10. Positions of the T-DNA inserts in the identified *cyp90c1* and *cyp90d1* mutants. In the sequences of insertion sites, nucleotides of *Arabidopsis* DNA are in boldface, while those of T-DNA origin are underlined. Coding sequences are indicated by upper-case letters. (In the *cyp90c1* mutant only one of the insertion sites was sequenced.) RB: right border, LB: left border.

Second generation plants carrying the mutations were recognized on the basis of their hygromycin resistance (carried by the T-DNA insert) and PCR test of the gene-specific insertion. To identify homozygous lines, individual plants carrying the *cyp90c1* or *cyp90d1* alleles were PCR-tested for the presence of the corresponding wild-type genes. The homozygous mutants obtained were maintained by self pollination. As anticipated, none of the two mutants had BR-deficient character. Homozygous *cyp90c1/rot3* plants showed the characteristic round-leaf phenotype of earlier *rot3* isolates (Kim et al., 1998), but the *cyp90d1* mutant was morphologically indistinguishable from wild-type plants.

4.7 **BR-dependent expression of the *Arabidopsis BRH1* gene**

During a differential-display RT-PCR screen for BR-regulated genes, Gergely Molnár identified a BR down-regulated transcript which encoded BRH1 (BRASSINOSTEROID-RESPONSIVE RING-H2, GeneBank AF134155), a 170-aa RHA-type RING-H2 protein with unknown function. In order to elucidate the nature and kinetics of BR effect on the *BRH1* transcript, following BL treatment we assayed its levels by Northern hybridization in wild-type *Arabidopsis*, as well as in BR-deficient *cpd* and BR-insensitive *bri1* mutants. In one-week-old wild-type seedlings the *BRH1* mRNA decreased to about 30% of the initial value within one hour of treatment with 1 μM BL, then remained unchanged upon further incubation (Fig. 11A). In the *cpd* mutant the same type of down-regulation was observed. The change, however, is much less spectacular, because both the initial and the final mRNA levels were substantially higher than in the wild-type plants (Fig. 11B). By contrast, in the BR-insensitive *cbb2* mutant RT-PCR assays did not detect any effect of BL treatment on *BRH1* expression (Fig. 11C).

The BR-dependent down-regulation of *BRH1* differs from that of the BR synthesis genes in that (i) repression was not prevented by simultaneous application of cycloheximide, and (ii) the transcript level did not decrease below 30% of the control value. On the other hand, the lack of BR response in the *cbb2* mutant (Fig. 11B) indicates that the regulation of *BRH1* also requires the BRI1 BR receptor function.

In order to find out if *BRH1* activity is also influenced by other phytohormones, we analyzed the effect of auxin (indoleacetic acid), cytokinin (kinetin), GA_3 (gibberellic acid), abscisic acid and ethylene on *BRH1* transcript accumulation. During the first four hours of treatment the mRNA level was not appreciably affected by any of these hormones (data not shown), indicating that the down-regulation of *BRH1* is a specific BR effect.

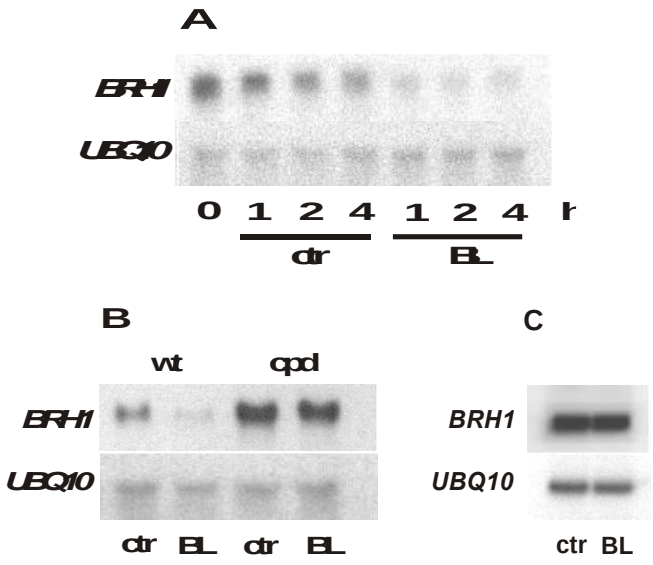


Figure 11. BR-dependent decrease in the abundance of *Arabidopsis BRH1* mRNA. A: Northern-blot showing the mRNA levels during a 4 h incubation period in 8-d-old BL-treated and control wild-type seedlings. B: Northern analysis of the transcript levels in 8-day-old wild-type (*wt*) and BR-deficient (*cpd*) seedlings following 1 h incubation with (BL) or without (ctr) 1 μM BL. The autoradiograms were made of the same Northern-blot, *BRH1* signals were overexposed to make each band visible. C: RT-PCR products obtained from total RNA of 8-day-old BR-insensitive *cbb2* seedlings, control (ctr) and BL-treated (BL, 1 μM, 2 h). In B and C, treatments were done in the presence of 100 μM cycloheximide. *UBQ10* served as constitutive control.

5 DISCUSSION

Plant cells must regulate their BR level in accordance with their developmental stage and environmental conditions. Such control is particularly important, because BRs must be supplied for many cellular functions throughout the life cycle of plants (reviewed by Clouse and Sasse, 1998). Maintenance of BR homeostasis, i. e. ensuring optimal hormone levels for the physiological functions requires efficient control mechanisms coordinating BR biosynthesis and metabolism.

5.1 Regulation of endogenous BR levels

The regulation of endogenous BR concentrations can take place at three main levels, namely biosynthesis, metabolism and transport. The data available about the transport of these hormones are scarce and sometimes contradictory (Adam and Schneider, 1999). By contrast, during the past few years an impressive amount of information has accumulated about the reactions and regulation of BR biosynthesis. It has been shown that synthesis, starting from campesterol and leading to the end product BL, occurs via two parallel routes: the early and late C-6 oxidation pathways (Fig. 2; Noguchi et al., 2000). Quantitative analyses of endogenous BR intermediates showed a considerable variation in the amounts of these compounds. Compared to their immediate precursors, the low amounts of cathasterone, teasterone and castasterone, as well as their 6-deoxo forms, indicated three rate-limiting steps in the pathway (Fujioka et al., 1995; Nomura et al., 2001). These reactions are catalyzed by the cytochrome P450 enzymes CYP90A1, CYP90B1 and CYP85A1. Since these conversion steps determine the flow-rate of

intermediates, the expression of the enzymes mediating these reactions can strongly influence the efficiency of BR biosynthesis.

5.1.1 The regulation of BR-biosynthetic genes

The expression of the gene responsible for C-23 hydroxylation was characterized by Mathur et al. (1998). They showed that the *CYP90A1/CPD* gene is negatively regulated by BL at the transcriptional level, in a concentration-dependent manner. Because gibberellin synthesis is also inhibited by its end-product through the repression of GA 20-oxidase and GA 3β-hydroxylase genes (Yamaguchi and Kamiya, 2000), the feedback control of biosynthetic genes seems to be a practicable course for the self-regulation of phytohormone levels.

In our study we demonstrated that, like *CYP90A1/CPD*, all other *CYP90* and *CYP85* genes of *Arabidopsis* are feedback-regulated by BL. The kinetics of the hormone effect (occurring within one hour) and the extent of repression (to ca. 10% of the initial expression level) was the same for all of these genes. Furthermore, the inhibition of these genes by BL could be abolished by the protein synthesis inhibitor cycloheximide, indicating that this process requires *de novo* synthesis of a negative regulatory protein. All these data suggest that the feedback control of BR-biosynthetic genes is well coordinated, and most probably utilizes the same transcriptional control mechanism.

It poses an intriguing question why multiple genes (and thereby conversions) of the same biosynthetic pathway need to be feedback-regulated, when the flow of syntheses could be determined by a single rate-limiting step. It is quite likely that the parallel regulation of consecutive reactions can make the control process more reliable, while also preventing futile expression of the enzymes catalyzing downstream reactions. Because BR

synthesis does not necessarily follow a linear route, coordination of the CYP90- and CYP85-mediated reactions may also control the effect of influx from stored or transported intermediates. Clearly, such multiple transcriptional regulation may have advantages in regulating phytohormone synthesis, because the feedback control of gibberellin biosynthesis also targets several biosynthetic genes (Helliwell et al., 2001).

In accordance with the repression of *CYP90* and *CYP85* genes by BRs, we also observed their derepression in the BR-deficient *cpd* mutant. These data show that at normal BR concentrations of wild-type plants the *CYP90* and *CYP85* genes are partially repressed. Hence, the feedback control circuit can be regarded as a physiological, rather than emergency, regulator of the hormone level.

Feedback control of the *CYP90* and *CYP85* genes requires a functional BRI1 BR receptor, because BL regulation is abolished in the BR-insensitive *bri1-2/cbb2* mutant. BR-insensitive mutants, such as *bri1-1*, were shown to accumulate high amounts of typhasterol, castasterone, and BL (Nomura et al., 1997; Noguchi et al., 1999). This is in agreement with our finding that intact BR signaling is necessary for the feedback control of steroid hormone level.

5.1.2 Developmental regulation of the *CYP90* and *CYP85* genes

BRs influence a wide spectrum of physiological responses (e.g. photomorphogenesis, cell division and elongation, etc.) which are essential for normal plant development. This regulation requires the presence and optimal concentration of these hormones at critical stages of the developmental cycle. The analysis of endogenous BR levels showed that castasterone and BL are most abundant in the siliques and seeds of *Arabidopsis* (Fujioka et al., 1998), indicating that active BR forms may have an important

function in germination. We showed that during the first week of development the expression of all *CYP90* and *CYP85* genes was transiently induced, but afterwards decreased to a much lower level. Since BL is accumulated in the seeds, induction of the BR-biosynthetic genes during germination and early seedling development clearly indicates a demand for *de novo* synthesized BRs at this stage. Because temporal expression profiles of the particular *CYP90* and *CYP85* genes showed small but characteristic differences during the experimental period, their induction appears to be regulated by distinct mechanisms. As some BR intermediates are already present in the seeds (Fujioka et al., 1998), this differential induction can be due to the demand for only some of the biosynthetic enzymes during the germination process. The high-level expression of *CYP90* and *CYP85* genes at the early seedling stage is not independent of the feedback control, because it can be efficiently down-regulated by exogenously applied BL. This also shows that, despite of the high biosynthetic activity, active BR levels are not allowed to rise substantially higher in seedlings than in mature plants.

5.1.3 Organ-specific expression of the BR-biosynthetic genes

Phytohormones elicit well coordinated cellular effects in various organs and tissues. Both quantitative and qualitative responses are largely determined by the sensitivity of the target cells and the local concentration of the hormone. Our results showing differential organ-specific expression of all *CYP90* and *CYP85* genes (except *CYP90B1/DWF4*) imply that in *Arabidopsis* the spatial regulation of BR synthesis may be an important means of adjusting proper hormone levels. It is interesting to note that, unlike the BR-biosynthetic genes, *DIM1* and *DET2*, encoding sterol-biosynthetic enzymes acting upstream of the BR pathway, are ubiquitously expressed in the plant. Compared to BRs,

phytosterols are more abundant by several orders of magnitude, therefore their synthesis (and availability) is not a limiting factor in BR synthesis (Yokota, 1997). By contrast, the expression of the enzymes acting closer to the active hormone may require stringent spatial regulation.

Because we thought that actual BR levels could determine or substantially influence the organ-specific expression of *CYP90* and *CYP85* genes, we performed experiments to test this possibility. These assays revealed that the difference between the shoot and root expression levels of *CPD* was maintained in the *bri1* BR-insensitive mutant, and that in wild-type plants the low root expression of this gene was not the result of high local BR concentration. Furthermore, the different organ-specificities of the uniformly feedback-regulated *CYP90* and *CYP85* genes also argue against any major involvement of the hormonal control in the determination of organ-specific expression. Therefore, the organ-specificity and the hormonal regulation of BR-biosynthetic genes seem to result from independently functioning regulatory mechanisms.

5.1.4 Organ-specific distribution of BR intermediates

Differential organ-specific activities of the *CYP90* and *CYP85* genes indicate different requirements for localized biosynthetic activity, and/or different local availability of particular substrates. This would imply that BR intermediates may also show an organ-specific distribution within the plant. GC-MS analyses of the shoots and roots of *Arabidopsis,* tomato and pea showed a tissue-specific distribution of BR compounds. Intriguingly, early BR biosynthesis intermediates, such as 6-deoxocathasterone, 6-deoxoteasterone, 3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol were more abundant in the roots, whereas the late intermediates 6-deoxocastasterone and castasterone

accumulated preferentially in the shoots. The level of BL was very low in the roots of *Arabidopsis* and pea, and not detectable in either of the tomato samples. Higher representation of the late BR intermediates in the shoot might be explained by the high sensitivity of root to active BRs, which can inhibit its growth at sub-nanomolar concentrations (Clouse et al., 1993). The similar distribution pattern of BR intermediates in *Arabidopsis*, pea and tomato suggests that the mechanism controlling the organ-specific levels of these compounds can be conserved in higher plants.

5.1.5 Complex transcriptional regulation of endogenous BR levels

In order to fulfill a signaling function, *in planta* hormone environments need fine, adaptive regulation. Phytohormones elicit responses in a concentration-dependent manner, exerting concerted effects on cells of susceptible tissues. The maintenance and adjustment of BR levels depends to a great extent on *de novo* hormone synthesis, which is influenced by the expression and stability of the biosynthetic enzymes, as well as their actual catalytic activity.

The expression of the *CYP90* and *CYP85* genes, encoding BR-biosynthetic enzymes, is subject to developmental, spatial and hormonal regulation. Its complexity seems to indicate a major role for transcriptional control in determining the availability of these P450 enzymes. In such a case, one might expect that the activity of particular *CYP90* or *CYP85* genes would correlate with the amount, and probably also the activity, of the encoded enzymes. Because C-23 hydroxylation is not a redundant function in *Arabidopsis*, and *CYP90A1/CPD* transcripts are much more abundant in shoots than in roots, a higher CYP90A1 activity could be anticipated in the above-ground part of the plant. Our data showing preferential accumulation of 6-dexocathasterone, the substrate of CYP90A1, in

roots is in agreement with this hypothesis. Therefore, it seems likely that transcriptional regulation of the *CYP90* and *CYP85* genes is important in determining the efficiency of BR biosynthesis.

These findings suggest that feedback control of the BR-biosynthetic genes is crucial in maintaining BR homeostasis. Interestingly, balanced BR levels are also ensured by an oppositely operating regulatory circuit, the feed-forward transcriptional mechanism inducing the expression of BR-inactivating P450s. The genes encoding the closely related CYP72B1/BAS1 (Neff et al., 1999) and CYP72C1/CHIBI2 (Sato et al., 2000), converting BL to a C-26-hydroxylated inactive form, are rapidly induced by BL treatment (Choe et al., 2001). Apparently, a secure control of active BR levels requires the functioning of both the feedback and feed-forward self-control mechanisms.

5.2. **The role of cytochrome P450 enzymes in BR biosynthesis**

5.2.1 Cytochrome P450 enzymes with known catalytic functions

In *Arabidopsis*, all identified BR-biosynthetic enzymes belong to the CYP90 or CYP85 families of cytochrome P450s (Fig. 2). The consecutive C-22 and C-23 hydroxylations of the steroid side chain are catalyzed by the CYP90B1 and CYP90A1 monooxigenases, respectively (Choe et al., 1998; Szekeres et al., 1996). The two-step C-6 oxidation of castasterone and some of its immediate precursors is carried out by CYP85A1 (AtBR6ox; Shimada et al., 2001). Both the conversion steps and the participating enzymes seem to be well conserved among higher plants (Nomura et al., 2001), and orthologs of CYP90A1 and CYP85A1 have been identified in several species (Bishop et al., 1996; Hong et al., 2002; Koka et al., 2000).

In addition to its preferred substrate campestanol, CYP90B1 was also shown to hydroxylate campesterol and some other early BR intermediates with low efficiency (Choe et al., 1998). On the other hand, in yeast expression system, CYP85A1 from both *Arabidopsis* and tomato oxidized multiple BR substrates, although at much lower rates than castasterone (Bishop et al., 1999; Shimada et al., 2001). These results show that, like many other P450s, the CYP90 and CYP85 enzymes have somewhat relaxed substrate-specificity. Accordingly, BR biosynthesis can be imagined as a network of reactions, rather than just consecutive reactions following the early or late C-6 oxidation pathways (Shimada et al., 2001). This would be similar to the organization of the gibberellin pathway, which is based on concurrent conversion steps (Phillips 1998). In such a scenario, the rate of synthesis is determined by substrate preferences of the participating enzymes and the availability of the particular intermediates.

In a recent paper Kang et al. (2001) reported that in etiolated pea yet another P450, CYP92A6/DDWF1 functions as BR C-2 hydroxylase. Presently it is not clear if in pea this is the main enzyme that synthesizes castasterone and 6-deoxocastasterone. Unlike CYP90 and CYP85, the CYP92 family is not ubiquitous, so plants like *Arabidopsis*, lacking such enzymes (The *Arabidopsis* Genome Initiative, 2000) obviously need a different type of P450 for C-2 hydroxylation.

5.2.2 CYP90 and CYP85 enzymes with uncertain roles in BR synthesis

Among the CYP90 and CYP85 enzymes of *Arabidopsis* the catalytic functions of three were unknown. Because they belong to P450 families of which all characterized members are involved in BR synthesis, and because their expression is subject to feedback regulation by BL, CYP90C1, CYP90D1 and CYP85A2 were also expected to participate in

BR biosynthesis. On the other hand, at least three reactions of the BR pathway, namely early C-6 oxidation, C-2 hydroxylation, and lactonization of the B ring, were thought to be mediated by yet unidentified P450s (Asami and Yoshida, 1999). Therefore it seemed possible that some of these conversion steps could be catalyzed by the above-mentioned three uncharacterized P450s.

Identification of the reactions catalyzed by CYP90A1 and CYP90B1 was facilitated by the availability of dwarf mutants defective in the genes encoding these enzymes. The lack of available CYP90C1-, CYP90D1-, or any CYP85-deficient mutants suggests that these functions, unlike CYP90A1 and CYP90B1, may be redundant in *Arabidopsis*. Indeed, a very recent work demonstrated that CYP85A2, sharing 82% aa sequence identity with CYP85A1, catalyzes C-6 hydroxylation of the same substrates as CYP85A1 in yeast expression system (Shimada et. al., 2003). We think that the closely related (55% identical) CYP90C1 and CYP90D1 may also have (at least partially) redundant roles. This seems to be in agreement with the very mild phenotype of *cyp90c1/rot3* mutants, and the lack of visible phenotype in our *cyp90d1* mutant. Because only these two CYP90 forms of *Arabidopsis* have no assigned functions, their redundancy would mean that *cyp90c1* and *cyp90d1* double mutants should be BR-deficient dwarfs. Rescue experiments with BR intermediate feeding and analyses of the endogenous BR content in such a double mutant would then reveal which particular step of BR synthesis is catalyzed by CYP90C1 and CYP90D1.

5.3 **BR-regulated gene expression**

BRs, like other phytohormones, are perceived by specific receptors, which then initiate intracellular signaling events that lead to well-defined changes in gene expression.

The molecular mechanism of BR signaling is intensely investigated, and only in the past two years eight new regulatory components have been identified (reviewed by Clouse, 2002). Our knowledge of the target genes is also rapidly expanding, but very few of the already described ones participate in early hormone responses. BR perception by the BRI1 receptor results in a phosphorylation cascade which activates and translocates to the nucleus BES1 and BZR1, two positive transcriptional regulators (Wang et al., 2002; Yin et al., 2002). Because these proteins do not possess DNA-interacting domains, the transcription factors recognizing BR-responsive promoter sequences still need to be discovered.

5.3.1 Identification of BR-regulated genes

BRs influence a wide spectrum of cellular responses. Understanding the molecular background of these effects requires the identification of specific target genes responding to the hormonal stimuli. The powerful cDNA microarray hybridization technique permitted extensive screening of transcripts which change their abundance in response to BR treatment. Recently two laboratories have assayed more than 12,000 of the nearly 25,000 *Arabidopsis* genes by this method, and identified several genes which were induced or repressed by the steroid hormone (Goda et al., 2002; Müssig et al., 2002). Although these screens also detected delayed, indirect transcriptional responses, both teams concluded that only a relatively small portion of the *Arabidopsis* genes (< 12%) were affected by BRs. Positively regulated genes included several auxin-responsive genes, xyloglucan endotransglucosylases and Myb-type transcription factors, while down-regulated genes included BR-biosynthetic genes and also some Myb transcription factors.

5.3.2 Different mechanisms of BR-dependent gene repression

The identification of BR-induced primary transcriptional responses can elucidate early regulatory events in the hormonal regulation of gene expression. Early responses utilize existing nuclear components, therefore they can be elicited in the absence of *de novo* protein synthesis (Koshiba et al., 1995). In a differential display RT-PCR screen for primary BR-response transcripts in *Arabidopsis* we isolated the cDNA of a small RING-H2 protein, BRH1, with unknown function. The *BRH1* gene is quickly down-regulated by BRs, and the effect is specific for this hormone group. However, the BR-dependent repression of *BRH1* can be clearly distinguished from those of the *CYP90* and *CYP85* genes. Upon steroid hormone treatment, *CYP90* and *CYP85* transcripts decreased to about 10% of the control levels, whereas the amount of *BRH1* mRNA declined more moderately, to one-third of the control value. Furthermore, in contrast to *BRH1*, the inhibition of BR-biosynthetic gene expression by the steroid hormone required the synthesis of a negative regulatory protein, indicating that this effect is not a primary BR response. These data show that the two types of BR-dependent repression act at different levels, and involve different regulatory processes.

The primary induction and the capacity of the RING motif to establish protein-protein interactions suggested a regulatory role for BRH1. Although the *Arabidopsis* genome contains several small RING-H2 gene families, the function of their products is largely unknown. An analogy with animal systems indicated that plant RING-H2 proteins may serve as specificity factors in E3 ubiquitin ligase complexes, targeting these to proteins destined for proteasome-mediated degradation (Joazeiro and Weissman, 2000). This degradative pathway was shown to be important in controlling the auxin response in

Arabidopsis (Schwechheimer et al., 2001). The assumed regulatory role of BRH1 seems to be in agreement with its primary response transcriptional control by BRs.

5.3.3 BR-responsive regulatory elements within the *CYP90* and *CYP85* promoters

The strong and specific BR response of the BR biosynthesis genes makes them ideal objects for studying the promoter elements mediating the steroid hormone effect. Mathur et al. (1998) showed that the hormonal down-regulation takes place primarily at the transcriptional level, because in transgenic *Arabidopsis* a 900 bp segment of the *CPD* promoter controlled the expression of a *GUS* reporter gene in the same, BR-sensitive manner. In order to localize the *cis* regulatory sequences required for the BR response, we generated fusion constructs between progressively truncated versions of the *CPD* promoter and the β-glucuronidase (*GUS*) reporter, and transformed these chimeric genes in *Arabidopsis*. Our preliminary results indicate that a roughly 150 bp promoter region, lying immediately upstream of the TATA transcriptional initiation signal, is sufficient for conferring BR-repressible expression. This promoter part contains a set of sequence segments that are conserved between all *CYP90* and *CYP85* promoters. The role of these elements in mediating the BR response is currently analyzed by using micro-deletion and *in vitro* mutagenized promoter constructs. Once the consensus sequence of the BR response element(s) is clarified, it might greatly facilitate the identification of its cognate transcription factor(s).

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ÖSSZEFOGLALÁS

A brasszinoszteroidok (BR-ok) a növényi hormonok nemrég felismert önálló osztályát alkotják. Ezek a polihidroxi-szteroid vegyületek valamennyi edényes növényben előfordulnak, és meghatározó szerepet játszanak ezek megnyúlásában, fotomorfogenezisében, fertilitásában és stressz-érzékenységében. Bár a biológiailag legaktívabb BR-nak tekintett brasszinolid (BL) szerkezetét csupán 1979-ben sikerült meghatározni, mára jórészt ismertté vált a BR-ok bioszintézise, másrészt megtörtént a hormonhatásért felelős jelátviteli komponensek többségének azonosítása.

A BR-ok pontos élettani szerepének, valamint a hormon szintézis génjeinek meghatározásában alapvető jelentősége volt az esősorban *Arabidopsis thaliana*-ban létrehozott BR-deficiens mutánsok vizsgálatának. Az ún. *constitutive photomorphogenesis and dwarfism* (*cpd*) mutáns nem szintetizál biológiailag aktív BR-okat. Az e mutáns segítségével azonosítható *CPD* gén egy citokróm P450 típusú szteroid hidroxilázt (CYP90A1) kódol, amely a BR oldallánc C-23-as pozíciójú hidroxilációjáért felelős. Ez az enzim a BR bioszintézis mindkét fő reakcióútjában szerepet játszik, ezért meghatározó lehet a szintézis hatékonyságának szabályozásában. Csoportunk korábban kimutatta, hogy a *CPD* gén működését a BR koncentráció egy negatív visszacsatolás révén szabályozza, és hogy ez a hatás elsősorban a transzkripció szintjén érvényesül. A hormon homeosztázis tehát biztosítható a CYP90A1/CPD enzim kifejeződésének kontrollján keresztül. Emellett a *CPD* gén aktivitása jellegzetes egyedfejlődési és szerv-specificitást is mutatott, ami a BR bioszintézis térbeli és időbeli organizáltságára utalt.

A BR szintézis összes eddig azonosított enzime a citokróm P450 monooxigenázok csoporjába, ezen belül a CYP90 és CYP85 családokba tartozik. *Arabidopsis*-ban a CYP90A1-en és a C-22-hidroxiláz funkciójú CYP90B1-en kívül két további, ismeretlen katalitikus aktivitású CYP90 enzim is jelen van. A C-6 keto-csoport kialakítását végző

CYP85A1 mellett előforduló CYP85A2 protein szerepéről szintén a legutóbbi időkig nem rendelkeztek információval. A BR bioszintézisre vonatkozó ismeretek bővülése ellenére annak szabályozása jórészt ismeretlen maradt. Tisztázásra várt, hogy a szintetikus folyamatok hol és mikor folynak a növény szervezetében, és ezek mennyiben befolyásolják a lokális hormon szintet. Eldöntésre szorult továbbá az is, hogy a bioszintetikus gének aktivitásától mennyiben függ a BR szintézis hatékonysága.

Célkitüzések

Munkánk célja a BR bioszintézisben résztvevő gének szabályozásának megismerése volt, hogy ezáltal információt nyerjünk a szintézis menetéről és az endogén hormon szintre gyakorolt hatásáról. Elsősorban a következő kérdésekre kívántunk választ kapni:

1. Mennyiben határozzák meg a BR bioszintézis génjeinek kifejeződését hormonális, fejlődési és szerv-specifikus tényezők? Mennyiben hasonlóak, ill. különbözőek e regulációs hatások az egyes gének esetében?
2. Kimutathatók-e szerv-specifikus különbségek a BR szintézis intermediereinek mennyiségében? Ha igen, ezek összhangban állnak-e a bioszintetikus gének lokális kifejeződésével?
3. Milyen molekuláris tényezők révén szabályozódik a BR bioszintézis génjeinek szabályozása, elsősorban is a *CPD* gén BR-ok általi negatív regulációja?

Főbb eredmények

1. A BR bioszintézisben résztvevő *Arabidopsis CYP90* és *CYP85* proteinek és génjeik struktúrális analízise alapján megállapítottuk, hogy e két citokróm P450 család tagjai egymással szorsos filogenetikai rokonságban állnak, és szétválásukra valószínúsíthetően a BR bioszintetetikus funkció kialakulását követően került sor. Aminosav-szekvencia azonosságuk alapján ezen P450 családok csak a gibberellin bioszintézisben résztvevő CYP88 családdal állnak közeli rokonságban. Az vizsgált citokróm P450 családok génjeinek esetében az exon-intron szerkezet konzerváltsága is hasonló rokonsági viszonyokat tükröz.

2. *Arabidopsis*-ban a CYP90A1, CYP90B1 és CYP85A1 enzimek BR bioszintetikus szerepe ismert volt, míg a CYP90 és CYP85 családok többi tagjának (CYP90C1, CYP90D1 és CYP85A2) funkciója tisztázása várt. Valamennyi *Arabidopsis CYP90* és *CYP85* mRNS szemikvantitatív RT-PCR analízisével megállapítottuk, hogy génjeik - a *CYP90A1/CPD*-hez hasonlóan - BL-függő negatív visszacsatolás által reguláltak. Ez a gátlás valüszínüleg egy egységes mechanizmus révén biztosítja a BR szintézis kontrollját. Kimutattuk továbbá, hogy e regulációs folyamat a BRI1 BR receptorról kiinduló szignáltól függ, mivel a receptor-deficiens *bri1-2/cbb2* mutánsban a *CYP90* és *CYP85* gének kifejeződése BL-dal nem gátolható.

3. A BR bioszintetikus gének transzkriptumainak RT-PCR analízise kiderítette, hogy a korai egyedfejlődés során a *CYP90* és *CYP85* családok valamennyi tagja magas szintű kifejeződést mutat. A csírázást és korai csíranövény stádiumot magában foglaló első hét során az mRNS mennyiségek gyors növekedése volt megfigyelhető, amely egy határozott csúcsérték elérése után annak kb. 10%-ára csökkent a vizsgálati periódus végére. A *CYP90*

és *CYP85* gének fokozott aktivitása összhangban áll a korai fejlődés folyamatainak magas BR igényével.

4. Az mRNS szintek vizsgálata fényt derített arra is, hogy a *CYP90* és *CYP85* gének kifejeződése (a *CYP90B1* kivételével) jellegzetes szerv-specifikus különbségeket mutat. A *CYP90A1* és *CYP85A2* esetében a föld feletti szervekben, míg a *CYP90C1, CYP90D1* és *CYP85A1* esetében a gyökérben észleltünk fokozott transzkriptum felhalmozódást. Adataink arra utalnak, hogy a *CYP90* és *CYP85* gének működésének térbeli szabályozása autonóm, a hormonális negatív visszacsatolástól alapvetően függetlenül ható folyamat. Ezt bizonyítja, hogy (1) a *CYP90A1* esetében az mRNS szint szerv-specifikus különbségei a BR-inszenzitív *bri1-2* mutánsban is kimutathatók, (2) vad típusú növények gyökerében a gyenge kifejeződés BL kezeléssel tovább csökkenthető, és hogy (3) az egyéges hormonális szabályozás ellenére a *CYP90* és *CYP85* gének különböző szerv-specificitást mutatnak.

5. Ezen eredmények tükrében feltehető volt, hogy a *CYP90* és *CYP85* gének aktivitásának térbeli kontrollja a BR bioszintézis szerv-specifikus különbségeivel járhat együtt. Ennek kiderítése céljából gáz-kromatográfiával kapcsolt tömegspekrtográfiás analízissel megvizsgáltuk a bioszintetikus intermedierek mennyiségi megoszlását *Arabidopsis*, borsó és paradicsom növények gyökerében és hajtásában. Ennek nyomán megállapítható volt, hogy az bár az egyes intermedierek szerv-specifikus felhalmozódásában markáns különbségek tapasztalhatók, az analizált három növényfaj eseténen a különbségek hasonló tendenciát mutatnak. Ez annak a bizonyítéka, hogy a BR szintézis térbeli regulációja konzervált a magasabbrendű növények körében. A korai bioszintetikus intermedierek felhalmozódása inkább a gyökérben, míg a BL-hoz közelebb eső prekurzoroké inkább a hajtásban volt észlelhető. Ennek oka feltehetően az, hogy a gyökerek fokozott BR-érzékenysége miatt fejlődésüket az utóbbi - esetleg már biológiailag aktív - BR alakok felhalmozódása gátolná. Figyelemre méltó a CYP90A1/CPD enzim

szubsztrátjának magas szintje a gyökerkben. Mivel a *CPD* gén gyökérben igen alacsony szinten fejeződik ki, ezért szubsztrátjának felhalmozódása arra utal, hogy a transzkripciós regulációnak meghatározó szerepe lehet a CYP90A1 enzimatikus aktivitásának, és ennek nyomán a BR bioszintézis hatékonyságának szabályozásában.

6. A tisztázatlan funkciójú CYP90C1, CYP90D1 és CYP85A2 esetében a BR bioszintetikus enzimekkel való strukturális rokonságuk és BR-regulált expressziójuk alapján valószínűsíthető, hogy ezek is a szteroid hormon szintézisben vesznek részt. Ez év folyamán ismertté vált, hogy a CYP85A2 a CYP85A1-gyel megegyező specificitású szteroid C-6 oxidáz. A továbbra is felderítetlen, valószinűleg redundáns szerepű CYP90C1 és CYP90D1 funkciójának tisztázása céljából Dr. Koncz Csaba (Max Planck Intézet, Köln) T-DNS-inszerciós *Arabidopsis* mutáns gyűjteményéből PCR alapú módszerrel e funkciókban defektív mutánsokat azonosítottunk. A homozigóta *cyp90c1* és *cyp90d1* vonalak létrehozása és ellenőrzése után az ezek keresztezésével kialakítandó kettős mutáns várhatóan BR-deficiens törpe fenotípusú lesz, és biokémiai karakterizálásával lehetőség nyílik a CYP90C1 és CYP90D1 fehérjék enzimatikus szerepének azonosítására.

7. Megállapítottuk, hogy a *CYP90* és *CYP85* gének BR-függő represszója markánsan eltér a laboratóriumunkban korábban azononosított, egy RING-finger proteint kódoló *BRH1* gén BR általi gátlásától. A BR bioszintetikus gének aktivitása BL kezelést követően egy nagyságrenddel csökkenthető, és a hatás kialakulásához *de novo* fehérjeszintézis szükséges. Ezzel szemben BL hatására a *BRH1* transzkriptum mennyisége csupán harmadára esik vissza, és a represszió fehérjeszintézis gátlókkal nem felfüggeszthető primér hormonválasznak tekinthető.