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Biochemical and genetic analysis of brassinosteroid metabolism and function in *Arabidopsis*

Miklós Szekeres[1,*] and Csaba Koncz[1,2]

[1] Institute of Plant Biology, Biological Research Center, Hungarian Academy of Sciences, H-6701 Szeged, Hungary.
[2] Max Planck-Institut für Züchtungsforschung, D-50829 Köln, Germany.
* Corresponding author (Fax 36-62-433434; E-mail szekeres@everx.szbk.u-szeged.hu)

Abstract Recent analysis of several constitutive photomorphogenic *Arabidopsis* mutants, defective in brassinosteroid biosynthesis or signaling, provided unequivocal evidence for the essential phytohormone function of brassinosteroids. From earlier studies a wealth of information is available regarding the occurrence of these steroids and their potential interaction with regulatory mechanisms controlled by other plant hormones. In addition, the major pathways of brassinosteroid biosynthesis and metabolism have been clarified in the past couple of years and significant efforts have been made to characterize structure-function relationships. This knowledge is now being confronted with data emerging from genetic and molecular analysis of the gene mutations and functions affecting brassinosteroid synthesis and signaling. Future progress in this field is expected to give insight into the evolution of steroid hormone regulation in eukaryotes, as well as the mechanisms by which brassinosteroids control basic functions, such as cell elongation, morphogenesis and stress responses. © Elsevier, Paris.

Key words Brassinosteroid, cell elongation, photomorphogenesis, biosynthesis, signaling, *Arabidopsis thaliana.*

Abbreviations ABA, abscisic acid; BR, brassinosteroid; GA, gibberellin; GC-MS, gas chromatography-mass spectroscopy.

Introduction

Brassinosteroids (BRs) are endogenous plant polyhydroxy-steroids eliciting remarkable growth-promoting and developmental effects at nanomolar concentrations (for reviews see Mandava, 1988; Sakurai and Fujioka, 1993). Their physiological role is distinct from, but well coordinated with those of auxins and gibberellins, each contributing on its own to a complex system of regulation also influenced by environmental stimuli such as light, stress factors, etc. Overlapping action spectra of these growth regulators presented a major obstacle in the way of delimiting the precise functions of BRs in the various developmental processes.

Recently several dwarf mutants of *Arabidopsis* and garden pea (*Pisum sativum*) have been isolated which proved to be defective in either the biosynthesis or perception of BRs (Takahashi *et al.*, 1995; Clouse *et al.*, 1996; Kauschmann *et al.*, 1996; Li *et al.*, 1996; Szekeres *et al.*, 1996; Nomura *et al.*, 1997). Analysis of these mutants revealed the influence of BRs on a variety of physiologically important traits, including photomorphogenesis and male fertility, pointing out their essential role in normal plant development. These findings, combined with their ubiquitous occurrence in higher plants, confirmed the position of BRs being a new class of *bona fide* plant hormones, which had been a center of debate in the past decade. The availability of numerous mutants and extensive genome information makes *Arabidopsis* a uniquely valuable tool for BR research. Biosynthetic mutants are crucial in uncovering the enzymatic background behind the impressively deduced schemes of BR biosynthesis and metabolism (for recent review see Fujioka and Sakurai, 1997), while analysis of insensitive mutants is expected to elucidate the mechanisms of BR perception and signaling. Both types of mutants

are expected to provide valuable information as to the role of BRs in the intricate network of various hormonal and intracellular signaling processes. In recent years the accumulation of a remarkable body of information about BR biosynthesis and the mutants signaled a major advance in BR research. This review offers an assessment of the current status of this rapidly unfolding field of plant biology, paying particular attention to the latest developments and their relevance to *Arabidopsis* studies.

Major areas of past BR research

Following the discovery and structural characterization of the first BR, brassinolide (Grove *et al.*, 1979), significant efforts were mounted to (*a*) find analogous steroid substances in the plant kingdom, (*b*) establish chemical synthesis and study the biosynthesis of BRs, (*c*) analyze their physiological action as potential phytohormones and (*d*) examine the applicability of BRs as growth regulators in agriculture. The enthusiasm of BR research stemmed from exciting early observations showing that brassinolide treatment significantly improved yield, growth rate, stress tolerance, and disease resistance of the major crops, vegetables, ornamentals and trees. In addition, BRs were found to be active as crop safeners in combination with several herbicides, offering an attractive target for the chemical industry (for review see Mandava, 1988). Application-oriented studies were quickly followed by physiological analyses of the action spectrum of brassinolide. Efficient new bioassays, such as the rice leaf lamina inclination test (Sakurai and Fujioka, 1993) were introduced to detect specific responses triggered by trace amounts (0.5 ng ml^{-1}) of brassinolide and related BRs. Measurements of physiological and mechanical parameters, such as CO_2 fixation, accumulation of pigments and reducing sugars, elongation and bending responses, etc., were thus correlated with the activity of externally provided BRs in diverse plant organs. Today, when plant hormones are known to differentially regulate the expression of hundreds of genes in a cell type specific manner, the application of such bioassays appears somewhat awkward. Nevertheless certain conclusions are worth to consider, especially because some of these assays are still widely used in plant physiology.

Physiological studies

Brassinolide stimulates the elongation of hypocotyl, epicotyl and stem segments in many plant species including several gibberellin (GA) deficient dwarf mutants. Nonetheless, brassinolide cannot fully restore the phenotype of GA-deficient mutants, and the effects of BRs and GAs seem to be additive in most bioassays, suggesting an independent mode of action. Induction of cell elongation by brassinolide correlates with increased cell wall extensibility, a cellular response thought to be controlled by auxin. Brassinolide, however, fails to stimulate characteristic auxin responses, such as fast auxin-induced gene expression in soybean epicotyls, and suppression of shoot lateral buds. Brassinolide was also reported to stimulate gravitropic bending of bean hypocotyls independently of auxin. On the other hand, brassinolide increased the sensitivity of certain cell types to externally provided auxin. This could result from the stimulation of ethylene production through the induction of aminocyclopropane-1-carboxylic acid (ACC) synthase by brassinolide. Retardation of apical hook opening and inhibition of root elongation in brassinolide-treated etiolated seedlings may thus reflect the characteristic triple response (*i.e.* inhibition of hypocotyl and root elongation with concomitant induction of exaggerated apical hook) caused by ethylene. Data are scarce and contradictory to judge whether the sensitization of auxin responses is due to the modulation of auxin synthesis and/or metabolism by brassinolide (for review see Clouse, 1996). Although BRs and auxins are proposed to act synergistically, in certain bioassays (*e.g.* the wheat leaf unrolling test) brassinolide also displays cytokinin-like activity. Some of these effects of brassinolide may reflect ethylene responses because cytokinins are also known to stimulate ethylene synthesis. Unlike cytokinins, brassinolide cannot induce the accumulation of anthocyanin pigments in dark-grown plants and fails to promote the opening of cotyledons in etiolated seedlings. Brassinolide was also reported to inhibit leaf senescence and abscission promoted by abscisic acid (ABA) in some bioassays. It is therefore generally believed that BRs and ABA act antagonistically and most BR-induced responses can be alleviated by ABA. From the view point of future studies, perhaps the most significant observation reached by bioassays was that the hypocotyl and epicotyl elongation responses promoted by BRs were found to be dependent on the spectral quality of light. Following inhibition of hypocotyl elongation of dark-grown

seedlings by irradiation with monochromatic red light (660 nm) BRs restored hypocotyl elongation. BRs failed to stimulate hypocotyl elongation in the dark or upon irradiation with far-red and blue light, indicating that hypocotyl elongation may be antagonistically controlled by BRs and phytochrome-dependent light signaling (reviewed by Mandava, 1988; Sakurai and Fujioka, 1993).

In carrot and tobacco cell suspension cultures depleted of external growth regulators, brassinolide induces cell enlargement but no cell division. Remarkably, brassinolide was reported to inhibit the proliferation of crown-gall tumor cells, although the molecular basis of this observation remained unknown. In correlation with immuno-histological localization of BRs in vascular bundles of stems and roots, brassinolide was demonstrated to stimulate the differentiation of xylem elements in mesophyll cells of *Zinnia elegans* and tuber tissues of Jerusalem artichoke. A specific role of BRs in xylogenesis was demonstrated by data showing that brassinolide-induced hypocotyl elongation is abolished by 2,6-dichlorobenzonitrile, an inhibitor of cellulose biosynthesis. In accordance, the BR-inducible *BRU1* gene of soybean was found to encode a xyloglucan endotransglycosylase involved in cell wall biosynthesis (Zurek and Clouse, 1994; Clouse, 1996). Cytological analysis of differentiating tracheary-elements in *Z. elegans* showed that BRs induce the aggregation of actin filaments, transverse bundling of microtubules, and lignification of the secondary cell wall. Cremart, an inhibitor of microtubule assembly, and uniconazole, the GA and steroid biosynthesis inhibitor, abolished cell elongation and xylem differentiation responses induced by brassinolide (Iwasaki and Shibaoka, 1991; Mayumi and Shibaoka, 1995). By controlling cell wall plasticity BRs may also influence pollen germination and the formation of cell to cell contacts.

In summary, apart from a unique function in the regulation of cell wall biosynthesis, classical physiological studies could not sufficiently distinguish the activity spectrum of BRs from those of other plant hormones such as auxins, gibberellins or ethylene. Because BR-induced cellular responses proved to be sensitive to auxin analogs, as well as to inhibitors of ethylene and gibberellin synthesis, it seemed plausible that BRs function as non-essential modulators of major plant hormones, particularly in developing pollen and seed where their concentration is highest. The fact that BRs were detected, if at all, at extremely low concentrations in vegetative plant organs seemed to support this conclusion.

Analytical approaches

Whereas physiologists raised doubts, analytical chemists provided sound arguments supporting the applicability and importance of steroidal plant growth factors. So far more than 40 natural BR forms have been identified in higher plants, but BRs were also detected in several gymnosperms, as well as in ferns and a green alga. Due to their low physiological concentration, the BR spectra detected in various plant species may rather reflect the limitations of available microanalytical methods than their real distribution (for detailed review see Fujioka and Sakurai, 1997).

All known BRs share a common 5α-cholestane skeleton and attain their structural diversity by carrying functional groups on rings A and B, as well as on the steroid side-chain. To correlate structural features with biological activity, Japanese and German laboratories devised elaborate chemical syntheses and established sensitive *in vivo* tests for BRs, such as the rice lamina inclination and wheat leaf unrolling assays. In the case of ring A, BRs carrying C-2α and C-3α vicinal hydroxyls proved to be more active than those with only C-3 substitution. The oxo-group at C-6 and particularly the 7-oxalactone structure further enhanced biological efficiency. With respect to the side-chain, vicinal diol substitution at C-22 and C-23 was found instrumental in eliciting significant BR response in the assay systems.

Experiments with brassinolide, the most effective BR, and its congeners, unveiled a gradual increase in biological activity according to the positions in the biosynthetic pathway (see *fig.* 1). Early precursors up to 6-oxocampestanol were essentially inactive, whereas relative activities between 6-oxocampestanol, cathasterone, teasterone, castasterone and brassinolide increased 500-fold, 20-fold, 12-fold and 50-fold, respectively (Fujioka *et al.*, 1995 *b*). The results of such bioassays, however, are difficult to interpret because externally applied BRs are readily converted to more active forms within the test plant. On the basis of biological activities and in accordance with analytical GC-MS (gas chromatography-mass spectroscopy) data, none of the biosynthetic precursors, only brassinolide and perhaps castasterone (Suzuki *et al.*, 1993 *b*; 1995 *a*) are believed to have genuine hormone function in the plant system.

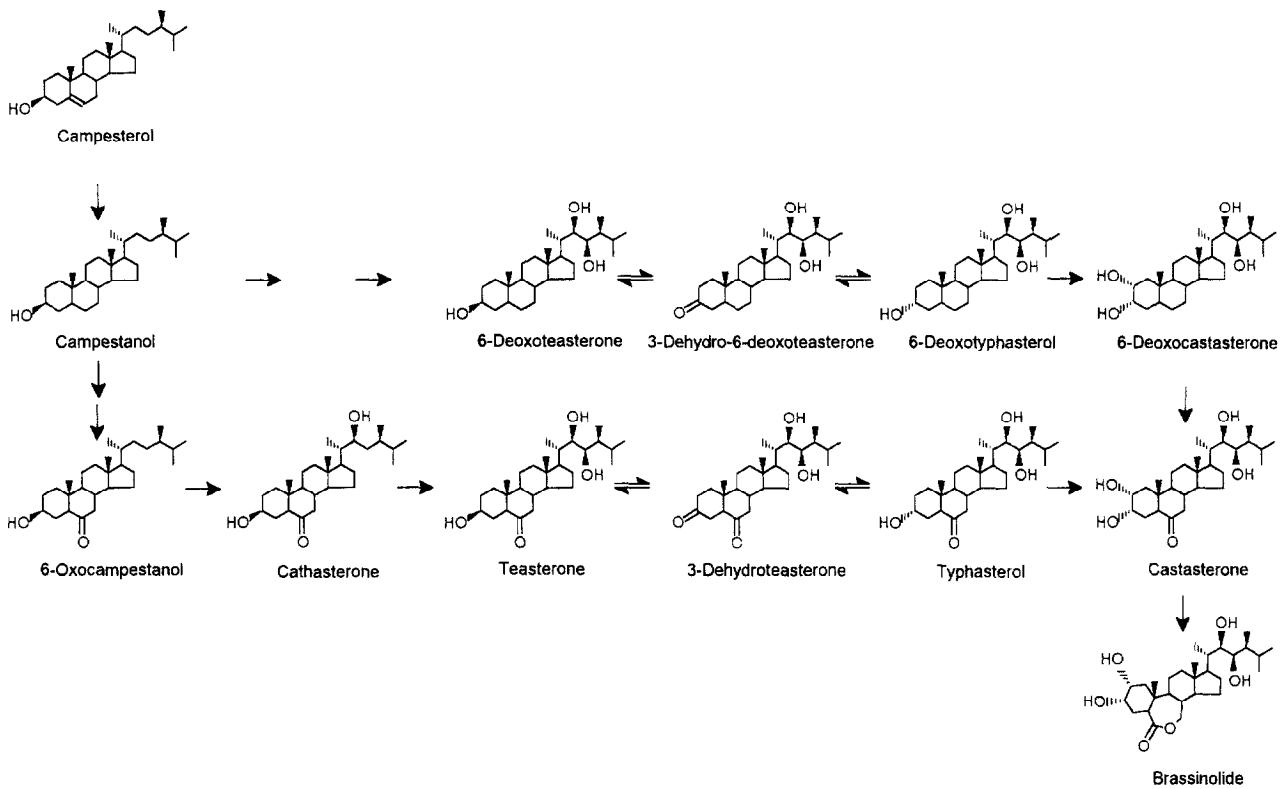

Figure 1. *Biosynthesis of brassinolide from campesterol.*

Extensive structure-activity relationship analyses were carried out by Brosa *et al.* (1996) using natural BRs and analogs in order to define the contribution of the various hydroxyl groups and *cis* or *trans* A/B ring junctions to biological efficiency. In rice lamina inclination tests explants were surprisingly responsive to some analogs with the non-natural *cis* steroid structure. Although such BR isomers do not exist in plants, these data offer valuable information about the versatility of hormone perception. Brosa *et al.* (1996) have suggested that stereochemical relation between parts of the BR molecule, rather than absolute configurations of substituents define biological activity. Detailed knowledge of the structural requirements of hormonal activity will be instrumental in designing synthetic analogs and biosynthetic inhibitors for BR perception and signaling studies, as well as agricultural applications.

Tracing BR biosynthesis and metabolism by precursor feeding

Side-chain structures of natural BRs, differing in the alkylation state of the C-24 position, suggest that they are synthesized from abundant phytosterols with the corresponding number of carbon atoms. Therefore it seems likely that more than one BR biosynthetic route exists in plants. The most significant pathway leading from campesterol to brassinolide (*fig.* 1) has been deduced from GC-MS data obtained in precursor feeding experiments which were carried out with cell suspension cultures of *Catharanthus roseus*, a plant containing an unusually high amount of BRs. The synthetic sequence between teasterone and brassinolide was established by monitoring the conversion of deuterated teasterone and typhasterol, while similar analyses using ^{14}C-labeled campesterol identified early precursors up to 6-oxocampestanol (Suzuki *et al.*, 1993 *a*; 1995 *b*). A link between these two par-

tial sequences was provided by the discovery of cathasterone which, being three orders of magnitude less abundant than 6-oxocampestanol, could not be detected as a conversion product (Fujioka *et al.*, 1995 *a*). These feeding experiments also suggested that the only reversible reaction in the process of brassinolide synthesis is the oxidative isomerization step between teasterone and typhasterol. This biosynthetic route is termed early C-6 oxidation pathway, to distinguish it from the recently discovered late C-6 oxidation pathway, which proceeds to castasterone through 6-deoxoteasterone, 6-deoxotyphasterol and 6-deoxocastasterone (Choi *et al.*, 1996; 1997). Similar feeding studies verified the existence of the early C-6 pathway in tobacco and rice, whereas simultaneous occurrence of 6-oxo and 6-deoxo intermediates in *Arabidopsis* and pea points out the parallel functioning of both synthetic routes in these organisms (Suzuki *et al.*, 1995 *a*; Fujioka *et al.*, 1996; Nomura *et al.*, 1997). At present the overall impression is that brassinolide biosynthesis in most higher plants proceeds according to the scheme presented in figure 1.

Currently available data regarding the abundance of BR intermediates offer some ideas about the flow-through kinetics of the above described biosynthetic pathway. In *C. roseus*, the pool size of cathasterone was found three orders of magnitude smaller than that of its precursor 6-oxocampestanol and comparable to those of castasterone and brassinolide. It is apparent therefore that the side-chain hydroxylation reaction leading to cathasterone may constitute a rate-limiting step in the synthetic cascade (Fujioka *et al.*, 1995 *a*). Nomura *et al.* (1997) showed that in garden pea plants the level of castasterone is about threefold higher than that of brassinolide. In other plants, such as tobacco, rice and *Arabidopsis*, no brassinolide could be detected (Suzuki *et al.*, 1995 *a*; Fujioka *et al.*, 1996) although these species are more responsive to brassinolide than to castasterone. These results suggest that lactamization of the B-ring may stringently control the availability of brassinolide. In shoots of garden pea the amount of 6-deoxocastasterone is nearly tenfold higher than that of castasterone (Nomura *et al.*, 1997) indicating a possible inefficiency of the oxidation step between these compounds. Following side-chain hydroxylation this is the only crossing point between the early and late C-6 oxidation pathways, so the latter synthetic route appears to play a subordinate role in vegetative tissues of pea.

Uptake experiments with tomato plants demonstrated that tritiated 28-homobrassinolide was readily metabolized to more hydrophilic substances (Schlagnhaufer and Arteca, 1991). Subsequent studies revealed that, like in the case of most other plant hormones, glucosylation plays a crucial role in the deactivation of BRs. In mung bean explants metabolization of radiolabeled brassinolide was primarily achieved by glucosylation of the C-23 hydroxyl. In rice lamina inclination tests the recovered glucoside was nearly as active as free brassinolide, indicating that *in vivo* the hormone may be released from the conjugate (Suzuki *et al.*, 1993 *b*). 24-Epicastasterone and 24-epibrassinolide were converted to C-25 and C-26 glucosylated compounds in tomato cell suspension cultures. The conjugation step was preceded by hydroxylation at the C-25 and C-26 positions by two distinct P450-type monooxygenases (Hai *et al.*, 1995). Glucosylation at the C-2 and the epimerized C-3 hydroxyls was also observed in the same cell system (Hai *et al.*, 1996). By studying the metabolism of 24-epicastasterone and 24-epibrassinolide in cell suspension of the legume *Ornithopus sativus*, C-25 hydroxylated derivatives and various C-3 acyl conjugates (myristilates, palmitates and laurates) were detected. These analyses revealed, for the first time, the formation of pregnane-type catabolites from BRs through a multi-step degradation pathway involving C-20 hydroxylation and subsequent side-chain cleavage (Kolbe *et al.*, 1996).

The physiological significance of BR metabolism is little understood. Anthers of *Lilium longiflorum* have been shown to accumulate C-3 acyl conjugates of teasterone which, during pollen maturation, released free teasterone in a developmentally regulated manner (Abe *et al.*, 1996; Asakawa *et al.*, 1996). C-23 glucosylated brassinolide is also believed to dissociate active hormone under physiological conditions (Suzuki *et al.*, 1993 *b*). Thus C-3 esterification and C-23 glucosylation seem to be physiologically important means of regulating the availability of free BRs. Furthermore, glucosylation makes the storage forms of BRs more hydrophilic, thereby facilitating their mobilization within the plant. By contrast, C-25, and C-26 hydroxylation, as well as cleavage of the side-chain, appear to ensure permanent inactivation of BRs. The various pathways of BR deactivation, both reversible and permanent, are summarized in figure 2.

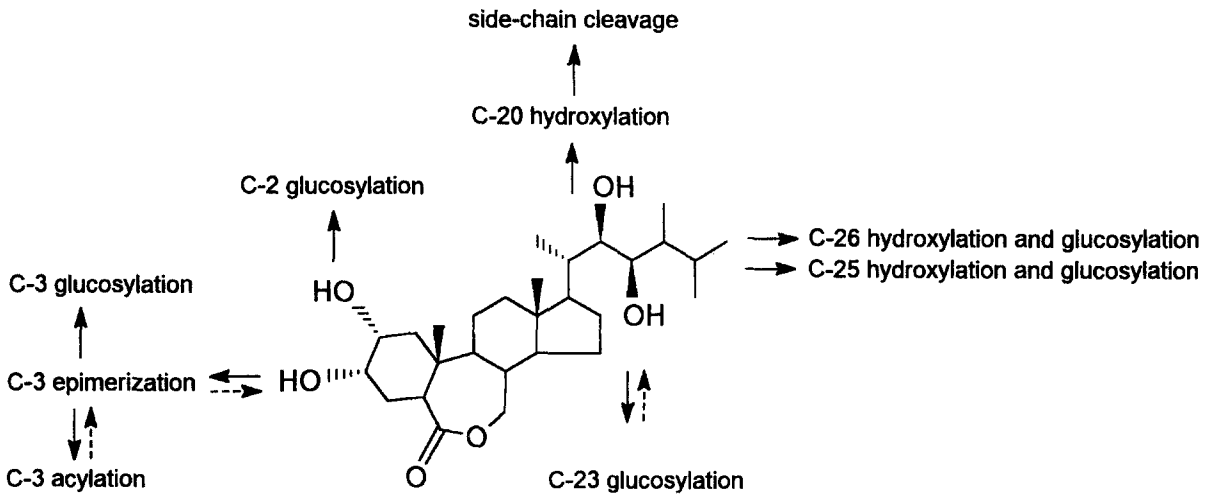

Figure 2. *Pathways of brassinosteroid metabolism.* Broken arrows indicate the possibility of reversible deactivation steps.

Genetic dissection of BR biosynthesis and signaling

Final proof for the essential hormonal function of BRs was provided by molecular genetic analysis of *Arabidopsis* mutants showing defects in photomorphogenesis and cell elongation. As discussed above, traditional bioassays with plant organs or seedlings are often biased because they monitor simple parameters reflecting complex responses controlled by multiple factors. Compared to light-grown plants, dark-grown etiolated seedlings provide a simplified model since many parameters resulting from interactions between light-dependent signaling and metabolic or hormonal pathways are excluded from the analyses. In the dark, plants undergo skotomorphogenic development. After germination, dicotyledonous species show a dramatic elongation of embryonic hypocotyl and root, and feature a closed apical hook of cotyledons. Hypocotyl elongation in the dark is inhibited by various stimuli, including red, far-red, and blue light, ethylene, cytokinin, etc. To genetically dissect the regulatory pathways controlling the elongation of different cell types in the hypocotyl, one may thus screen for mutants which show either normal hypocotyl elongation in the presence of an inhibitory factor (*e.g.* ethylene), or defective hypocotyl elongation in the absence of any inhibitor in the dark. Because seedlings also synthesize growth regulators in the dark, the latter screening approach will score those mutants which have either lost an intrinsic factor promoting cell elongation or are sensitized towards an intrinsic inhibitor of cell elongation in the hypocotyl. Since light is excluded, any mutation which activates light-regulated gene expression in addition to inhibiting cell elongation in the dark will identify regulatory functions acting downstream of the primary photoreceptors. Mutations affecting the biosynthesis, perception, and signaling of ethylene, auxin, and cytokinin (ABA and GA can be excluded because their synthesis is light-dependent) may further help the classification of mutants impaired in hypocotyl elongation. For this purpose, however, only mutations causing auxin insensitivity are available in *Arabidopsis*, whereas the isolation of mutations increasing the sensitivity to ethylene or cytokinin, or abolishing the biosynthesis of these hormones is still awaited.

Thus far, genetic screens for hypocotyl elongation defects in the dark yielded several, distinct types of *Arabidopsis* mutants which appear to conform the above described predictions. Reduction of hypocotyl elongation was thus observed in ethylene overproducing (*eto*) mutants which show the characteristic triple response (see above), as well as in auxin resistant mutants (*axr*) which develop to dwarf or semidwarf, fertile or partially fertile plants in the light. Other mutants feature more dramatic hypocotyl elongation defects with or without opening of the apical hook in

the dark. These mutants are known as constitutive photomorphogenic (*cop*) or de-etiolated (*det*) mutants because they also display activation of different light-regulated genes and the onset of chloroplast development characteristic for the induction of photomorphogenesis. In one class of the *cop/det* mutants a high level of anthocyanin production is detected during embryogenesis and germination. *Fusca* (*fus*) and embryo-lethal (*emb*) mutants selected on the basis of anthocyanin overproduction, resulting from constitutive expression of chalcone synthase, were found allelic to *det1* and several *cop* mutations. Strong alleles of these mutations (*cop1*, *det1*, *fus1* to *12*) cause embryo or early seedling lethality because the growth of plants is arrested after germination in both dark and light. Weak alleles, however, permit full maturation in the light, but cause various levels of growth retardation and occasionally pale-green leaf color. A second class of de-etiolated mutants, including *det2*, *cpd/cbb3*, *dim/cbb1*, *det3*, and *cbb2/bri1* produce normal levels of anthocyanin. Based on their phenotype in the light these mutants fall into two groups: *det2*, *dim*, *cpd*, and *bri1* develop to extreme-dwarf and male-sterile plants with dark-green epinastic leaves, resembling the phenotype of gibberellin deficient (*ga*) and insensitive (*gai*) mutants; plants carrying the *det3* mutation are fertile and show less extreme dwarfism.

Dwarf mutants were traditionally classified as gibberellin responsive dwarfs (*ga*), showing defects in GA biosynthesis, and gibberellin insensitive (*i.e.* genetic) dwarfs (*gai*) possibly affected in GA perception, which are not restored to wild type by GA-treatment. However, neither *ga* nor *gai* mutants display constitutive photomorphogenic and de-etiolated phenotypes in the dark. The key to the physiological background of the extreme dwarfism of *det2*, *dim*, *cpd*, *bri1*, and *det3* was provided by the molecular analysis of the *DET2* and *CPD* loci of *Arabidopsis* which turned out to encode proteins showing homology to animal enzymes involved in steroid biosynthesis (see later). Because *bri1* (brassinosteroid-insensitive) has been isolated by selecting for mutants capable of normal root elongation in the presence of brassinolide (Clouse *et al.*, 1993), all other *bri1*-like mutants were tested for their response to BRs. These experiments showed that inhibition of hypocotyl elongation of the *det2*, *cpd*, and *dim* mutants could be restored to wild type by BRs (but no other plant hormones) indicating that these mutants are impaired in BR biosynthesis. By contrast, BR treatment did not alter the phenotype of the *bri1/cbb2* and *det3* mutants, suggesting that these mutations affect either BR perception or other processes controlling hypocotyl elongation (Kauschmann *et al.*, 1996; Li *et al.*, 1996; Szekeres *et al.*, 1996). Remarkably, castasterone and brassinolide were found to trigger hypocotyl elongation of *axr2* and the *fusca*-type, anthocyanin overproducing, seedling lethal *cop/fus/det1* mutants in the dark, but not in the light. This observation was interpreted to suggest that BRs can overcome the defect of certain regulatory functions required for cell elongation in the dark, but fail to suppress these defects in the light due to a possible antagonistic interaction between BR and light signaling. It was therefore proposed that some of the *COP/FUS/DET1* functions either directly influence BR biosynthesis or interact with BR signaling (Szekeres *et al.*, 1996). Further possible connections between the regulation of BR and GA biosynthesis or signaling were proposed by Kauschmann *et al.* (1996). These suggestions incited a debate since previous models proposed that the *det2* and *det1* (as well as other *cop/fus*) mutations define two parallel pathways specifically involved in light signaling (Chory *et al.*, 1996). Because of the type of genetic screens they derived from, all these mutants are affected in functions acting downstream of photoreceptors, as outlined above. Thus currently it is difficult to assess which of these mutations identify specific lesions in signaling pathways controlling cell elongation by light, BRs, or yet unknown hormonal (*e.g.* ethylene, auxin, cytokinin), stress, metabolic factors, etc. The question how cytokinin can phenocopy some *cop/fus/det1* mutations thus also awaits an answer. To clarify these open questions, it is necessary to determine how the *cop/fus/det1* mutations control BR biosynthesis and/or perception (particularly because earlier data show that BR-promoted cell elongation responses are dependent on the spectral quality of light), as well as how the *COP/FUS/DET1* functions are controlled by BRs and their signaling pathway(s). The existence of such interactions is suggested by the capability of *hy5*, a mutation promoting hypocotyl elongation in the light, to overcome at least partially the hypocotyl elongation defects of *det1* and *det2* mutants in the dark (Chory, 1992). It is apparent that BR-promoted cell elongation responses are saturated in the dark because BR treatment of etiolated wild type plants does not trigger significant hypocotyl elongation response. This also explains earlier observations which indicated the inactivity of BRs in the dark. Regulation of ethylene bio-

synthesis by BRs also implies that ethylene may counterbalance BR-induced hypocotyl elongation in the dark. The fact that nearly all ethylene resistant mutants (*e.g. etr1*) show an enhanced hypocotyl elongation in response to BRs in the dark seems to support this model.

In addition to raising intriguing questions about the control of cell elongation, recent genetic approaches provided a unique opportunity for the molecular analysis of BR biosynthesis. Isolation and sequence analysis of the *DET2* gene revealed that it encodes a protein sharing 40% identity with mammalian steroid 5α-reductases. This, together with the reversion of *det2* phenotype by brassinolide, suggested that *det2* may affect the reduction of campesterol (Li *et al.*, 1996). Evidence for the steroid-reductase activity of DET2 came from expression studies showing interchangability with its human homolog. When expressed in human embryonic kidney cells, DET2 converted progesterone to 4,5-dihydroxyprogesterone and, conversely, the human reductase hS5R was capable of rescuing the *det2* phenotype (Li *et al.*, 1997). Involvement of DET2 in campesterol reduction was confirmed by GC-MS data which, however, indicated that the campestanol content of *det2* null-mutants is still about 10% of the wild type level (Chory *et al.*, 1996). Thus in *Arabidopsis* DET2 may not be the only enzyme capable of producing campestanol. Functional analogy of DET2 and hS5R, as well as *in vitro* conversion analyses suggest that DET2, like other steroid 5α-reductases, requires a $\Delta^{5,6}$-3-ketone substrate for the saturation of the $\Delta^{5,6}$ double-bond, therefore campesterol may not be reduced to campestanol in a single enzymatic step (Li *et al.*, 1997).

The extreme dwarf *Arabidopsis* mutant *cpd* (constitutive photomorphogenic dwarf) mutant and its allele *cbb3* resemble *det2* in many of its phenotypic defects including de-etiolation in the dark. Compared to the wild-type, *cpd* plants are 20 to 30-times smaller due to the severely reduced length of leaves, petioles and peduncle (*fig.* 3). In addition to an overall inhibition of cell elongation, the *cpd* mutation was also demonstrated to cause male sterility and inhibition of xylem differentiation. The *CPD* gene encodes a P450-type hydroxylase (CYP90) showing sequence similarity to mammalian steroid hydroxylases (Szekeres *et al.*, 1996). Treatment with C-23-hydroxylated BRs restored the *cpd* mutant phenotype to wild-type, suggesting that the most likely function of CYP90 is the conversion of cathasterone to teasterone. Bishop *et al.* (1996) found that a dwarf mutant of tomato (*d*) is also deficient in a P450 hydroxylase (CYP85) which is so

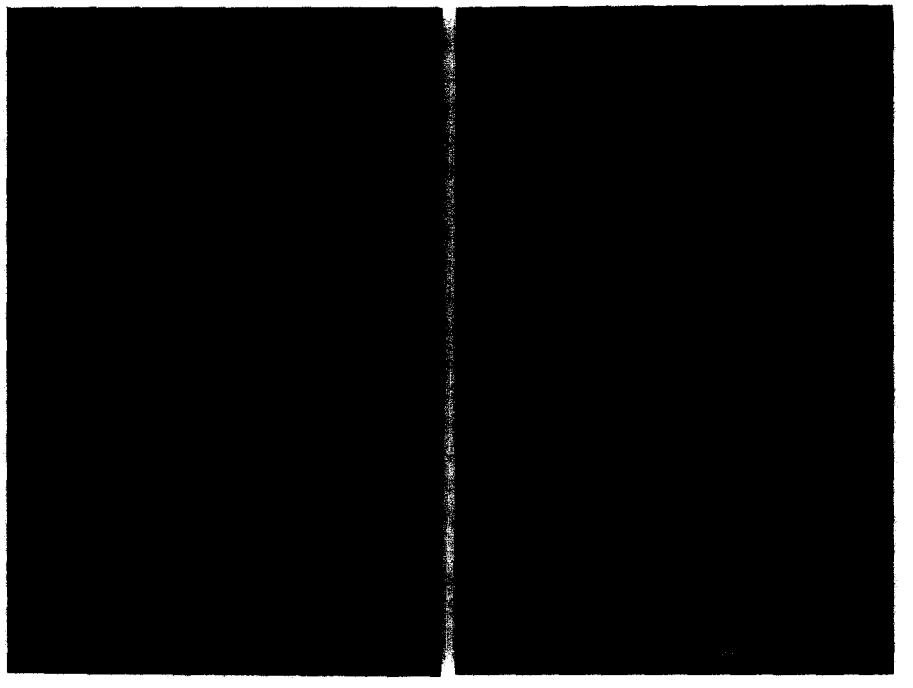

Figure 3. *Phenotypes of wild-type and* cpd Arabidopsis *plants.* **A**, 30-day-old light-grown *cpd* (left) and wild-type (right) plants; **B**, 7-day-old dark-grown *cpd* (right) and wild-type (left) seedlings. Scale bars represent 1 cm.

far the closest relative of CYP90, although at present it is unclear whether the tomato enzyme participates in BR biosynthesis. The severe phenotypic effects of the *cpd* mutations imply that *cpd* plants do not contain biologically active BRs. Hence, C-23 hydroxylation of the side-chain seems to be blocked in both the early and late C-6 oxidation routes indicating a shared function of the CYP90 enzyme between the two parallel branches of the biosynthetic pathway. The possibility that some conversion steps may be carried out by enzymes capable of accepting both oxo- and deoxo-substrates points out potential interdependence of the early and late C-6 oxidation pathways.

The phenotype of another *Arabidopsis* mutant, *dim* (diminuto) can also be suppressed by external application of BRs (Szekeres *et al.*, 1996). In its morphology the mutant is very similar to *cpd* but shows normal repression of light-regulated genes in the dark (Takahashi *et al.*, 1995). The morphology of *dim* and its alleles *cbb1/dwf1-6* is identical with those of *det2* and *cpd* mutants but shows normal repression of light-regulated genes in the dark (Takahashi *et al.*, 1995). The *DIM* gene encodes a protein of unknown function with a putative FAD-binding domain (Mushegian and Koonin, 1995). This sequence motif suggests a possible oxidative role, perhaps in the isomerization reaction leading to typhasterol. Feeding experiments also indicate that the *dim* mutations affects a biosynthetic step before the formation of typhasterol (Szekeres *et al.*, 1996). Whereas the putative FAD-binding domain indicates a possible enzymatic function, a nuclear targeting motif in DIM (Takahashi *et al.*, 1995) could equally suggest a role in BR signaling. Recently two gibberellin insensitive dwarf mutants of garden pea, *lka* and *lkb*, have been shown to be BR deficient or insensitive. These mutants display shortened internodes but otherwise normal appearance in the light and, unlike BR deficient or insensitive *Arabidopsis* mutants, do not undergo de-etiolation in the dark. GC-MS analyses in combination with feeding tests using synthetic BRs suggest a possible lesion in BR biosynthesis prior to the formation of teasterone in the *lkb* mutant (Nomura *et al.*, 1997). The relatively mild phenotypic consequences suggest, however, that the *lkb* mutation either causes only partial impairment of an enzyme function in BR biosynthesis or affects the regulation of the biosynthetic pathway. The other mutant, *lka*, was found partially insensitive to BRs, with a 100-fold weaker brassinolide response than seen in the wild-type.

Studies of BR deficient and insensitive mutants have already provided some insight into the functioning of target genes that are controlled by BR signaling. By analyzing the expression of the BR-responsive *MERI5* and *TCH4* genes in the *cpd/cbb3* and *dim/cbb1/dwf1-6* mutants, Kauschmann *et al.* (1996) found that steady state transcript levels were lower than those in the wild type, but could be normalized by externally applied 24-epibrassinolide. Both *MERI5* and *TCH4* belong to the group of *XET* (xyloglucan endotransglycosylase) genes that control cell wall extensibility (Xu *et al.*, 1995; 1996). These data are in accordance with earlier studies which demonstrated that mRNA of *BRU1* (brassinosteroid up-regulated), a member of the *XET* gene family and a soybean homolog of *MERI5*, accumulates during BR-treatment (Zurek and Clouse, 1994). The *TCH4* transcript was not detectable in the *cbb2* mutant which proved to be allelic to *bri1* (Clouse *et al.*, 1996; Kauschmann *et al.*, 1996). By contrast, *MERI5* is transcribed in the *cbb2* mutant, but shows reduced steady-state transcript levels upon treatment with BRs. Remarkably, *MERI5* mRNA accumulates to high levels in the *cbb2* mutant following treatment with gibberellin. These data suggest the independence of BR and GA action and, as discussed by Clouse (1996), a differential control of the expression of *XET* genes by brassinolide. Analysis of steady-state mRNAs from a wide variety of genes in the *cpd* mutant indicates that BR signaling may play a role in the negative regulation of light- and stress-responsive gene expression in plants. In comparison to the *fus*-type *cop/fus/det1* mutants, deficiency of BR biosynthesis in *det2* and *cpd* results only in slight transcriptional induction of light-regulated genes coding for the small subunit of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b-binding proteins. This may be explained by the observation that the *cop/fus/det1* mutations affect the regulation of several gene sets (Mayer *et al.*, 1996) while *det2* and *cpd* influence more defined signaling functions. By contrast, numerous genes (*e.g.* chalcone synthase, alcohol dehydrogenase, lipoxygenase, etc.) known to be controlled by light, as well as stress factors (*e.g.* anaerobiosis and heat), hormones (*e.g.* cytokinin and ABA) and carbon/glucose catabolic repression show de-repressed expression in both BR biosynthesis and *cop/fus/det1* mutants. As outlined above, further genetic studies in conjunction with the molecular analysis of *cis*-regulatory elements and *trans*-acting factors of well-defined target genes are required to clarify the role played by

BRs in vital regulatory functions, such as the control of cell elongation, xylem differentiation, and photomorphogenesis.

Apart from plants, steroid hormones are also important signal molecules in vertebrates, invertebrates and fungi. Steroid hormone receptors in these organisms are structurally well conserved soluble proteins which control the transcription of their target genes by binding to specific hormone response elements of the promoters. Such a phylogenetically preserved perception mechanism tempted speculations whether BRs also exert their regulatory function through soluble nuclear receptors (Clouse, 1996). So far, however, homology searches of plant genomic and cDNA sequences available in databanks failed to uncover genes that would encode members of the steroid/thyroid hormone receptor family (Li *et al.*, 1997), thus it is possible that BR perception and signaling is mediated by an entirely different mechanism. This intriguing question is expected to be answered by molecular analysis of the BR insensitive *bri1* and *cbb2* mutants (Clouse *et al.*, 1993; 1996; Kauschmann *et al.*, 1996).

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