The Plant Journal (1996) **9**(5), 701–713

Genetic evidence for an essential role of brassinosteroids in plant development

Annette Kauschmann[1], Alison Jessop[1,†], Csaba Koncz[2,3], Miklos Szekeres[3], Lothar Willmitzer[1,‡] and Thomas Altmann[1,‡,*]

[1]Institut für Genbiologische Forschung Berlin GmbH, Ihnestraße 63, D-14195 Berlin, Germany,
[2]Max-Planck-Institut für Züchtungsforschung, Carl-von Linné-Weg 10, D-50829 Köln, Germany, and
[3]Institute of Plant Biology, Biological Research Center of Hungarian Academy of Sciences, H-6701 Szeged, P.O. Box 521, Temesv ri krt. 62, Hungary

Summary

Brassinosteroids which show high structural similarity to animal steroid hormones elicit a variety of growth responses when exogeneously applied to plant tissues. Thus far however, the function of endogeneous brassinosteroids in higher plants has been unclear. This paper describes three extremely dwarfed *Arabidopsis thaliana* mutants, *cbb1* (*dwf1-6*), *cbb2* and *cbb3*, which are impared in cell elongation controlled by brassinosteroids. While *cbb1* (*dwf1-6*) and *cbb3* can be phenotypically normalized to wild-type by feeding with brassinosteroids indicating deficiencies of brassinosteroid biosynthesis, *cbb2* is brassinosteroid-insensitive and defines a function required for further metabolic conversion necessary for biological activity or for perception/signal transduction of these growth-regulating plant steroid hormones. Expression of the *meri5* and *TCH4* genes is low in all three *cbb* mutants and can be restored to wild-type levels by brassinosteroid treatment in the *cbb1* (*dwf1-6*) and *cbb3* mutants but are unaffected in the *cbb2* mutant. These data indicate that brassinosteroids are essential for proper plant development and play an important role in the control of cell elongation.

Introduction

In 1970 a new class of putative plant growth factors was isolated from pollen extracts of *Brassica napus* and named 'brassins' (Mitchell *et al.*, 1970). Brassinolide, a compound exhibiting structural similarity to animal steroid hormones, was shown to be responsible for the growth promoting activity of 'brassins' (Grove *et al.*, 1979). Today over 30 brassinosteroids are known in a wide variety of plant species (Adam *et al.*, 1996; Mandava, 1988; Marquardt and Adam, 1991). Like animal steroids such as estrogen, testosterone or ecdysone, brassinosteroids are composed of a typical steroidal skeleton. The most active brassinosteroids carry either a 6-oxo function or a lactone B ring and are hydroxylated at specific positions. The 7-oxalactone type compounds such as brassinolide or 24-epibrassinolide (see Figure 3, no. 1) appear to be more active in bioassays than the 6-keto type compounds such as castasterone or 24-epicastasterone (see Figure 3, no. 2) and other synthetic brassinosteroids which are often used in biological experiments (e.g. (22S,23S) homobrassinolide, (see Figure 3, no. 3); Adam and Marquardt, 1986; Marquardt and Adam, 1991). However, brassinosteroid activities determined by bioassays, such as the rice lamina inclination test (Wada *et al.*, 1984), the bean second internode bioassay (Mitchell and Livingston, 1968), the *Raphanus sativus* test (Takasuto *et al.*, 1983), or the tomato test (Takasuto *et al.*, 1983) are not always comparable. For example, the relative activities of brassinolide, 28-homobrassinolide, and 28-norbrassinolide as determined in the rice lamina inclination test are different from those observed in the *Raphanus* test (Adam and Marquardt, 1986). It is not clear, whether these differences in sensitivity to the various compounds are species-specific, cell type-specific, or due to a different physiological status of the treated tissues including possible differences in the ability to further metabolize the applied substances.

Physiological studies show that a broad spectrum of cellular responses could be elicited by exogenous application of brassinosteroids to either intact plants or hypocotyl or epicotyl segments. Brassinosteroids stimulate growth through enhanced cell divisions and cell elongation (Mandava, 1988; Marquardt and Adam, 1991). In several systems they act synergistically with auxins (Katsumi, 1985; Kim *et al.*, 1990; Yopp *et al.*, 1981) and additively with gibberellins (Katsumi, 1985; Mandava *et al.*, 1981). Further observed responses to brassinosteroid application include enhancement or retardation of root growth, unrolling of leaves, differentiation of xylem vessels, enhanced ethylene production, membrane hyperpolarization, increased ATPase activity, enhanced DNA, RNA and protein synthesis, stimulation of photosynthetic activity, and changes in the balance of other endogeneous phytohormones (Arteca

Received 7 December 1995; revised 21 February 1996; accepted 7 March 1996.
*For correspondence (fax +49 331 977 2301; e-mail: altmann@mpimg-berlin-dahlem.mpg.de).
†Present address: C.I.D.-C.S.I.C., Dept. Genetica Molecular, Jordi Girona 18-26, 08034 Barcelona, Spain.
‡Present address: Max-Planck-Institut für Molekulare Pflanzenphysiologie, Karl-Liebknecht Straße 25, Haus 20, D-14476 Golm, Germany.

et al., 1988; Braun and Wild, 1984; Cerana *et al.*, 1983; Clouse and Zurek, 1991; Clouse *et al.*, 1993; Eun *et al.*, 1989; Henry *et al.*, 1981; Iwasaki and Shibaoka, 1991; Kalinich *et al.*, 1985; Romani *et al.*, 1983; Schlagnhaufer *et al.*, 1984; Wada *et al.*, 1985; Yopp *et al.*, 1981). Although differential synthesis of many proteins has been detected in elongating soybean epicotyles treated with brassinolide (Clouse *et al.*, 1992), thus far only one gene, *BRU1* which shows significant sequence homology to xyloglucan endotransglycosylase, is known to be specifically regulated by brassinosteroids (Zurek and Clouse, 1994).

Although the presence of brassinosteroids in plant tissues, their efficient elicitation of growth responses, and the induction of specific physiological changes were documented, no information about the importance of this group of steroidal compounds with respect to the regulation of developmental processes in plants is currently available. In contrast, the role of 'classical' plant hormones such as auxins (Davies, 1988; Evans, 1984), gibberellins (Hooley, 1994), cytokinins (Brzobohaty' *et al.*, 1994; Kaminek, 1992), ethylene (Abeles *et al.*, 1992; Zarembinski and Theologis, 1994), or abscisic acid (Giraudat *et al.*, 1994) in plant development has been well proven through studies of mutants with biosynthetic or perception/signal transduction deficiencies (Hobbie and Estelle, 1994; Hobbie *et al.*, 1994). Further evidence has been obtained through the modification of endogenous hormone levels by expression of bacterial genes in transgenic plants (Klee and Estelle, 1991) and the exogenous application of these substances or their antagonists in addition to the determination of the endogenous hormone levels. In particular, the extension growth of plant cells was shown to be regulated by auxins (Rayle and Cleland, 1992) and gibberellins (MacMillan and Phinney, 1987).

Here we describe the characterization of three extremely dwarfed mutants, *cbb1* (*dwf1-6*), *cbb2*, and *cbb3*, isolated by transposon mutagenesis in *Arabidopsis thaliana* (Altmann *et al.*, 1995) and demonstrate that the *cbb* mutations affect the biosynthesis and perception of brassinosteroids which are required for the proper control of plant development.

Results

De-etiolation and reduced cell expansion in the mutants cbb1, cbb2, *and* cbb3

Three mutants named *cabbage1*, *cabbage2*, and *cabbage3* (*cbb1*, *cbb2*, *cbb3*) exhibiting a strongly stunted axis, a reduced root system, small dark green leaves and a very compact rosette structure (Figure 1) were isolated from an *Arabidopsis* mutant collection generated by transposon mutagenesis (Altmann *et al.*, 1995). Soil-grown *cbb1* mutant plants illuminated with white light showed a maximum height of 6.18 ± 0.74 cm in comparison with 28.68 ± 1.67 cm for the wild-type plants, while *cbb2* and *cbb3* plants only reached a size of less than 1 cm. Furthermore, *cbb1*, *cbb2*, and *cbb3* plants exhibited a strong reduction of leaf expansion, the latter two being most dramatically affected. Plants carrying the *cbb2* or *cbb3* mutation also barely survived in soil and they rarely developed visible inflorescences which were extremely stunted and contained sterile flowers (Figure 1). The reduction in elongation growth was even more pronounced during germination and growth in darkness (skotomorphogenesis). In comparison with wild-type plants which developed a long hypocotyl and small, closed cotyledons in the dark (Wei *et al.*, 1994), dark-grown *cbb1*, *cbb2* and *cbb3* seedlings displayed a short hypocotyl, opened cotyledons and the emergence of primary leaves after prolonged growth in darkness (Figure 1e) thus resembling other *de-etiolated* (*det*) (Chory, 1993) or *constitutive photomorphogenesis* (*cop*) (Deng, 1994) *Arabidopsis* mutants. In the dark, the hypocotyl elongation of the *cbb1* mutant was about 40% of the wild-type and the *cbb2* and *cbb3* seedlings developed even shorter hypocotyls (Table 1). These skotomorphogenetic characteristics of the *cbb* mutants were in contrast to those of other dwarfed *Arabidopsis* mutants impaired in gibberellin biosynthesis (*ga4-1* and *ga5-1*) and gibberellin perception (*gai-1*) which were tested in parallel. They exhibited wild-type skotomorphogenesis with closed cotyledons and only a minor reduction of hypocotyl elongation. When grown for 11 days in darkness *ga4-1* showed 78% (16 ± 2.2 mm), *ga5-1* 67% (13.9 ± 2.1 mm), and *gai-1* 56% (11.5 ± 0 mm) of the length of the wild-type hypocotyl.

Microscopic measurements of the size and number of cells in longitudinal sections through the cotyledons of 7-day-old wild-type, *cbb1*, *cbb2*, and *cbb3* seedlings grown in the light revealed that the reduced size of the mutant seedlings was primarily due to a reduction of cell size rather than the presence of fewer cells (Table 2). The *cbb1* mutant showed an intermediate size of cotyledonary mesophyll cells in comparison with the wild-type and *cbb2* and *cbb3*. A similar reduction in cell size was also seen in the hypocotyl of *cbb1*, *cbb2*, and *cbb3* mutant seedlings. In the case of *cbb2* and *cbb3*, the cells were rather isodiametric in contrast to the longitudinally elongated cells of wild-type seedlings (Figure 2). Although it can not be excluded that in the later stages of development the number of cells within certain organs might be reduced, it is clear that a major consequence of the genetic defects in the *cbb* mutants is reduced cell expansion growth.

Genetic analysis of the cbb *mutants*

Allelism tests revealed that the *cbb1*, *cbb2*, and *cbb3* mutants were affected at different genetic loci (for details see Experimental procedures). Through crosses of hetero-

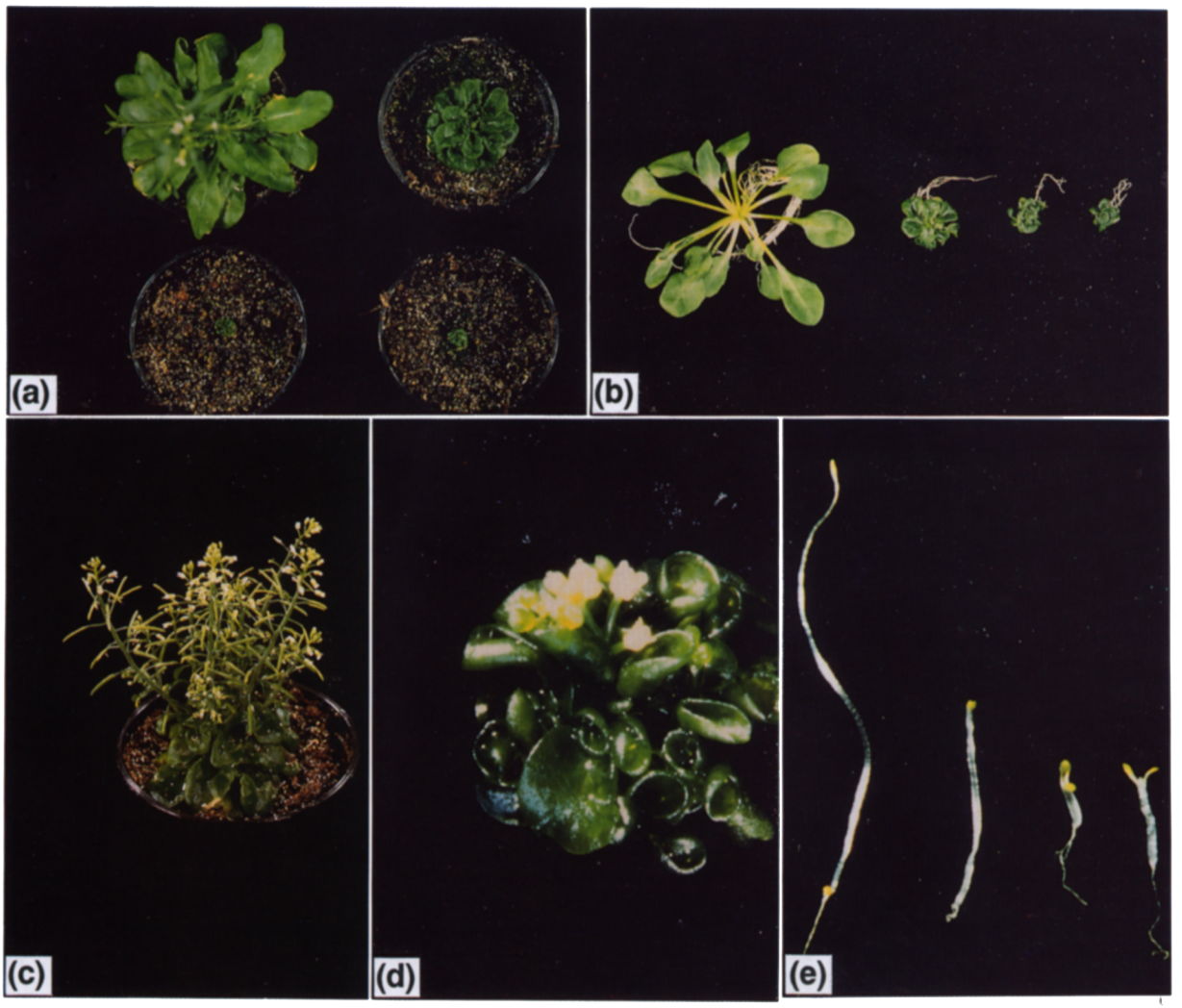


Figure 1. Morphological characteristics of *A. thaliana* wild-type and *cabbage* mutants.
(a) Top left to bottom right: wild-type, *cbb1* (*dwf1-6*), *cbb2*, *cbb3*, 3 weeks old, grown in soil.
(b) Left to right: wild-type, *cbb1* (*dwf1-6*), *cbb2*, *cbb3*, 3 weeks old, grown in half-concentrated MS medium.
(c) *cbb1* (*dwf1-6*), 5 weeks old with a fertile inflorescence, grown in soil.
(d) *cbb2*, 6 weeks old with a sterile and stunted inflorescence, grown in half-concentrated MS medium.
(e) Skotomorphogenesis of wild-type and mutant plants. Left to right: wild-type, *cbb1* (*dwf1-6*), *cbb2*, *cbb3*. Seedlings were germinated and grown for 11 days in darkness in half concentrated MS medium (Murashige and Skoog, 1962). While wild-type plants produce an elongated hypocotyl with unopened, etiolated cotyledons, the *cbb2* and *cbb3* mutant plants show almost no hypocotyl elongation and fully opened cotyledons. The *cbb1* (*dwf1-6*) mutant exhibits an intermediate phenotype.

Table 1. Hypocotyl-length of illuminated and non-illuminated *A. thaliana* wild-type and *cabbage* mutant seedlings

	Light-grown plants		Dark-grown plants	
Seedling type	mm	% of wt	mm	% of wt
Wild-type	3.0 ± 0.63	100	18 ± 0.14	100
cbb1 (*dwf1-6*)	1.2 ± 0.24	40	8 ± 0.08	44.44
cbb2	0.51 ± 0.13	17	1.1 ± 0.11	6.1
cbb3	0.54 ± 0.10	18	1.98 ± 0.03	10.55

Seedlings were germinated and grown for 11 days on half-concentrated MS (Murashige and Skoog, 1962) medium under a 16 h day/8 h night regime (light-grown plants) or in continuous darkness (dark-grown plants) before measurement of the hypocotyl length was performed. Each value represents the mean of 10 independent measurements with standard deviation; wt: wild-type.

Table 2. Cell number and cell size of cotyledonary mesophyll cells of *A. thaliana* wild-type and *cabbage* mutant seedlings

Seedling type	Cell length (μm)	Cell width (μm)	Number of cell layers vertical	Number of cells longitudinal
Wild-type	74.25 ± 4.34	66.66 ± 10.08	6	60.75 ± 3.92
cbb1 (*dwf1-6*)	71.25 ± 8.53	48.00 ± 5.71	6	53.14 ± 2.60
cbb2	44.50 ± 4.04	36.75 ± 5.37	6	57.00 ± 3.60
cbb3	50.00 ± 7.07	35.75 ± 3.73	6	65.00 ± 2.90

Seven-day-old seedlings grown under a 16 h day/8 h night regime on half-concentrated MS (Murashige and Skoog, 1962) medium were embedded in plastic (Technovit 7100) and sections were used for light microscopic analysis. The sizes of cotyledonary mesophyll cells of at least six individuals of each mutant and the wild-type were measured. In addition, the number of mesophyll cells in longitudinal sections of cotyledons and the number of cell layers including the adaxial and abaxial epidermis were determined. The values represent means with standard deviation.

Table 3. Treatment of *A. thaliana* wild-type and *cabbage* mutant plants with different phytohormones and phytohormone inhibitors

		Reaction of wild-type and *cbb* mutants	
Substance/activity	Concentration range	Similar	Different
GA3, Gibberellin	10^{-4} – 10^{-8} M	+	
GA4, Gibberellin	10^{-6} – 10^{-8} M	+	
IAA, Auxin	10^{-6} – 10^{-8} M		+[a]
2,4-D, Auxin	10^{-6} – 10^{-8} M	+	
Kinetin	10^{-6} – 10^{-8} M		+[b]
Jasmonic acid	7 μM	+	
pCIB, antiauxin	10^{-7} – 10^{-6} M		+[c]
TIBA, auxin transport inhibitor	10^{-6} – 10^{-8} M	+	
Ethrel, ethylene releasing compound	10^{-3} – 10^{-4} M	+	
AIB, blocker of ethylene biosynthesis	10^{-6} – 10^{-8} M	+	
$AgNO_3$, blocker of ethylene perception	100 μM	+	

Summary of the treatments of wild-type and mutant plants with phytohormones and phytohormone inhibitors (all substances were purchased from Sigma, Deisenhofen). Seeds were germinated in half-concentrated MS (Murashige and Skoog, 1962) medium and seedlings were transferred after 1 week to fresh medium containing different concentrations of the indicated substances. Phenotypic alterations were monitored over a period of 2 weeks. Only minor differences in the reactions of mutant plants in comparison to wild-type were detected.

[a, b]wild-type more pale than *cbb1* (*dwf1–6*), *cbb2*, and *cbb3*. [c]Petioles of *cbb1* (*dwf1–6*) plants slightly elongated.

zygous plants and phenotypic analysis of the F_1 progeny, allelism between *cbb3* and another extreme dwarf mutant, *cpd*, recovered by T-DNA insertion mutagenesis (Szekeres *et al.*, unpublished) was detected. Using molecular genetic markers (Bell and Ecker, 1994; Konieczny and Ausubel, 1993), the *cbb1* locus was mapped to the upper arm of chromosome 3 (*cbb1*: 16.71 ± 6.9 cM to nga 172, 3.9 ± 2.3 cM to nga 162, 21.18 ± 9.3 cM to GAPA). Similarly, the *cbb2* mutation was mapped to the lower end of chromosome 4 (*cbb2*: 2.9 ± 1.6 cM to DHS1), and the *cbb3* mutation was localized on the upper arm of chromosome 5 (*cbb3*: 7.6 ± 3.1cM to nga 151, 11.29 ± 6.6 cM to nga 106). These genetic mapping data indicate that the *cbb* mutants were different from the *de-etiolated* mutants *det1*, *det2*, and *det3* and the *constitutive photomorphogenesis* mutants *cop1* and *cop9* (as deduced from map positions compiled in the AIMS database, Michigan State University, and the AAtDB data base, Massachusetts General Hospital, Boston).

The cbb *mutants can not be normalized by known phytohormones or their antagonists*

Mutations affecting gibberellin biosynthesis or perception (Koornneef and van der Veen, 1980; Koornneef *et al.*, 1985), auxin signaling (Wilson *et al.*, 1990), or the control of ethylene response (Kieber *et al.*, 1993) were demonstrated to cause dwarfism in *Arabidopsis*. Therefore, the *cbb* mutants were tested for their response to a set of known phytohormones and their inhibitors including auxins, auxin antagonists, gibberellins, ethrel (an ethylene-releasing compound), ethylene inhibitors, cytokinins, and jasmonic acid (Table 3). When added to MS medium (Murashige and Skoog, 1962) used for aseptic germination of the mutant or wild-type seeds none of these substances restored a wild-type phenotype in any of the mutants, nor did any of them result in a *cbb* mutant phenocopy of the wild-type. Furthermore, the reactions of seedlings of all three *cbb*

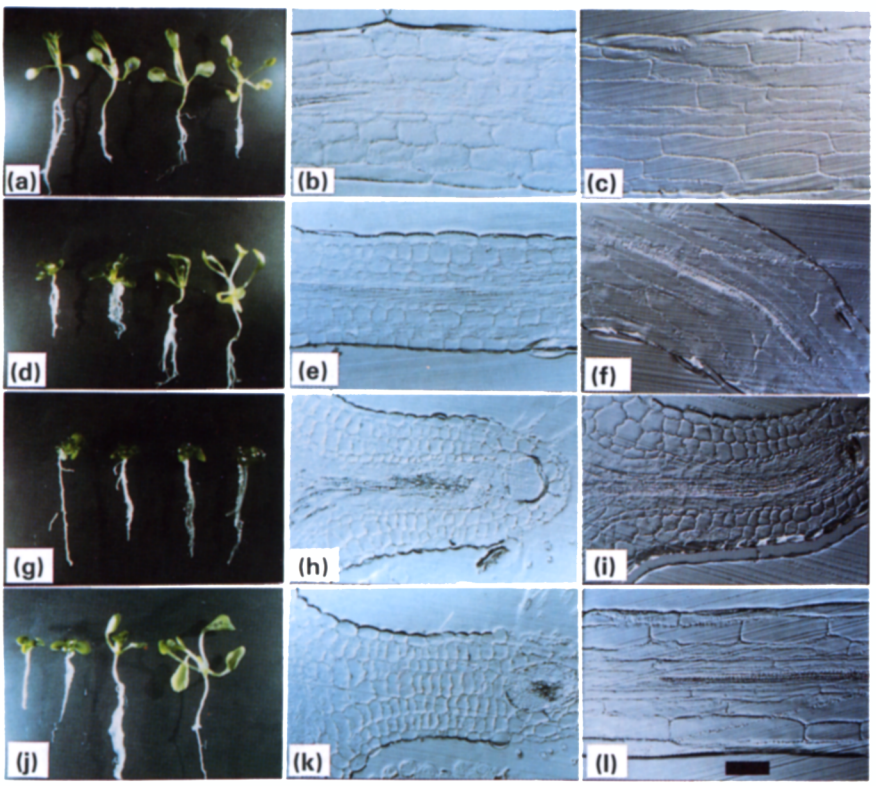
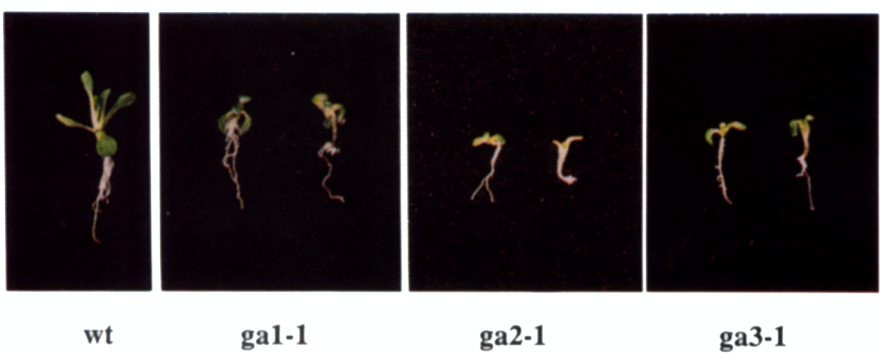


Figure 2. Influence of the brassinosteroid 24-epicastasterone on the (cell) morphology of *A. thaliana* wild-type and *cabbage* mutants. The plants were grown in a 16 h day/8 h night regime for 14 days on half-concentrated MS medium in the absence or presence of different concentrations of 24-epicastasterone (a, d, g, j: from left to right: 0, 0.01, 0.1, 1.0 μM; b, e, h, k: 0 μM; c, f, i, l: 1 μM). For microscopical analysis the plantlets were embedded in plastic (Technovit 7100) and sections were prepared with a Reichert-Jung Biocut microtome and examined under a Zeiss Axiophot light-microscope. The bar represents 100 μm.
a,b,c: wild-type; d, e, f: *cbb1* (*dwf1-6*); g, h, i: *cbb2*; j, k, l: *cbb3*.



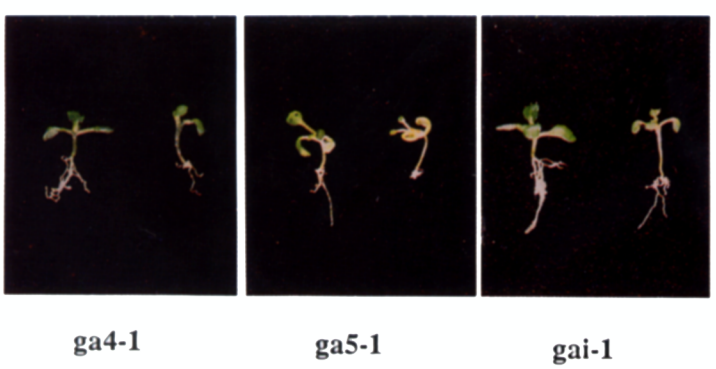


Figure 6. Brassinosteroid treatment of gibberellin-deficient and -insensitive mutants of *A. thaliana*.
Gibberellin-deficient (*ga1-1, ga2-1, ga3-1, ga4-1*, and *ga5-1*) and gibberellin-insensitive (*gai-1*) mutant plants were germinated and grown for 11 days in a 16 h day/8 h night regime on half-concentrated MS medium supplemented with (plant on the right) or without (plant on the left) 0.5 μM 24-epibrassinolide. An untreated wild-type plant grown under the same conditions is shown for comparison (wt).

mutants to these treatments were similar to those of wild-type plants (Table 3). The dwarfism of the *cbb* mutants therefore could not be attributed to a consequence of a deficiency in the biosynthetic pathway (or an overproduction in the case of ethylene) of any of these phytohormones. Also, a general insensitivity to these growth regulators (or a hypersensitivity to ethylene) could be excluded. In particular, the GA_3 and GA_4 treatments failed to normalize the three *cbb* mutants to wild-type phenotype but elicited responses from the mutant plants visible as a limited elongation of the leaf petioles and a pale leaf coloration. The *cbb* mutants are therefore different from the gibberellin-deficient (Koornneef and van der Veen, 1980) and gibberellin-insensitive (Koornneef *et al.*, 1985) *A. thaliana* mutants which were tested under similar conditions.

The cbb *mutations affect growth responses to brassinosteroids*

In sharp contrast to the above-mentioned hormone/hormone antagonist treatments, a conversion of *cbb1* and *cbb3* mutant plants to a phenotype nearly indistinguishable from that of wild-type plants grown under the same conditions was achieved by the addition of brassinosteroids to the germination medium (Figure 2). Both mutants reacted in a dose-dependent manner by elongation of their hypocotyls and petioles, expansion of their leaves, and shortening of their roots (a response to brassinosteroids previously described for *A. thaliana* by Clouse *et al.*, 1993). Histological analysis of hypocotyl segments of 14-day-old *cbb1* and *cbb3* seedlings germinated and grown in the presence of 1 μM 24-epicastasterone, revealed the presence of longitudinally expanded cells similar in size and shape to the cells of brassinosteroid-treated wild-type plants (Figure 2). Hypocotyl cells of 24-epicastasterone-treated wild-type were also increased in size in comparison with the untreated wild-type plants. The phenotype of the *cbb2* seedlings, in contrast, was not influenced by the 24-epicastasterone treatment. Similar results were observed when seedlings were transferred to brassinosteroid-containing medium after germination and growth for 7 days on medium without brassinosteroids, following the procedure used in the aforementioned hormone / hormone antagonist treatments (data not shown). In this case, leaves of *cbb1* and *cbb3* mutant plants which were formed after the onset of the brassinosteroid supply showed strong petiole elongation and leaf blade expansion. In order to define the specificity of the responses of the three mutants, a set of different steroidal compounds was tested for activity with respect to the normalization of the mutant phenotypes. These included three active brassinosteroids ((22S-23S) homobrassinolide (Figure 3, no. 3); 24-epicastasterone (Figure 3, no. 2); 24-epibrassinolide (Figure 3, no. 1)) which are frequently used in studies on brassinosteroid action, plant sterols that do not show activity in brassinosteroid bioassays (β-sitosterol (Figure 3, no. 7); stigmasterol (Figure 3, no. 8); stigmastanol (Figure 3, no. 9); stigmasta-4,22-dien-3on (Figure 3, no. 10)), as well as the fungal sterol ergosterol (Figure 3, no. 6), the insect hormones α-ecdysone (Figure 3, no. 4) and β-ecdysone (Figure 3, no. 5), and cholesterol (Figure 3, no. 11). While *cbb2* seedlings retained their mutant morphology regardless of which of these substances was added to the growth medium, *cbb1* and *cbb3* seedlings were converted to wild-type growth by (22S-23S) homobrassinolide (Figure 3, no. 3), 24-epicastasterone (Figure 3, no. 2), and 24-epibrassinolide (Figure 3, no. 1) but not by any of the other sterols (Figure 3, no. 4–11). The degree of conversion was concentration-dependent as shown by the gradual increase of hypocotyl length of *cbb1* and *cbb3* seedlings when germinated and grown for 11 days on media containing different concentrations of 24-epicastasterone (Figure 4). Furthermore, when added to the media different efficacies of the three brassinosteroids were observed with optimum concentrations for phenotypic normalization of *cbb1* and *cbb3* of 0.1–0.5 μM for (22S–23S) homobrassinolide (Figure 3, no. 3), 0.1–1 μM for 24-epicastasterone (Figure 3, no. 2), and 0.01–0.1 μM for 24-epibrassinolide (Figure 3, no. 1), respectively. The naturally occurring brassinosteroid 24-epicastasterone (Figure 3, no. 2), was found to restore the growth of *cbb1* and *cbb3* in a manner that most resembled untreated wild-type seedlings (Figure 2). Interestingly, a shortening of the roots of wild-type, *cbb1*, and *cbb3* seedlings was only observed upon treatment with (22S–23S) homobrassinolide (Figure 3, no. 3), and 24-epibrassinolide (Figure 3, no. 1; data not shown). In addition to the restoration of the growth defect of light-grown *cbb1* and *cbb3* seedlings, brassinosteroid feeding also stimulated the hypocotyl elongation of the *cbb1* and *cbb3* mutants in the dark. The *cbb3* mutant exhibited wild-type hypocotyl length in the presence of 0.5 μM 24-epibrassinolide. In comparison, the *cbb1* mutant showed a reduced hypocotyl elongation response to 24-epibrassinolide in the dark (Figure 5). In these experiments, opening of the cotyledons in mutant seedlings was not suppressed by the brassinosteroid treatment (data not shown).

Gibberellin-deficient or -insensitive mutants are not normalized by brassinosteroid feeding

To provide further evidence that the *cbb* mutants were different from the known dwarfed *A. thaliana* mutants affected in gibberellin biosynthesis or perception, seeds of the *ga1-1, ga2-1, ga3-1, ga4-1, ga5-1,* and the *gai-1* mutants were germinated and cultivated in the presence of 0.5 μM 24-epibrassinolide for 11 days (Figure 6). Despite different degrees of increased hypocotyl length observed for all mutants (data not shown), no phenotypic normalization

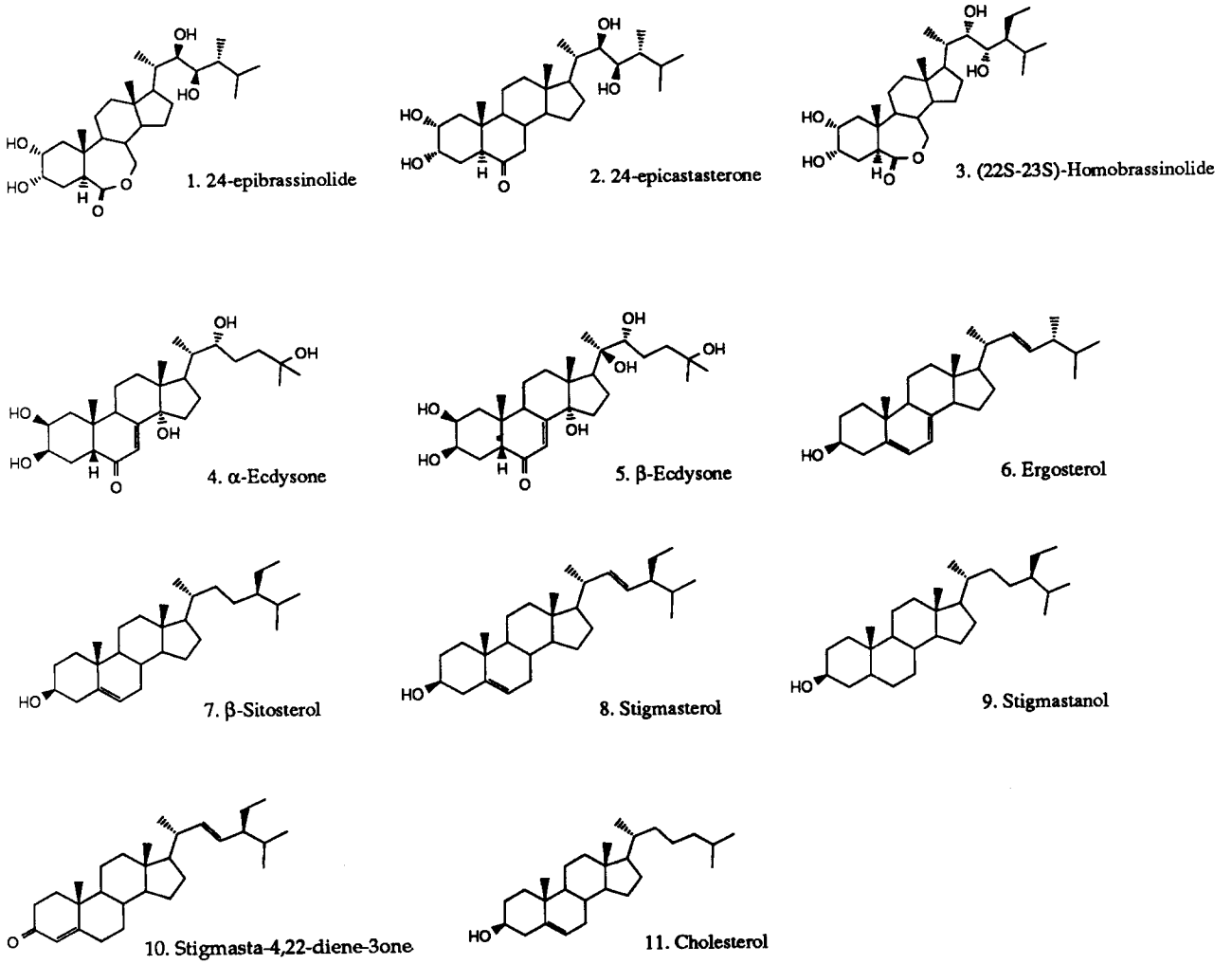


Figure 3. Structural formulas of applied steroids.
Active brassinosteroids: 1, 2, 3.
Other sterols: 4, 5, 6, 7, 8, 9, 10, 11.

(petiole elongation, leaf blade expansion) was achieved through this treatment in any case.

The cbb *mutations affect the expression of* TCH4, meri5 *and* γ-TIP

The brassinosteroid feeding experiments strongly suggested that a deficiency in endogenous brassinosteroids (in the case of *cbb1* and *cbb3*) or an insensitivity to brassinosteroids (in the case of *cbb2*) was responsible for the reduced cell expansion in the mutants. The *TCH4* (Braam and Davis, 1990) and the *meri5* (Medford *et al.*, 1991) genes which show strong sequence homology to xyloglucan endotransglycosylase, an activity potentially involved in cell-wall loosening, were therefore tested for their expression. Total RNA was prepared from seedlings of wild-type and the three *cbb* mutants grown for 13 days in the light on half-concentrated MS medium supplemented with or without 0.5 μM 24-epibrassinolide. Both genes were expressed in brassinosteroid-treated and untreated wild-type seedlings (Figure 7a). In comparison, all three *cbb* mutants grown in the absence of brassinosteroids showed lower levels of *meri5* expression and considerable reduction in the amounts of steady-state *TCH4* mRNA. Brassinosteroid treatment restored the levels of *TCH4* and *meri5* mRNA in the *cbb1* and *cbb3* mutants. Seedlings of the *cbb2* mutant, however, were again clearly different in their reponse to the brassinosteroid application as transcript levels of *TCH4* and *meri5* were unaffected.

A similar RNA hybridization analysis performed with 3-week-old wild-type and *cbb* mutant seedlings grown for 1 week in the presence of 1 μM GA_3 indicated that the *meri5* gene is also inducible by gibberellin (Figure 7b). The levels of steady-state *meri5* mRNA were increased in both wild-type and *cbb* mutant seedlings, including *cbb2*, by GA_3-treatment in comparison with the GA_3 untreated con-

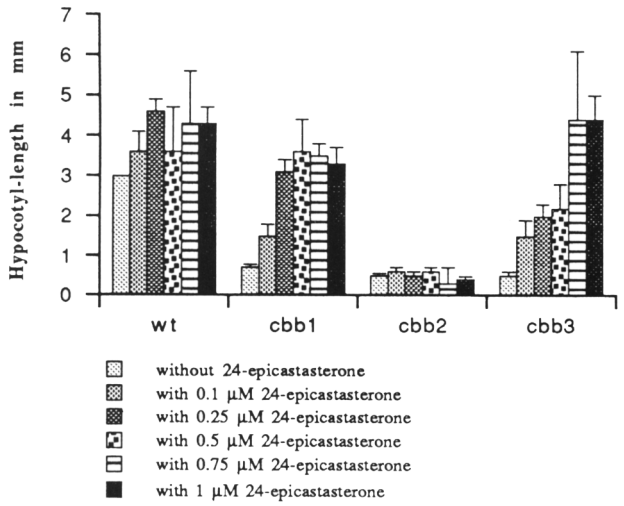


Figure 4. Hypocotyl length of 11-day-old plants treated with 24-epicastasterone.
The plants were grown in a 16 h day/8 h night regime on half-concentrated MS medium supplemented with different concentrations of 24-epicastasterone as indicated in the figure. After 11 days the hypocotyl length of the plantlets was measured. The values represent means with standard deviation.

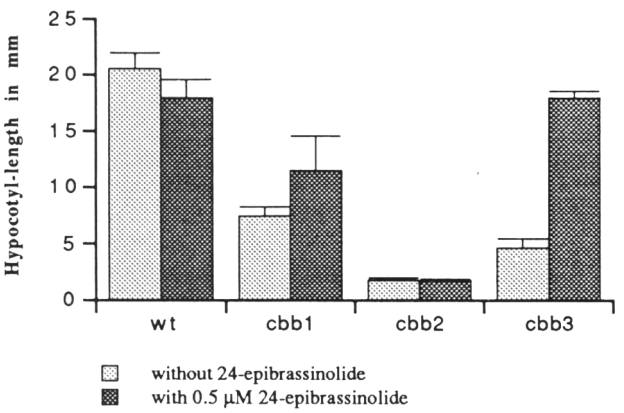


Figure 5. Hypocotyl length of 11-day-old dark-grown of *A. thaliana* wild-type and *cabbage* mutant plants treated with 24-epibrassinolide.
The plants were grown in darkness on half-concentrated MS medium supplemented with or without 0.5 µM 24-epibrassinolide. After 11 days the hypocotyl length of the plantlets was measured. The values represent means with standard deviation.

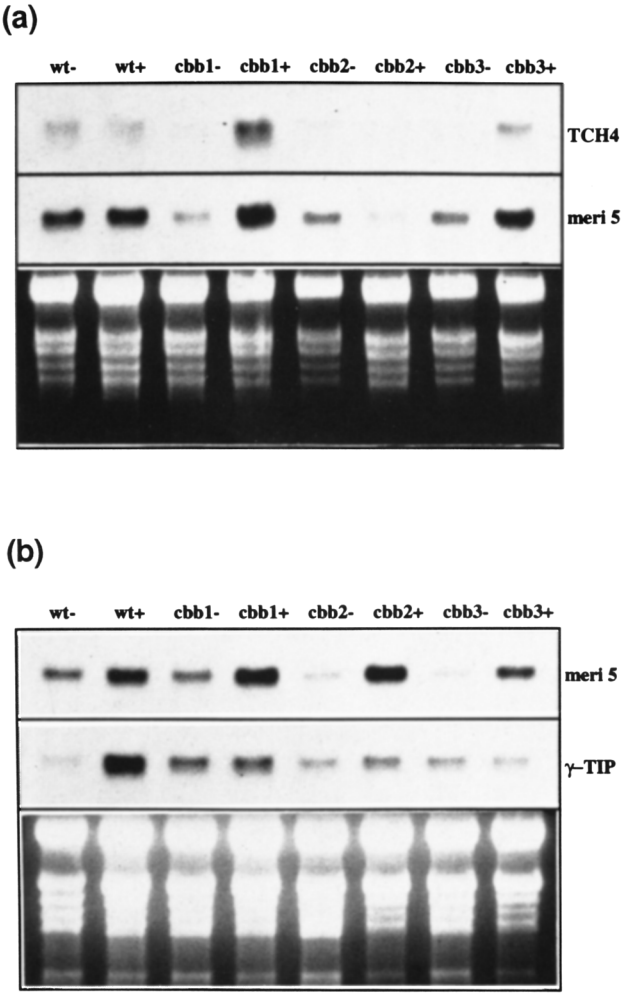


Figure 7. RNA blot analysis of 24-epibrassinolide-(a) and gibberellin-(b) induced expression of *TCH4*, *meri5* and γ-*TIP* genes of *A. thaliana* wild-type and *cabbage* mutants.
(a) RNA was isolated from 13-day-old plants grown on half-concentrated MS medium supplemented with (+) or without (–) 0.5 µM 24-epibrassinolide. Hybridization was performed with probes derived from the *TCH4* gene and the *meri5* gene. The ethidium bromide-stained gel is shown to demonstrate that equal amounts of total RNA were loaded in each lane.
(b) RNA was isolated from 3-week-old plants cultivated on half-concentrated MS medium, which during the last week were grown in the presence (+) or absence (–) of 1 µM GA_3. Probes derived from the *meri5* gene and the γ-*TIP* gene were used for hybridization. The ethidium bromide-stained gel is shown to demonstrate that equal amounts of total RNA were loaded in each lane.

trols (Figure 7b). Intriguingly, the transcription of the gibberellin induced γ-*TIP* gene (Höfte *et al.*, 1992; Phillips and Huttly, 1994) showed a characteristic de-repression in the *cbb* mutants (Figure 7b). In the absence of GA_3 the steady state mRNA levels from the γ-*TIP* gene were elevated in the *cbb* mutants in comparison with wild-type. In the wild-type, the expression of the γ-*TIP* gene was strongly induced by the GA_3 treatment, which resulted only in a slight increase of γ-*TIP* mRNA levels in the *cbb* mutants.

Discussion

A set of three dwarfed mutants, *cbb1*, *cbb2*, *cbb3*, is described which are mainly affected in cell expansion. While *cbb1* showed an intermediate reduction in size between the wild-type and *cbb2* and *cbb3*, both macroscopically as well as on the cellular level, the latter two exhibited a rather extreme dwarf phenotype. The strong reduction in cell expansion was apparent in plants grown in the light but was even more obvious after germination

and growth in darkness. The observed inhibition of hypocotyl elongation, the opening of the cotyledons, and the emergence of primary leaves are characteristic features of *de-etiolated* (*det*) or *constitutive photomorphogenesis* (*cop*) mutants. The genetic mapping, however, excludes that any of the *cbb* mutants would be allelic to either *det1*, *det2*, *det3*, *cop1*, or *cop9*. Furthermore, the characteristic accumulation of anthocyanins and the adult lethality of the *cop8*, *cop10*, and *cop11 mutants (Wei et al.*, 1994) clearly distinguishes them from the *cbb* mutants since these do not show this purple coloration, complete a full life cycle (although being infertile), and have an extended longevity in comparison with wild-type plants (data not shown). Other *constitutive photomorphogenesis* mutants such as *cop2*, *cop3*, and *cop4* also differ from the *cbb* mutants because they show almost no reduction in hypocotyl length in the dark and they are morphologically nearly identical to wild-type at the adult stage (Hou *et al.*, 1993).

Arabidopsis mutants defective in gibberellin biosynthesis (Koornneef and van der Veen, 1980), mutants insensitive to gibberellins (Koornneef *et al.*, 1985) or auxins (Wilson *et al.*, 1990), and mutants with constitutive ethylene response (Kieber *et al.*, 1993) have been shown to exhibit a dwarf phenotype. Phytohormone/phytohormone antagonist feeding experiments performed with the *cbb* mutants in the present study, however, demonstrated that none of these three mutants were either deficient in or insensitive to auxins, gibberellins, cytokinins, or jasmonic acid, or exhibit ethylene overproduction or hypersensitivity. In the latter two cases the appearance of an exaggerated apical hook would have been expected (Guzman and Ecker, 1990; Kieber *et al.*, 1993), the opposite of which was observed. The *cbb* mutants are thus different from all other known *A. thaliana* mutants impaired in phytohormone biosynthesis or perception. These observations corroborate previous reports on the characteristics of the *dim* mutant (Takahashi *et al.*, 1995), an allele of *dwf1* (Feldmann *et al.*, 1989), which was shown to be allelic to *cbb1* (Altmann *et al.*, 1995). In accordance with accepted procedures of *Arabidopsis* nomenclature *cbb1* was therefore renamed and should furthermore be referred to as *dwf1-6*.

Indications about the biochemical defect resulting from the genetic lesion in *dwf1-6* (*cbb1*) and *cbb3* were obtained by a set of steroid feeding experiments which showed a normalization of the phenotype of *dwf1-6* (*cbb1*) and *cbb3*, both morphologically and on the level of gene expression (see below), through the administration of brassinosteroids. In agreement with previous reports (Adam and Marquardt, 1986), 24-epibrassinolide (Figure 3, no. 1) exhibited a higher activity than 24-epicastasterone (Figure 3, no. 2) or (22S–23S) homobrassinolide (Figure 3, no. 3). Normalization of the phenotype with respect to hypocotyl elongation was also achieved with dark-grown seedings. In these treatments *cbb3* showed a slightly stronger response than *dwf1-6* (*cbb1*). The observation that wild-type seedlings were not changed in the degree of hypocotyl elongation in darkness through brassinosteroid feeding is in agreement with previous reports indicating a lack of growth response to brassinosteroids in darkness (Mandava, 1988). The finding, that both *dwf1-6* (*cbb1*) and *cbb3* seedlings were responsive to 24-epibrassinolide in darkness, however, points rather to a predominant role of brasssinosteroids in the regulation of skotomorphogenesis. This apparent contradiction might be explained by the assumption that the growth response is saturable and a full induction is already achieved by the dark stimulus in the wild-type. To elucidate this topic further, the use of a recently described inhibitor of brassinosteroid action (Kim *et al.*, 1995) would be useful and complementary to a further analysis of the mutants described here. Also, the observed lack of normalization of the cotyledon phenotype of the mutants in darkness, which might be due to insufficient transport of the brassinosteroids taken up from the medium to the cotyledons or a requirement of brassinosteroids prior to germination, needs further investigation.

The results of the steroid feeding experiments strongly suggest a specific defect in brassinosteroid biosynthesis in the *dwf1-6* (*cbb1*) and *cbb3* mutants. This conclusion is further supported by the observation that the deduced amino acid sequence of the *CBB3* gene product shows significant homology to mammalian cytochrome P450 proteins including steroid hydroxylases (Szekeres *et al.*, unpublished). The CBB3 protein might therefore act as a catalyst for a specific hydroxylation reaction in the biosynthesis of brassinosteroids. The recently isolated maize *DWARF3* gene, coding for a cytochrome P450 enzyme involved in a hydroxylation step in gibberellin biosynthesis, provides an example of this type of activity (Winkler and Helentjaris, 1995). The data presented here, however, exclude an involvement of the CBB3 protein in the gibberellin biosynthetic pathway. To further elucidate the function of the *CBB3* gene, biochemical analysis of the spectrum and the relative contents of the brassinosteroids of wild-type *A. thaliana* and of mutants lacking *CBB3* activity are currently being performed.

Sequence information for the *DWF1* gene may also indicate a direct enzymatic role as a FAD-dependent oxidase through the presence of a putative FAD-binding motif (Mushegian and Koonin, 1995). Alternatively, the presence of a potential nuclear targeting signal may point to a possible regulatory function of the DWF1 protein (Takahashi *et al.*, 1995). The less severe phenotypes of the different known alleles of *dwf1* (*dwf1-1*; *dim*, which should be considered as *dwf1-2*; and *dwf1-6*/*cbb1*) in comparison with *cbb3* leads to the assumption that a defect in the *DWF1* gene might result in strongly reduced levels but not in a complete lack of endogenous brassinosteroids. The data thus far collected, however, are also consistent with

the interpretation of the *dwf1-6* (*cbb1*) phenotype as being due to a partial insensitivity to brassinosteroids that could be overcome to a large extent by excessive amounts of brassinosteroids as supplied by external feeding. In line with this interpretation is the observation that normalization of *dwf1-6* (*cbb1*) mutant seedlings occurred to a high degree in the light and partially in darkness.

The third mutant studied, *cbb2*, which was phenotypically almost indistinguishable from *cbb3* seedlings grown in the absence of brassinosteroids, appeared insensitive to brassinosteroid treatment both at the morphological level and with respect to gene expression (see below). Such a complete lack of response to an active hormone might be due to two types of defects: there might be a lack of a functional hormone receptor as for instance proposed in the case of the *etr1* mutant (Chang *et al.*, 1993), or a component of a signal transduction pathway might be affected as in the cases of the *ctr1* mutant (Kieber *et al.*, 1993), the *abi3* mutant (Giraudat *et al.*, 1992), and the *abi1* mutant (Leung *et al.*, 1994; Meyer *et al.*, 1994). In contrast to the situation for ethylene or abscisic acid, however, the principal active brassinosteroid has not been unequivocally defined yet. This is due to the fact that in all activity tests (bioassays) used hitherto conversion of the applied substance could not be excluded. Indication of such a metabolic conversion into a compound of higher biological activity has been obtained recently for 24-epibrassinolide which in tomato cell cultures was converted into 25-hydroxy-24-epibrassinolide (Adam *et al.*, 1996). This novel compound showed a 10-fold higher activity in the rice lamina inclination test compared with 24-epibrassinolide. Insensitivity to the three brassinosteroids tested, 24-epicastasterone, (22S,23S) homobrassinolide, and 24-epibrassinolide, as in the case of *cbb2*, might thus also be due to the inability to convert the applied compounds (which actually might be analogs of biosynthetic intermediates) into a yet undefined biologically active substance. No information about the nature of the *CBB2* gene at the molecular level is so far available.

The conclusion of an insensitivity to brassinosteroids in the case of *cbb2* and a deficiency for endogenous brassinosteroids in the case of *dwf1-6* (*cbb1*) and *cbb3* is further supported by the gene expression data obtained. A reduced expression of the *TCH4* gene (Braam and Davis, 1990) and the *meri5* gene (Medford *et al.*, 1991) in all three *cbb* mutants and a specific induction by brassinosteroids was observed in *dwf1-6* (*cbb1*) and *cbb3* but not in *cbb2*. This strongly supports the conclusion that expression of these genes is brassinosteroid-dependent and that this regulatory activity is missing in *dwf1-6* (*cbb1*) and *cbb3*. These data are consistent with previous reports on the brassinosteroid inducibility of *TCH4* (Xu *et al.*, 1995) and the strong homology of *TCH4* and *meri5* to the soybean *BRU1* gene which has been identified by virtue of its induction by brassinolide (Zurek and Clouse, 1994). The lack of *meri5* inducibility in the *cbb2* mutant was specific for the brassinosteroid stimulus as the expression of this gene could be induced by GA_3 in the same manner as in the wild-type and in *dwf1-6* (*cbb1*) and *cbb3*. These results are in good agreement with previous observations which showed additive effects of brassinosteroids and gibberellins (Gregory and Mandava, 1982; Mandava *et al.* 1981), indicating separate modes of action for these two classes of phytohormones. The results observed with the gibberellin-inducible γ-*TIP* gene (Höfte *et al.*, 1992; Phillips and Huttly, 1994) might however indicate an interaction of brassinosteroids and gibberellins. As the data presented here were derived from long-term incubations, it can not be deduced whether the observed alterations of gene expression cause (at least in part) the corresponding phenotypic changes or were rather a consequence of the changed physiological status of the plants. Nevertheless, the observed reduction of *TCH4* and *meri5* expression, which both (potentially) encode xyloglucan-endotransglycosylases, might serve as a direct link between the brassinosteroid deficiency/insensitivity and the observed phenotype of reduced cell expansion. This activity is proposed to catalyze a 'molecular grafting' reaction between xyloglucan molecules and thus might be involved in plant cell-wall loosening (Albersheim, 1976; Fry, 1989; Fry *et al.*, 1992; Nishitani and Tominaga, 1992). Previous observations of increased cell-wall extensibility of brassinolide-treated soybean epicotyl sections (Zurek *et al.*, 1994) support the assumption that changes in the mechanical properties of the cell wall are regulated through brassinosteroids. Further work is required to prove the hypothesis that brassinosteroids are essential signal compounds for the expression of 'wall-loosening factors' which are necessary to allow cell-wall expansion and thus cell growth.

The identification of mutants which are brassinosteroid deficient or insensitive provides clear evidence that this class of compounds plays a major role in the development of plants and that they are essential as endogenous regulators of cell elongation. These mutants might contribute to the further elucidation of the pathway of brassinosteroid biosynthesis in plants and to the identification of factors involved in plant cell elongation that are regulated by brassinosteroids. Furthermore, together with the other known phytohormone-deficient/insensitive *A. thaliana* mutants they will allow a genetic analysis of the proposed complex interaction with other phytohormones (Mandava, 1988; Marquardt and Adam, 1991). Mutants insensitive to the known brassinosteroids such as *cbb2* and the recently described *bri1* (Clouse *et al.*, 1995) will be important tools to understand brassinosteroid signal perception/transduction and may lead to novel information about the biologically active brassinosteroid hormone molecule.

Experimental procedures

Plant material and isolation of mutants

Arabidopsis thaliana ecotype C24 was grown in soil (Einheitserde Typ P/Einheitserde Typ T/sand (2:1:1), Gebrüder Patzer, Simtal-Jossa, Germany) or in half-concentrated MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose under a 16 h day (3000 lux fluorescent light, 20°C)/8 h night (17°C) regime at a relative humidity of 70%.

Cabbage mutants were isolated in the course of a two-component *Ac/Ds* transposon mutagenesis as described by Altmann *et al.* (1995).

Genetic analysis

Mapping of the *cabbage* loci relative to molecular markers was done with segregating F_2 populations from crosses of heterozygous *cabbage* plants (ecotype C24) with wild-type plants (ecotype Columbia, Col-0). Genomic DNA for PCR was prepared from leaves as described by Rogers and Bendich (1985). Co-dominant cleaved amplified polymorphic sequence markers (CAPS) were used as described by Konieczny and Ausubel (1993) and simple sequence-length polymorphism markers (SSLP) as described by Bell and Ecker (1994). Restriction enzymes were obtained from Boehringer Mannheim, Germany, *Taq* polymerase was supplied by Gibco BRL, Eggenstein, Germany. PCR products were separated by agarose gel electrophoresis (Seakem LE Agarose, FMC Bio Products, Rockland, ME). The map positions of the CAPS and SSLP markers were taken from the *Arabidopsis* map generated through the Landsberg/Columbia recombinant inbred lines by Lister and Dean (1993), released June 29 1995. The map distances relative to the CAPS and SSLP markers in centimorgans were calculated according to Kosambi (1944).

The allelism test between the *cbb2* and *cbb3* mutant was performed through crosses of five heterozygous plants of each line and between *cbb1* and *cbb2* with four heterozygous plants of each line. Furthermore, crosses were performed in eight combinations between three heterozygous *cbb1* plants and three heterozygous *cbb3* plants. Of these crosses all F_1 progeny showed wild-type phenotype. The allelism test between *cbb3* and the *cpd* mutant (Szekeres *et al.*, unpublished) was done with four individual heterozygous plants of each line. Each of the resulting F_1 populations segregated for mutant plants. Of the total of 619 F_1 plants 142 were mutant and 477 were wild-type, a ratio of 1 : 3.3 as expected in the case of allelism.

Light microscopy

Plantlets (7–11 days old) were fixed for 12 h in 0.2% glutaraldehyde, 4% formaldehyde, 0.5% triton x-100, 0.1 M sodium phosphate buffer pH 7.0, followed by a 24 h dehydration with 75, 85, 90 and 2× 100% ethanol. The tissue was then pre-incubated for 6–8 h in equal parts of ethanol (100%) and Technovit (7100, Heraeus, Kulzer, Wehrheim, Germany), incubated overnight in 100% Technovit with hardener I and then embedded in Technovit with hardener II. Sections of 5 μm thickness were prepared with a Reichert Jung Biocut microtome and examined under a Zeiss Axiophot light-microscope.

Treatment with phytohormones and steroids

Seeds were germinated on half-concentrated MS medium, solidified with 0.7% agarose and seedlings were transferred after 1 week to fresh medium containing different concentrations of phytohormones and phytohormone inhibitors as indicated in Table 3.

For steroid treatment, seeds were directly plated on steroid-containing half-concentrated MS medium (Murashige and Skoog, 1962) with 0.7% agarose. Then 24-epibrassinolide, 24-epicastasterone and (22S-23S) homobrassinolide were applied in concentrations ranging from 0.01 to 1 μM. All other steroids including stigmasterol, stigmastanol, stigmasta-4,22-dien-3on, β-sitosterol, ergosterol, α-ecdysone, β-ecdysone, and cholesterol were added to the medium at a concentration of 1 μM.

All substances were purchased from Sigma (Deisenhofen, Germany), with the exception of 24-epibrassinolide, 24-epicastasterone, and ergosterol which were kindly provided by Dr G. Adam (Halle, Germany).

RNA extraction and RNA blot analysis

Total RNA was extracted from 1 to 4-week-old seedlings as described by Logemann *et al.* (1987). Samples were electrophoretically separated on MOPS-formaldehyde agarose gels, blotted on to Hybond N membranes (Amersham) and hybridized with radiolabeled DNA probes using the Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany). Hybridizations were performed at 65°C in sodium phoshate buffer (0.25 M, pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA.

The following probes were used: a partial cDNA of the *TCH4* gene (Braam and Davis, 1990) which was kindly provided by Dr J. Braam (Rice University, Houston, TX); a partial cDNA of the *meri5* gene (Medford *et al.*, 1991) which was PCR amplified and sequenced, and a PCR amplified and sequenced cDNA of the γ-*TIP* tonoplast water channel (Höfte *et al.*, 1992).

Acknowledgments

We thank Dr H. Peña-Cortés for his valuable comments during discussions of the project and we are very grateful to Professor Dr G. Adam for providing us with the synthetic 24-epibrassinolide, 24-epicastasterone and ergosterol. The *A. thaliana* mutants *ga1-1, ga2-1, ga3-1, ga4-1, ga5-1,* and *gai-1* were gratefully received from the Nottingham *Arabidopsis* Stock Center. This work was supported by a grant of the Bundesministerium für Forschung und Technologie BCT 0389 'Molekular- und zellbiologische Untersuchungen an höheren Pflanzen und Pilzen'.

References

Abeles, F.B., Morgan, P.W. and Salveit, Jr, M.E. (1992) *Ethylene in Plant Biology.* New York: Academic Press.

Adam, G. and Marquardt, V. (1986) Brassinosteroids. *Phytochemistry,* **25,** 1787–1799.

Adam, G., Porzel, A., Schmidt, J., Schneider, B. and Voigt, B. (1996) New developments in brassinosteroid research. In *Studies in Natural Products Chemistry* (Atta-ur-Rahmann, S.T., ed.). Amsterdam: Elsevier, in press.

Albersheim, P. (1976) The primary cell wall. In *Plant Biochemistry* (Bouner, J. and Varner, J.E., eds). New York: Academic Press, pp. 225–274.

Altmann, T., Felix, G., Jessop, A., Kauschmann, A., Uwer, U., Peña-Cortés, H. and Willmitzer, L. (1995) *Ac/Ds* transposon mutagenesis in *Arabidopsis thaliana*: mutant spectrum and

frequency of *Ds* insertion mutants. *Mol. Gen. Genet.* **247**, 646–652.

Arteca, R.N., Bachman, J.M. and Mandava, N.B. (1988) Effects of indole-3-acetic acid and brassinosteroid on ethylene biosynthesis in etiolated mung bean hypocotyl segments. *J. Plant Physiol.* **133**, 430–435.

Bell, C.J. and Ecker, J.R. (1994) Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. *Genomics*, **19**, 137–144.

Braam, J. and Davis, R.W. (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell*, **60**, 357–364.

Braun, P. and Wild, A. (1984) The influence of brassinosteroid on growth and parameters of photosynthesis of wheat and mustard plants. *J. Plant Physiol.* **116**, 189–196.

Brzobohaty', B., Moore, I. and Palme, K. (1994) Cytokinin metabolism: implications for regulation of plant growth and development. *Plant Mol. Biol.* **26**, 1483–1497.

Cerana, R., Bonetti, A., Marré, M.T., Romani, G., Lado, P., and Marré, E. (1983) Effects of brassinosteroid on growth and electrogenic proton extrusion in Azuki bean epicotyls. *Physiol. Plant.* **59**, 23–27.

Chang, C., Kwok, S.F., Bleecker, A.B. and Meyerowitz, E.M. (1993) *Arabidopsis* ethylene-response gene *ETR1*: similarity of product to two-component regulators. *Science*, **262**, 539–544.

Chory, J. (1993) Out of darkness: mutants reveal pathways controlling light-regulated development in plants. *Trends Genet.* **9**, 167–172.

Clouse, S.D. and Zurek, D.M. (1991) Molecular analysis of brassinolide action on plant growth and development. In *Brassinosteroids* (Cutler, H.G., Yokota, T. and Adam, G., eds). Washington, DC: American Chemical Society, pp.122–140.

Clouse, S.D., Zurek, D.M., McMorris, T.C. and Baker, M.E. (1992) Effect of brassinolide on gene expression in elongating soybean epicotyls. *Plant Physiol.* **100**, 1377–1383.

Clouse, S.D., Hall, A.F., Langford, M., McMorris, T.C. and Baker, M.E.J. (1993) Physiological and molecular effects of brassinosteroids on *Arabidopsis thaliana*. *J. Plant Growth Regul.* **12**, 61–66.

Clouse, S.D., Langford, M. and McMorris, T.C. (1995) Brassinosteroids as signals in plant growth and development. *J. Cell. Biochem.* **Suppl. 21A**, 479.

Davies, P.J. (1988) *Plant Hormones and their Role in Plant Growth and Development*. Dordrecht: Kluwer Academic Publishers.

Deng, X.-W. (1994) Fresh view of light signal transduction in plants. *Cell*, **76**, 423–426.

Eun, J.-S., Kuraishi, S. and Sakurai, N. (1989) Changes in levels of auxin and abscisic acid and the evolution of ethylene in squash hypocotyls after treatment with brassinolide. *Plant Cell Physiol.* **30**, 807–810.

Evans, M.L. (1984) Functions of hormones at the cellular level of organization. In *Hormonal Regulation of Development II. Encyclopedia of Plant Physiology*, Volume 10 (Scott, T.K., ed.). Berlin: Springer Verlag, pp. 23–79.

Feldmann, K.A., Marks, M.D., Christianson, M.L. and Quatrano, R.S. (1989) A dwarf mutant of *Arabidopsis* generated by T-DNA insertion mutagenesis. *Science*, **243**, 1351–1354.

Fry, S.C. (1989) Cellulases, hemicelluloses and auxin-stimulated growth: a possible relationship. *Physiol. Plant.* **75**, 532–536.

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K. and Matthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochem. J.* **282**, 821–823.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and Goodman, H.M. (1992) Isolation of the *Arabidopsis ABI3* gene by positional cloning. *Plant Cell*, **4**, 1251–1261.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.-C., Bouvier-Durand, M. and Vartanian, N. (1994) Current advances in abscisic acid action and signalling. *Plant Mol. Biol.* **26**, 1557–1577.

Gregory, L. E. and Mandava, N. B. (1982) The activity and interaction of brassinolide and gibberellic acid in mung bean epicotyls. *Physiol. Plant.* **54**, 239–243.

Grove, M.D., Spencer, G.F., Rohwedder, W.K., Mandava, N., Worley, J.F., Warthen, Jr, J.D., Steffens, G.L., Flippen-Anderson, J.L. and Cook, Jr, J.C. (1979) Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. *Nature*, **281**, 216–217.

Guzman, P. and Ecker, J.R. (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. *Plant Cell*, **2**, 513–523.

Henry, E.W., Dungy, L.J. and Bracciano, D.M. (1981) The effect of brassinolide on growth and enzyme activity in mung bean (*Phaseolus aureus* Roxb). In *8th Proceedings of the Plant Growth Regulator Society of America*, Volume 1981 (Cooke, A.R. and Abdelrahman, M., eds). Lake Alfred: Plant Growth Regulator Soc. America, pp. 146–160.

Hobbie, L. and Estelle, M. (1994) Genetic approaches to auxin action. *Plant Cell Environ.* **17**, 525–540.

Hobbie, L., Timpte, C. and Estelle, M. (1994) Molecular genetics of auxin and cytokinins. *Plant Mol. Biol.* **26**, 1499–1519.

Höfte, H., Hubbard, L., Reizer, J., Ludevid, D., Herman, E.M. and Chrispeels, M.J. (1992) Vegetative and seed-specific forms of tonoplast intrinsic protein in the vacuolar membrane of *Arabidopsis thaliana*. *Plant Physiol.* **99**, 561–570.

Hooley, R. (1994) Gibberellins: perception, transduction and responses. *Plant Mol. Biol.* **26**, 1529–1555.

Hou, Y., von Arnim, A. and Deng, X.-W. (1993) A new class of *Arabidopsis* constitutive photomorphogenic genes involved in regulating cotyledon development. *Plant Cell*, **5**, 329–339.

Iwasaki, T. and Shibaoka, H. (1991) Brassinosteroids as regulators of tracheary-element differentiation in isolated *Zinnia* mesophyll cells. *Plant Cell Physiol.* **32**, 1007–1014.

Kalinich, J.F., Mandava, N.B. and Todhunter, J.A. (1985) Relationship of nucleic acid metabolism to brassinolide-induced responses in beans. *J. Plant Physiol.* **120**, 207–214.

Kaminek, M. (1992) Progress in cytokinin research. *Trends Biotechnol.* **10**, 159–164.

Katsumi, M. (1985) Interaction of a brassinosteroid with IAA and GA_3 in the elongation of cucumber hypocotyl sections. *Plant Cell Physiol.* **26**, 615–625.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. (1993) *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the Raf family of protein kinases. *Cell*, **72**, 427–441.

Kim, S.-K., Abe, H., Little, C.H.A. and Pharis, R.P. (1990) Identification of two brassinosteroids from the cambial region of scots pine (*Pinus silvestris*) by gas chromatography-mass spectrometry, after detection using a dwarf rice lamina inclination bioassay. *Plant Physiol.* **94**, 1709–1713.

Kim, S.-K., Asano, T. and Marumo, S. (1995) Biological activity of brassinosteroid inhibitor KM-01 produced by a fungus *Drechslera avenae*. *Biosci. Biotechnol. Biochem.* **59**, 1394–1397.

Klee, H. and Estelle, M. (1991) Molecular genetic approaches to plant hormone biology. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 529–551.

Konieczny, A. and Ausubel, F.M. (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant, ecotype-specific PCR-based markers. *Plant J.* **4**, 403–410.

Koornneef, M. and van der Veen, J.H. (1980) Induction and analysis

of gibberellin sensitive mutants in *Arabidopsis thaliana*. *Theor. Appl. Genet.* **58**, 257–263.

Koornneef, M., Elgersma, A., Hanhart, C.J., van Loenen-Martinet, E.P., van Rijn, L. and Zeevaart, J.A.D. (1985) A gibberellin insensitive mutant of *Arabidopsis thaliana*. *Physiol. Plant.* **65**, 33–39.

Kosambi, D.D. (1944) The estimation of map distance from recombinant values. *Ann. Eugen.* **12**, 172–175.

Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F. and Giraudat, J. (1994) *Arabidopsis* ABA response gene *ABI1*: features of a calcium-modulated protein phosphatase. *Science*, **264**, 1448–1452.

Lister, C. and Dean, C. (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant J.* **4**, 745–750.

Logemann, J., Schell, J. and Willmitzer, L. (1987) Improved method for the isolation of RNA from plant tissue. *Anal. Biochem.* **163**, 16–20.

MacMillan, J. and Phinney, B.O. (1987) Biochemical genetics and the regulation of stem elongation by gibberellins. In *Physiology of Cell Expansion during Plant Growth* (Cosgrove, D.J. and Knievel, D.P., eds). Rockville, MD: American Society of Plant Physiologists, pp. 156–171.

Mandava, N.B. (1988) Plant growth-promoting brassinosteroids. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 23–52.

Mandava, N.B., Sasse, J.M. and Yopp, J.H. (1981) Brassinolide, a growth promoting steroidal lactone. II. Activity in selected gibberellin and cytokinin bioassays. *Physiol. Plant.* **53**, 453–461.

Marquardt, V. and Adam, G. (1991) Recent advances in brassinosteroid research. In *Chemistry of Plant Protection*, Volume 7 (Ebing, W., ed.-in-chief). Berlin: Springer Verlag, pp. 103–139.

Medford, J.I., Elmer, J.S. and Klee, H.J. (1991) Molecular cloning and characterisation of genes expressed in shoot apical meristems. *Plant Cell*, **3**, 359–370.

Meyer, K., Leube, M.P. and Grill, E. (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. *Science*, **264**, 1452–1445.

Mitchell, J.W. and Livingston, G.A. (1968) *Methods of Studying Plant Hormones and Growth-regulating Substances. Agriculture Handbook, US Department of Agriculture No. 336.* Washington, DC: US Government Printing Office.

Mitchell, J.W., Mandava, N., Worley, J.F., Plimmer, J.R. and Smith, M.V. (1970) Brassins—a new family of plant hormones from rape pollen. *Nature*, **225**, 1065–1066.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Mushegian, A.R. and Koonin, E.V. (1995) A putative FAD-binding domain in a distinct group of oxidases including a protein involved in plant development. *Protein Sci.* **4**, 1243–1244.

Nishitani, K. and Tominaga, R. (1992) Endo-xyloglucan transferase, a novel class of glycosyltransferase that catalyzes transfer of a segment of xyloglucan molecule to another xyloglucan molecule. *J. Biol. Chem.* **267**, 21 058–21 064.

Phillips, L.P. and Huttly, A.K. (1994) Cloning of two gibberellin-regulated cDNAs from *Arabidopsis thaliana* by subtractive hybridization: expression of the tonoplast water channel, *γ-TIP*, is increased by GA_3. *Plant Mol. Biol.* **24**, 603–615.

Rayle, D.L. and Cleland, R.E. (1992) The acid growth therory of auxin-induced cell elongation is alive and well. *Plant Physiol.* **99**, 1271–1274.

Rogers, S.O. and Bendich, A.J. (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissue. *Plant Mol. Biol.* **5**, 69–76.

Romani, G., Marre, M.T., Bonetti, A., Cerana, R., Lado, P. and Marre, E. (1983) Effects of brassinosteroid on growth and electrogenic proton extrusion in maize root segments. *Physiol. Plant.* **59**, 528–532.

Schlagnhaufer, C., Arteca, R.N. and Yopp, J.H. (1984) A brassinosteroid–cytokinin interaction on ethylene production by etiolated mung bean segments. *Physiol. Plant.* **60**, 347–350.

Takahashi, T., Gasch, A., Nishizawa, N. and Chua, N.-H. (1995) The *DIMINUTO* gene of *Arabidopsis* is involved in regulating cell elongation. *Genes Devel.* **9**, 97–107.

Takasuto, S., Yazawa, N., Ikegawa, N., Takematsu, T., Takeuchi, Y. and Koguchi, M. (1983) Structure–activity relationship of brassinosteroids. *Phytochemistry*, **22**, 2437–2441.

Wada, K., Marumo, S., Abe, H., Morishita, T., Nakamura, K., Uchiyama, M. and Mori, K. (1984) A rice lamina inclination test—a micro-quantitative bioassay for brassinosteroids. *Agric. Biol. Chem.* **48**, 719–726.

Wada, K., Kondo, H. and Marumo, S. (1985) A simple bioassay for brassinosteroids: a wheat leaf-unrolling test. *Agric. Biol. Chem.* **49**, 2249–2251.

Wei, N., Kwok, S.F., von Armin, A.G., Lee, A., McNellis, T.W., Piekos, B. and Deng, X.-W. (1994) *Arabidopsis COP8, COP9*, and *COP11* genes are involved in repression of photomorphogenic development in darkness. *Plant Cell*, **6**, 629–643.

Wilson, A.K., Pickett, B.F., Turner, J.C. and Estelle, M. (1990) A mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. *Mol. Gen. Genet.* **222**, 377–383.

Winkler, R.G. and Helentjaris, T. (1995) The maize *Dwarf3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. *Plant Cell*, **7**, 1307–1317.

Xu, W., Purugganan, M., Polisensky, D.H., Antosiewicz, D.M., Fry, S.C. and Braam, J. (1995) *Arabidopsis TCH4*, regulated by hormones and the enviroment, encodes a xyloglucan endotransglycosylase. *Plant Cell*, **7**, 1555–1567.

Yopp, J.H., Mandava, B. and Sasse, J.M. (1981) Brassinolide, a growth-promoting steroidal lactone. I. activity in selected bioassays. *Physiol. Plant.* **53**, 445–452.

Zarembinski, T.I. and Theologis, A. (1994) Ethylene biosynthesis and action: a case of conservation. *Plant Mol. Biol.* **26**, 1579–1597.

Zurek, D.M. and Clouse, S.D. (1994) Molecular cloning and characterization of a brassinosteroid-regulated gene from elongating soybean (*Glycine max* L.) epicotyls. *Plant Physiol.* **104**, 161–170.

Zurek, D.M., Rayle, D.L., McMorris, T.C. and Clouse, S.D. (1994) Investigation of gene expression, growth kinetics, and wall extensibility during brassinosteroid-regulated stem elongation. *Plant Physiol.* **104**, 505–513.