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Review

Molecular physiology of brassinosteroids revealed by the analysis of mutants

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Introduction

In 1970 a new class of putative plant growth factors was detected in pollen extracts of *Brassica napus* and named "brassins" (Mitchell et al. 1970). Brassinolide (Fig. 1), a novel steroid of unique structure, was shown to be responsible for the growth-promoting activity of "brassins" (Grove et al. 1979). Today over 40 brassinolide-like brassinosteroids (BRs) have been identified in a wide variety of plant species (reviewed in: Mandava 1988; Marquardt and Adam 1991; Adam et al. 1996; Sasse 1997).

Like steroids such as estrogen, testosterone or ecdysone known from animals, BRs consist of a typical steroidal skeleton with specific substitutions required for biological activity. Several bioassays, including the bean second-internode bioassay (Mitchell and Livingston 1968), the *Raphanus sativus* test (Takasuto et al. 1983), the tomato test (Takasuto et al. 1983) and the rice lamina inclination test (Wada et al. 1981; Arima et al. 1984), have been used to determine the bioactivity of either naturally occurring substances isolated from plant tissues or chemically synthesised analogs. The most active BRs exhibit either a 6-oxo function (as in the case of castasterone) or a lactone structure (as in the case of brassinolide) at the B ring which in the latter case is seven-numbered. Furthermore, most of the naturally occurring BRs thus far identified are hydroxylated at the C-2, C-3, C-22 and C-23 positions, the latter two being in R configuration. Structural variations other than the substitution patterns of rings A and B lie in the different alkylations of C-24. The most highly active BRs include the 7-oxalactone-type compounds brassinolide, 24-epibrassinolide and 28-homobrassinolide (Fig. 1), while the corresponding 6-keto-type substances like castasterone, 24-epicastasterone and ethylbrassinone show reduced biological activity (Takasuto et al. 1983; Adam and Marquardt 1986; Mandava 1988; Marquardt and Adam 1991).

Since the first discovery of BRs in rape pollen, numerous studies have proved the ubiquitous presence of representatives of this class of steroids in as many as 36 plant species, including angiosperms, gymnosperms, a fern and a green alga (reviewed in: Sasse 1997). With the exception of roots, in which BRs have not yet been detected biochemically, BR contents of aerial plant tissues usually range from ng/kg to μg/kg with the highest levels present in seeds and pollen (Adam and Marquardt 1986; Adam et al. 1996). Only very few studies have addressed the tissue- or cell-type-specific or the subcellular localisation of BRs (e.g. Kim et al. 1990; Taylor et al. 1993).

Information on the biosynthesis of BRs has been obtained through extensive step-wise feeding studies performed with radiolabelled presumed precursors of brassinolide and the careful analysis of their bioconversion products (summarised in: Fujioka and Sakurai 1997; Yokota 1997). Thus, a branched biosynthetic pathway (Fig. 2) from the phytosterol campesterol to brassinolide has been established for *Catharanthus roseus* for which most of the reactions were monitored in vivo: after the reduction of campesterol to campestanol, a series of hydroxylation and oxidation steps occurs in the steroid body and in the side chain, with the hydroxylation and oxidation to the keto group at position C-6 occurring either early or late, i.e. before or after the modifications at positions C-22, C-23, C-3 and C-2. The two branches, termed early or late C-6 oxidation pathways, respectively, convene at the formation of castasterone, the (proposed) immediate precursor of brassinolide. Similarly, a subset of these reactions has also been directly demonstrated to occur in tobacco, rice and lily (Suzuki et al. 1995; Choi et al. 1996; Abe et al. 1994, 1996). Potential variations in the order of the

Abbreviations: BR = brassinosteroid; WT = wild type

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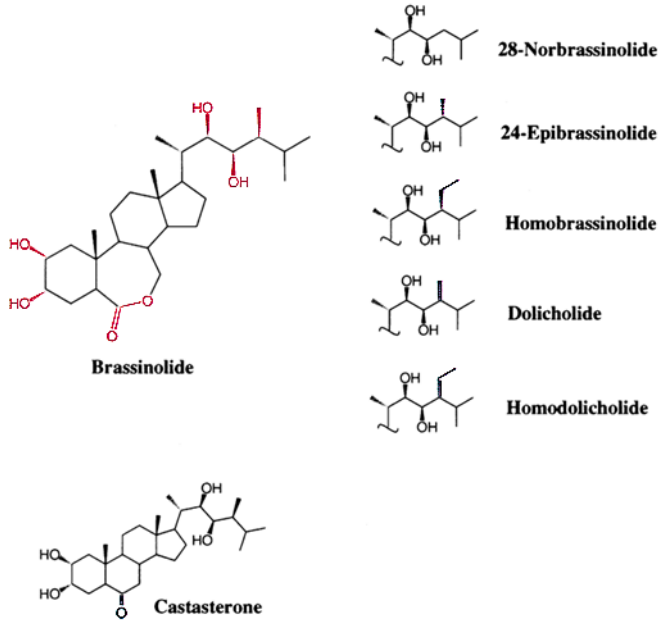

Fig. 1. Structural formulae of brassinolide and castasterone, the most common representatives of the 7-oxalactone type and the 6-keto type BRs, respectively. Sites of structural variation between various BRs are highlighted (*red*) in the brassinolide structural formula. Side-chain modifications present in several naturally occurring BRs are displayed. Differences from the brassinolide structure are marked (*blue*)

modification reactions and possible interconnections between different steps remain to be elucidated, as does the unequivocal demonstration that brassinolide is an endproduct of BR anabolism. Another level of complexity is provided by the natural occurrence of brassinolide analogs, e.g. with differences in the side-chain structure (such as 24-epibrassinolide with an R-configured methyl group at C-24, homobrassinolide with an ethyl group at C-24, norbrassinolide without a substituent at C-24, dolicholide with a methylene group at C-24 and homodolicholide with an ethylene group at C-24) (Fig. 1). The frequent co-occurrence in the same plant species of several BRs with different side chains (Marquardt and Adam 1991), which are presumed to be synthesised from the corresponding phytosterols, indicates the potential existence of additional (parallel) pathways. Furthermore, knowledge about the enzymology of the various steps in the biosynthesis has hitherto been very scarce, a topic which now receives strong support through the identification of genes coding for biosynthetic factors (see below). To understand the mechanisms through which the levels of biologically active BRs are regulated, knowledge about the metabolic conversions of the presumed biosynthetic endproducts is of equal importance. Thus, feeding of radiolabelled 24-epibrassinolide, or 24-epicastasterone to suspension cultures of *Lycopersicon esculentum* or *Ornithopus sativus* has revealed a variety of modifications,

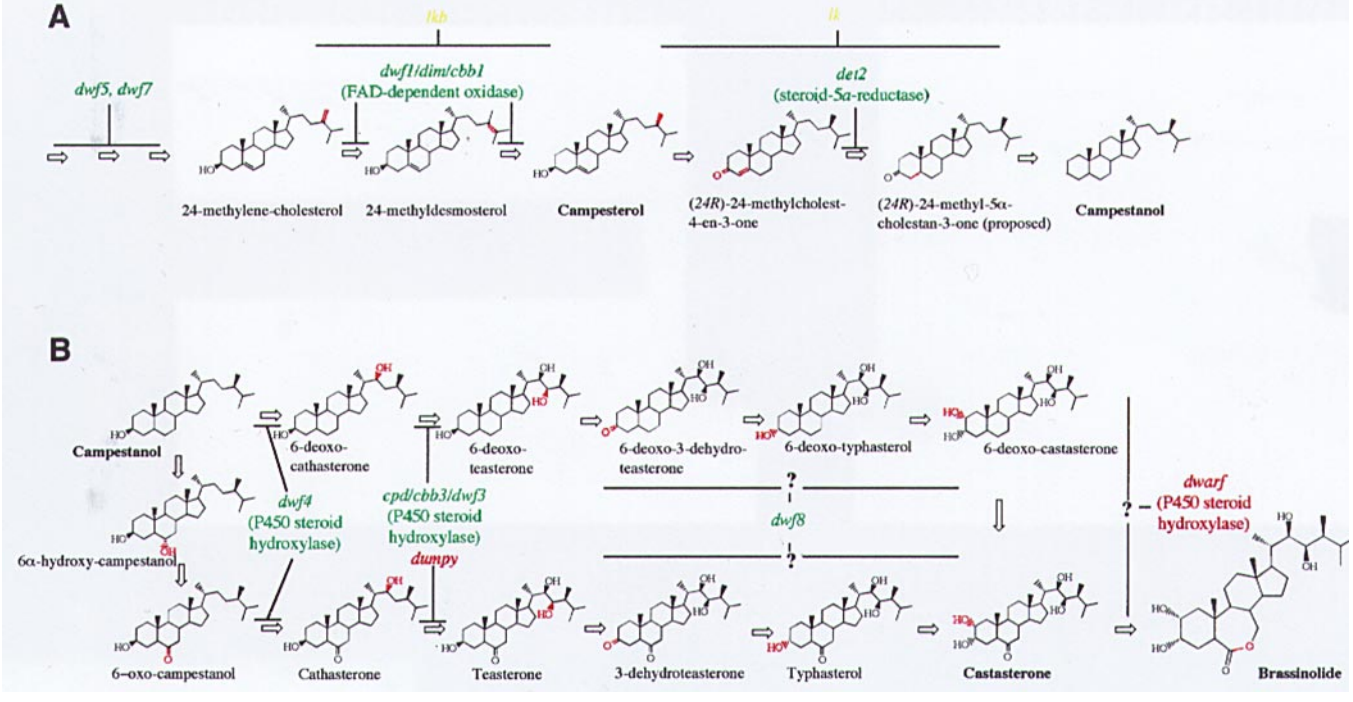

Fig. 2A, B. Proposed biosynthetic pathway of brassinolide marked with the steps blocked in the various BR-deficient mutants. Gene/allele symbols in *green* mark *Arabidopsis thaliana* mutants, *red* symbols are used for *Lycopersicon esculentum* mutants, and *yellow* symbols denote *Pisum sativum* mutants. The (proposed) function of the hitherto isolated genes corresponding to the *A. thaliana* mutants is mentioned. Limited information is available about the metabolic blocks caused by the *dwf8* and *dwarf* mutations (question marks). **A** Biosynthetic steps leading to the formation of an early BR metabolic intermediate, campestanol, via synthesis of a bulk phytosterol, campesterol. **B** Branched biosynthetic pathway from campestanol to castasterone via the late C-6 oxidation pathway (*top*) or the early C-6 oxidation pathway (*bottom*) and the final conversion of castasterone to brassinolide. The modifications in the molecules occurring in each of the reactions are highlighted (*red*)

including hydroxylation at C-25 or C-26 followed by glucosylation, epimerisation at C-3 followed by glucosylation at C-2, or C-3, hydroxylation and oxidation at C-20 (involving side-chain cleavage), and fatty acid conjugation at C-3 (summarised in: Adam et al. 1996). Furthermore, disaccharide conjugates were identified after feeding 24-epiteasterone to tomato suspension cultures (Kolbe et al. 1997). Again, with the exception of the C-25 and C-26 hydroxylations, which have been studied in more detail (Winter et al. 1997), very little is known about the enzymology of these reactions.

High interest in the function of BRs has been elicited through the strong responses of intact plants and explants observed upon application of exogenous BRs. Including the changes monitored in the bioassays mentioned above, a broad spectrum of reactions of plant tissues could be triggered by the exogenous application of BRs, of which only a limited number of examples can be mentioned here (for comprehensive reviews, see Mandava 1988; Marquardt and Adam 1991; Sakurai and Fujioka 1993; Clouse 1997; Sasse 1997; Yokota 1997). When applied at nanomolar concentrations to either explants such as hypocotyl or epicotyl segments or to intact plants, BRs were shown to exert strong growth-promoting effects through the stimulation of both cell division and cell elongation (summarised in: Mandava 1988; Marquardt and Adam 1991). In several systems, synergistic effects of auxins and BRs have been observed (e.g. Yopp et al. 1981) with an increased sensitivity of tissues to auxins induced by BRs pretreatment (e.g. Cohen and Meudt 1983; Katsumi 1985) or vice versa (Kim et al. 1990). In contrast, rather additive effects on elongation growth have been recorded by gibberellins and BRs (e.g. Mandava et al. 1981; Katsumi 1985). Furthermore, induction of cell enlargement (e.g. Sala and Sala 1985), enhancement or retardation of root growth (e.g. Yopp et al. 1981; Romani et al. 1983; Roddik and Guan 1991; Clouse et al. 1993), unrolling of leaves (e.g. Wada et al. 1985), bending of leaves at the joint (e.g. Wada et al. 1984), and the differentiation of xylem vessels (Clouse and Zurek 1991; Iwasaki and Shibaoka 1991; Fukuda 1997) have been reported. Observed physiological responses to BR application include ethylene production through activation of 1-aminocyclopropane-1-carboxylic acid synthesis (e.g. Schlagnaufer et al. 1984; Arteca et al. 1988), membrane hyperpolarisation through enhanced proton extrusion (e.g. Cerana et al. 1983), increased ATPase activity (e.g. Henry et al. 1981), enhanced DNA, RNA and protein synthesis (e.g. Kalinich et al. 1985), increased invertase activity (e.g. Schilling et al. 1991), stimulation of photosynthetic activity (e.g. Braun and Wild 1984), and changes in the balance of other endogenous phytohormones (e.g. Eun et al. 1989). Impressive reports on increased yield (e.g. Ikegava and Zhao 1991) and enhanced tolerance to biotic or abiotic stress (e.g. Wilen et al. 1995; Kamuro et al. 1997) obtained upon BR applications in field trials indicated the great potential for commercial applications.

Although the presence of BRs in plant tissues, their efficient elicitation of growth responses and of specific physiological changes at very low concentrations, and their movement within plants (Yokota et al. 1992) have been documented, their phytohormonal status (Sasse 1991) and their importance for the regulation of plant growth and development have not been as widely accepted as for "classical" plant hormones such as auxins, gibberellins, cytokinins, ethylene and abscisic acid (Kende and Zeevaart 1997). This situation has changed dramatically over the past three years during which several reports on the identification and characterisation of BR-deficient or -insensitive mutants have been published. The information gathered through the analysis of these mutants will be the major topic of this review.

Identification and characterisation of BR mutants

While the phenotype of mutants deficient for BR biosynthesis or the response to BRs could not readily be predicted from previous knowledge about BR action, a lack of BR or a strong reduction in endogenous BR levels could be expected to result in dwarfism (according to a lack of a growth-promoting activity) and a lack of BR sensitivity could result in a loss of the inhibition of root growth, a response previously observed for wild type (WT) plants (Roddick and Guan 1991; Clouse et al. 1993). Accordingly, the two studies specifically directed towards the identification of BR-related mutants (Clouse et al. 1996; Nomura et al. 1997) made use of these criteria. The majority of the isolated BR mutants, however, were initially selected for detailed analysis due to other features such as extreme dwarfism (Feldmann et al. 1989; Kauschmann et al. 1996; Szekeres et al. 1996) or de-etiolated growth in darkness (Chory et al. 1991) and it was the identification of the affected genes (see below) which triggered further experiments to test for BR-related defects.

Hitherto, BR mutants have been identified in three plant species, *Arabidopsis thaliana*, *Pisum sativum*, and *Lycopersicon esculentum* with a total of eight different loci affected in *Arabidopsis*, three loci in pea and at least four candidate loci in tomato. In all cases, the defects lead to dwarfism, albeit expressed to different extents in the various mutants. These defects are discussed in the following paragraphs according to the order of their gene products in the proposed BR-biosynthesis pathway (Fig. 2).

Biosynthetic mutants and corresponding genes. The earliest step in BR biosynthesis affected by any of the known mutations actually involves the formation of the phytosterol precursor to the specific BR pathway, campesterol. As shown for the *dim* allele (Takahashi et al. 1995), the two-step conversion of 24-methylenecholesterol to campesterol is blocked in *A. thaliana* upon mutation of the *DWF1* locus (Feldmann et al. 1989; Klahre et al. 1998). The defect results in a strong increase in the level of 24-methylenecholesterol and in contrast to the WT, mutant seedlings are unable to convert deute-

rium labelled 24-methylenecholesterol or the reaction intermediate 24-methyldesmosterol to campesterol (Klahre et al. 1998). Consistent with the redox reaction involved in the saturation of the side-chain double bond during this conversion, the corresponding gene encodes a protein which contains a domain possibly involved in FAD binding and which is conserved in a group of proteins including several FAD-dependent oxidases (Mushegian and Koonin 1995). The enzymic function of the DWF1 protein has not yet been demonstrated directly. The phenotype of the *dim* seedlings (Takahashi et al. 1995) and of seedling carrying other allelic mutations, *dwf1-6* (Kauschmann et al. 1996) and *dwf1-1* (Feldmann et al. 1989) could be normalised by feeding BR but not by feeding any other phytohormone (Kauschmann et al. 1996; Klahre et al. 1998). This mutant was therefore classified as BR deficient and it was concluded that the corresponding gene most probably encodes a factor involved in BR biosynthesis. In the light of the aforementioned data it should, however, rather be regarded as a phytosterol biosynthetic mutant with associated drastically reduced BR levels. Loss of the DWF1 function results in a strong dwarfism due to reduced cell size in all parts of the plant, with the hypocotyl, the petioles and the axis most dramatically affected. Leaves are less expanded, short, round, dark green, and epinasticly curled. The mutants show an extended longevity with delays in flower induction and onset of senescence and reduced fertility, but they are able to produce viable seeds. Another prominent feature of the mutants is the "de-etiolated" or "constitutive photomorphogenic" phenotype (Chory et al. 1989; Deng et al. 1991) of seedlings grown in darkness, i.e. the development of a short hypocotyl, lack of an apical hook, opened cotyledons and the emergence of primary leaves. In contrast to other mutants in this class such as *det1* (Chory et al. 1989), *cop1* (Deng et al. 1991) and *cop9* (Wei and Deng 1992), *dwf1* mutants do not show chloroplast differentiation and the levels of light-inducible genes such as *CAB* or *RBCS* are not elevated (Takahashi et al. 1995). Consistent with the reduction in cell expansion, the expression of several genes encoding factors potentially involved in cell wall growth (for details on this topic, see Cosgrove 1997) is changed in the mutants. Thus, steady-state mRNA levels of xyloglucanendotransglycosylase (XET) and expansin genes are very low but inducible upon BR-feeding (Kauschmann et al. 1996), the levels of arabinogalactan proteins are reduced, and the mRNA level of one specific β-tubulin gene, *TUB1*, is decreased (Takahashi et al. 1995). The dwarf *lkb* mutant of pea, which contains drastically reduced endogenous levels of brassinolide, castasterone, and 6-deoxocastasterone and which has thus been shown to be BR-deficient (Nomura et al. 1997) is, like the *dwf1* mutants, impaired in the conversion of 24-methylenecholesterol to campesterol (Yokota et al. 1997). Interestingly, the *lkb* mutant has been analysed for parameters related to stem elongation (Behringer et al. 1990). This study of wall stress relaxation (a technique that monitors the rate and the degree of wall modification leading to loosening) revealed that wall properties of cells in the epicotyl growing zone are altered, showing a dramatically increased wall yield threshold (Y). According to Lockhart's growth equation (Lockhart 1965), plastic extension of the cell wall would only occur if the turgor pressure (P) of the cell exceeds this value, and the volumetric growth rate would be proportional to the difference between P and Y. As the turgor pressure of growing cells is not decreased but even increased in the *lkb* mutant (Behringer et al. 1990), the apparent inability to loosen the cell walls to the normal extent is the major cause of the reduced cell size i.e. dwarfism. A role for BRs in the regulation of cell wall properties has previously been indicated by the identification of *BRU1*, an XET-encoding gene that is BR-inducible in soybean epicotyls. The timing of the onset of its mRNA accumulation coincides with changes in the cell wall extensibility of BR-treated epicotyl segments (Zurek and Clouse 1994; Zurek et al. 1994). Furthermore, alteration in the expression of an *XET* gene, *MERI5* (Verical and Medford 1997), and changes in the activity of arabinogalactan proteins (Willats and Knox 1996) have both been shown to influence cell (wall) growth. Based on this information it is tempting to speculate that BRs control cell wall growth through the regulation of the expression of genes encoding factors involved in cell wall weakening. It is not yet clear, however, if all or a subset of the changes in gene expression observed in the mutants are the cause or rather the consequence of the reduced cell expansion. The experiments performed hitherto do not allow us to discriminate between the possibility that the BR-mediated expression of these genes is the major mechanism through which the necessary cell wall weakening is provided, or the alternative scenario according to which the induction of the genes is the consequence of an enhanced cell wall growth mediated via a different BR-dependent reaction. In soybean, gibberellin- and auxin-induced epicotyl elongation occurs without induction of *BRU1* expression (Zurek and Clouse 1994), which provides a hint that the latter possibility is less likely. Brassinosteroids may furthermore modulate cell wall synthesis through re-orientation of cortical microtubules (Mayumi and Shibaoka 1995) which, in turn, affect the orientation of the cellulose microfibrils in the cell wall and thus the direction of cell expansion.

Loss of the *LKB* gene function not only affects the synthesis of campesterol but also that of other phytosterols such as sitosterol, sitostanol and stigmasterol, the levels of which are also drastically reduced in the pea mutant *lkb* (Yokota et al. 1997). The latter three sterols are synthesised from isofucosterol which is converted to sitosterol in a reaction analogous to the conversion of 24-methylenecholesterol to campesterol. Accordingly, the level of isofucosterol was found to be increased in the mutant, resulting in an unusual sterol composition of its membranes (Yokota et al. 1997). Conclusions about the role of BRs drawn from analysis of these mutants require consideration of this change. As in the case of the *Arabidopsis dwf1* mutant, the *lkb* mutant shall be regarded as a BR-deficient phytosterol mutant.

Conversion of campesterol to campestanol, a reaction that proceeds via a recently identified intermediate,

(24*R*)-24-methylcholest-4-en-3-one (Fujioka et al. 1997), is the first biosynthetic reaction specific to the BR biosynthetic pathway. Mutants impaired in this reaction have been identified in *Arabidopsis* and pea, *det2* and *lk*, respectively (Li et al. 1996; Yokota et al. 1997). The DET2 protein is a plant homolog to mammalian steroid 5α-reductases; through expression in human cells it was shown to catalyse 5α-reduction of several animal steroids including testosterone and progesterone (Li et al. 1997). Conversely, complementation of the *det2* mutation, resulting in rescue of the *det2* phenotype to WT, was achieved by expression of human steroid 5α-reductase in transgenic *Arabidopsis* plants (Li et al. 1997). The natural substrate of the DET2 protein in plants appears to be (24*R*)-24-methylcholest-4-en-3-one, the level of which is increased 3-fold in *det2* mutants (Fujioka et al. 1997). The contents of campestanol are reduced to about 10% of the WT levels (Fujioka et al. 1997). The BR-deficiency of *det2* was furthermore confirmed by the analysis of endogenous BR levels, which revealed a reduction of castasterone and 6-deoxocastasterone to less than 10% of the WT level and of typhasterol and 6-deoxothyphasterol to undetectable levels (Fujioka et al. 1997). For the *lk* mutant of pea which is also impaired in the conversion of campestanol to campesterol, reduced levels of endogenous castasterone and 6-deoxocastasterone were observed (Yokota et al. 1997).

Mutation of the *DET2* locus results in extreme dwarfism (Chory et al. 1991; Li et al. 1996) which is even more severe than in the case of *dwf1* mutants. The *det2* mutants were, however, initially isolated through their de-etiolated phenotype showing several characteristics of light-grown plants when cultivated in darkness (Chory et al. 1991). These include a short hypocotyl, opened and expanded cotyledons, development of primary leaf buds and a de-repression of several light-inducible genes such as *rbcS*, *cab*, and *psbA* (Chory et al. 1991). This phenotypic class is represented by at least 10 different mutations (Kwok et al. 1996) that affect two or more signal transduction pathways. The *det1*, *cop1* and *cop9* mutations, for instance, have been shown to act in a pathway different from that in which *det2* is involved (Chory 1992). In contrast to deficiencies for *DET1*, *COP1* or *COP9*, which result in chloroplast differentiation and full derepression of chloroplast genes (Chory et al. 1989; Deng et al. 1991; Wei and Deng 1992), loss of *DET2* function does not affect plastid differentiation and leads to only a 10% expression level of light-inducible plastid genes as compared to their expression levels in light-grown WT plants (Chory et al. 1991). Similarly, the mRNA steady-state levels of light-inducible nuclear genes are higher in dark-grown *det1* seedlings than in dark-grown *det2*. The BR deficiency of *det2* is thus associated with a (partial) de-etiolation of mutant seedlings grown in darkness. The precise role that BRs play in the regulation of photo- or skotomorphogenesis remains to be resolved (see below), as does the nature of the proposed interaction between the *det1* and *det2* signalling pathways (Szekeres et al. 1996).

The two *Arabidopsis* loci, *DWF4* and *CPD*/*CBB3*/*DWF3* (Kauschmann et al. 1996; Szekeres et al. 1996; Azpiroz et al. 1998; Choe et al. 1998), code for BR biosynthetic factors that introduce hydroxyl groups into the BR side chain. Both genes encode cytochrome P450 monooxygenases. Thus, through BR-feeding experiments to the *dwarf4* (*dwf4*) mutant, the corresponding gene product (CYP90B) has recently been shown to be responsible for the hydroxylation of the C-22 atom (Choe et al. 1998). According to the strikingly increased bioactivity of the C-22-hydroxylated cathasterone as compared to the corresponding precursor, 6-oxocampestanol, this reaction has been proposed to constitute a rate-limiting step in BR biosynthesis (Fujioka et al. 1995). The C-22-hydroxylated intermediates of both the early C-6 oxidation and the late C-6 oxidation pathways rescued the dwarf phenotype of the *dwf4* mutant, demonstrating a role for DWF4 in both branches of the pathway (Choe et al. 1998). Additional feeding experiments involving the synthetic C-22 hydroxylated compound 22-hydroxy-campesterol resulted in rescue of *dwf4*. These results are indicative of a relaxed substrate specificity of the enzymes usually converting campesterol to campestanol and furthermore to 6-oxo-campestanol, or of a possible existence of another BR biosynthetic subpathway. Further indication for a broader substrate specificity of the C-6 oxidase was provided by feeding 6-hydroxycathasterone, another synthetic C-22 hydroxylated compound, which also normalised the *dwf4* growth defect. In general, these observations may suggest that the various modifications of the BR biosynthetic intermediates do not occur in a particular fixed order. If this holds true, the BR-biosynthetic pathway may be viewed as a network of reactions allowing various different routes to be followed by the metabolised molecules rather than a (branched) (co-)linear arrangement of conversions.

The *Arabidopsis thaliana CPD* gene product (CYP90A) mediates the second hydroxylation reaction in the BR side chain, at C-23, as has been demonstrated by BR-feeding experiments (Szekeres et al. 1996). Thus, teasterone and all further metabolites in the (early C-6 oxidation) pathway normalised the growth defect of the *constitutive photomorphogenesis and dwarfism* (*cpd*) mutant, which like *det2* and *dwf4* is an extreme dwarf. More recently (C. Koncz, personal communication), the 23-hydroxylated intermediates of the late C-6 oxidation pathway have also been shown to normalise the *cpd* mutant, indicating a role for the *CPD*-encoded cytochrome P450 (CYP90A) in this branch of the pathway (or this reaction of the biosynthetic network) as well. Indications of a similar defect in C-23-hydroxylation were obtained for the tomato *dumpy* (*dpy*) mutant, again through BR-feeding experiments (S.D. Clouse, personal communication). This mutant may therefore carry a defect in the corresponding tomato CYP90A ortholog.

As happened for *dwf1*, other *cpd* alleles (*cpd*, *cbb3*, *dwf3*) have been independently isolated in *Arabidopsis* (Kauschmann et al. 1996; Szekeres et al. 1996; Choe et al. 1998). The phenotypic changes associated with a loss of CPD activity closely resemble that of *det2*. As in the case of *dwf1-6*, the growth defect of the *cbb3* allele of *CPD* could only be normalised by supplementing the growth

medium with active BRs, but not with any other phytohormone or phytohormone antagonist (Kauschmann et al. 1996). Light-grown *cpd* and *cbb3* mutants are extremely dwarfed due to drastically reduced cell expansion with dark-green, poorly expanded and epinastically curled leaves, a strongly stunted inflorescence and male-sterile flowers (Kauschmann et al. 1996; Szekeres et al. 1996). Extranumerary phloem cell files that apparently developed at the expense of xylem cells were observed in stem cross-sections (Szekeres et al. 1996). This observation is consistent with previous demonstrations of an important role for BRs in xylogenesis (Clouse and Zurek 1991; Iwasaki and Shibaoka 1991; Fukuda 1997). When germinated and grown in darkness, *cpd* and *cbb3* mutant plants also exhibit a de-etiolated phenotype with short hypocotyls and opened cotyledons (Kauschmann et al. 1996; Szekeres et al. 1996), and a de-repression of light-inducible genes such as *rbcS* and *cab* (Szekeres et al. 1996). Furthermore, several changes in the expression of genes were observed in light-grown mutant plants: similar to the observations on *dwf1-6*, and consistent with the reduced cell expansion growth in the mutant, *xyloglucanendotransglycosylase* and *expansin* genes were very low but inducible upon BR-feeding in the *cbb3* mutant (Kauschmann et al. 1996; A. Kauschmann, D.J. Cosgrove, and T. Altmann, unpublished results). In the *cpd* mutant, a subset of genes known to be responsive to stress conditions such as *chalcone synthase* (*chs*), *alcohol dehydrogenase* (*adh*), *lipoxygenase* (*lox2*), *S-adenosylmethionine synthase* and *heat shock protein 18.2* (*HSP 18.2*) showed elevated steady-state mRNA levels, while the pathogenesis-related genes *PR1*, *PR2*, and *PR5* were reduced in expression (Szekeres et al. 1996). Conversely, overexpression of the *CPD* gene caused induction of the three *PR* genes (Szekeres et al. 1996). The expression of the *CPD* gene with respect to tissue specificity and feedback-regulation by BRs has recently been studied by means of analysis of mRNA steady-state levels and the use of *CPD* promoter-*uidA* (β-glucuronidase) reporter gene fusions introduced into transgenic *Arabidopsis* plants (Mathur et al. 1998). In etiolated seedlings, expression was confined to cotyledons, which in light-grown plants showed transient activity of the *CPD* promoter. At later stages of development, expression shifted to developing leaves and finally decayed in ageing leaves. In the shoot, expression appeared to be mostly confined to leaves and no activity was observed in roots. As *CPD* is necessary for BR biosynthesis, its expression pattern is probably indicative of the regions in the plant competent for BR synthesis. Other organs such as the hypocotyl, shoot axis and floral organs (except for the sepals), which, according to the phenotypes of the mutants clearly require BRs for normal development may not be able to synthesise BRs and may thus depend on BR import. This work also uncovered a striking analogy of the regulation of the *CPD* gene to the end-product regulation of steroidogenic P450 genes in animals. Expression of the *CPD* gene was specifically repressed by BRs in a protein-synthesis-dependent manner. Other plant growth factors such as auxin, ethylene, gibberellin, cytokinin, jasmonic acid and salicylic acid, however, did not influence the activity of the *CPD* gene (Mathur et al. 1998). It will be interesting to see if the signalling involved in this feedback regulation depends on the *BRI* gene product required for all other aspects of BR perception/signalling hitherto analysed (see below) and whether a lack of BR synthesis, e.g. due to loss of *DWF1*, *DET2*, or *DWF4* function, would lead to *CPD* overexpression.

Further BR-responsive *Arabidopsis dwarf* mutants, including *dwf5*, *dwf7* and *dwf8* (Choe et al. 1998; K.A. Feldmann, personal communication) await detailed analysis. Initial results indicate that BR (phytosterol) metabolism may be blocked prior to the formation of 24-methylenecholesterol in *dwf5* and *dwf7* while the *dwf8* mutant seems to be impaired in a reaction late in the BR-specific pathway (K.A. Feldmann, personal communication Fig. 2).

Another cytochrome P450 (CYP85) most probably involved in BR biosynthesis has been identified in tomato (CYP85; Bishop et al. 1996). The corresponding mutant, *dwarf* (*d*), has recently been shown to be BR-responsive and to be deficient for endogenous castasterone (G.J. Bishop, personal communication). Although the precise step in the BR biosynthetic pathway catalysed by the *D*-encoded CYP85 has not been identified yet, these data indicate a function of CYP85 in BR biosynthesis. Interestingly, the *D* gene has been isolated via insertion of the transposable element *Ac* into transgenic tomato plants (Bishop et al. 1996). The co-occurrence of mutant and revertant sectors (the latter caused by restoration of the gene function through excision of *Ac*) on the same leaf seems to indicate limitations in the degree to which, and/or the range over which, transport of BRs (at least the products of the CYP85-catalysed reaction and further derived metabolites) occur in tomato leaves. According to data obtained with reporter-gene fusions to the *D* gene promoter introduced into transgenic tomato plants, expression of the *D* gene is confined to submeristematic elongating regions of tomato seedlings (Bishop et al. 1998). Another three dwarfed tomato mutants, *Crk* (*crinkled*), *cb-2* (*cabbage-2*), and *tbr* (*tomato brassinosteroid responsive*) have been shown to respond strongly to BR treatment (G.J. Bishop, personal communication) and await further detailed physiological, genetic and molecular characterisation.

In summary, a set of *Arabidopsis* mutants affected at four different steps in BR, or BR-precursor (phytosterol) biosynthesis have been identified and the corresponding genes isolated. The majority of these mutants still await a detailed biochemical characterisation, i.e. determination of the changes in endogenous BR levels as well as alterations in the contents of other phytohormones and metabolites caused by the genetic defects. Furthermore, the demonstration of the enzymic function of (most of) the corresponding gene products in vitro or in heterologous expression systems is still missing and is one of the future tasks in the molecular analysis of BR biosynthesis, as is the identification and characterisation of the genes coding for the factors involved in the remaining steps in BR biosynthesis, especially in the later parts of

the pathway. For a subset of the lesions characterised in *Arabidopsis*, corresponding BR-deficient mutants of pea and tomato have been identified and analysed to various extents at the biochemical or molecular genetic level. Further completion of the collection of BR biosynthetic mutants and the corresponding genes will provide indispensable tools for the elucidation of the BR biosynthetic pathway(s) or networks and the possible existence of parallel pathways that convert different phytosterols into BRs.

The role of BRs as regulators of physiological processes requires precise temporal and spatial control of their endogenous levels. As indicated by the negative feedback regulation observed for the expression of *CPD*, at least part of this control is exerted on the anabolism of BRs. A similar importance can be expected for the regulation of deactivating or degrading activities. Although a variety of reactions that (most probably) lead to BR inactivation, including epimerisation, hydroxylation/oxidation, side-chain cleavage, and/or conjugation to sugars or fatty acids, have been identified (summarised in Adam et al. 1996), neither the genes coding for the corresponding enzymic factors nor any mutants deficient for these activities have yet been identified in any plant.

The phenotypic alterations caused by the loss of the various biosynthetic activities are remarkably consistent among the different mutants, at least within the collection of *Arabidopsis* mutants characterised in detail and with the exception of the *dwf1* alleles which appear to be less severely affected than those of the other mutants. Common features of the BR-deficient mutants are a dramatic dwarfism caused by reduced cell expansion occurring in light- and dark-grown plants and a de-etiolated development in darkness with corresponding changes in gene expression patterns. Further detailed phenotypic analysis of the mutants will be required to learn about the full range of consequences caused by the BR deficiency and to distinguish between primary and secondary effects. This may, in particular, apply to the proposed role of BRs in light-regulated development, as the de-etiolated phenotype of the dark-grown mutants has recently been proposed to be a secondary consequence of the reduced elongation growth (Azpiroz et al. 1998). Furthermore, the effects that BRs exert on the levels of other phytohormones and vice versa require detailed biochemical analysis of the BR-deficient mutants in order to extend the initial information gathered on the pea *lkb* mutant (and the BR-insensitive pea *lka* mutant, see below) which showed reduced endogenous auxin contents but no change in gibberellin levels (Lawrence et al. 1992; McKay et al. 1994).

Brassinosteroid perception/signal transduction mutants and corresponding genes. Loss of activity of factors involved in the primary perception of the BR signal (receptor mutants), in essential components of a (hypothetical) signal transduction pathway, or in effectors (target genes) that are responsible for the expression of major components of the BR response is expected to cause insensitivity to BRs. Accordingly, a second class of BR-related mutants (BR-insensitive mutants) has been identified for *Arabidopsis*, pea, and tomato (Fig. 3). In pea, the *lka* mutant, which displays a dwarfed phenotype similar to that of the BR-deficient *lkb* mutant, was shown to be 100 times less responsive to brassinolide application as compared to *lkb* and was therefore classified as BR insensitive (Nomura et al. 1997). Furthermore, the endogenous contents of brassinolide were reduced to only 50% and the castasterone levels were increased 5-fold in *lka* in comparison to the WT (Nomura et al. 1997). Brassinosteroid insensitivity has been observed recently for the *cu-3* mutant of tomato (S.D. Clouse, personal communication). A large collection of BR-insensitive *Arabidopsis* mutants has been isolated which all appeared to be allelic and affected in the *BRI1* locus (Clouse et al. 1996; Kauschmann et al. 1996; Li and Chory 1997; Choe et al. 1998). It will be interesting to see whether, in *Arabidopsis*, this is due to the presence of only one factor which is central to and specific for the mediation of the known BR responses. Alternatively, genetic redundancy of the genes coding

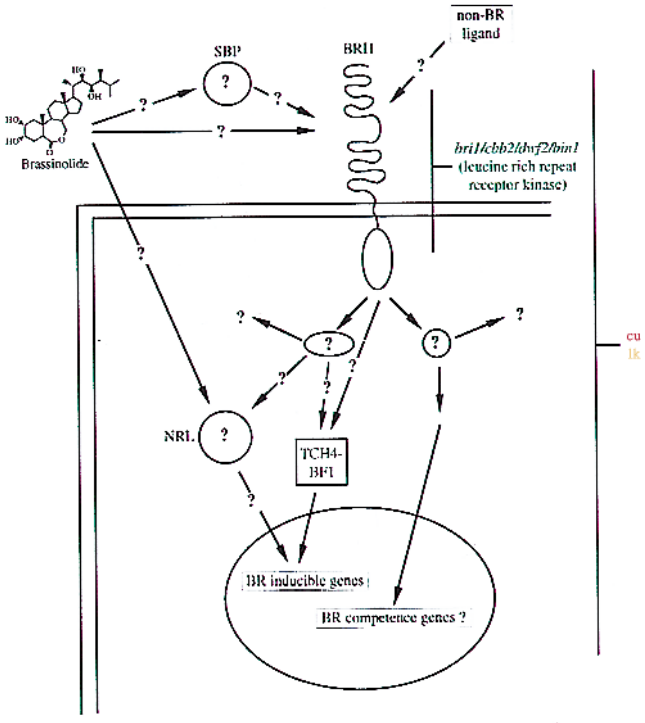

Fig. 3. Potential BR signal transduction pathway involving BRI1, a putative receptor kinase with leucine-rich repeats. BRI1 may bind BRs directly or indirectly (potentially via steroid-binding proteins, *SBP*) and, after ligand binding may phosphorylate other signal transduction intermediates that finally activate expression of BR-inducible genes (potentially via factors such as TCH4-BF1 or other transcripton factors). Alternatively, BRI1 activation may lead to the induction or activation of genes or factors that provide competence to BR perception to the cell. The existence of additional, BRI-independent BR-signalling pathways, e.g. via nuclear-receptor-like (*NRL*) factors similar to animal steroid receptors cannot yet be excluded. The allelic series of the BR-insensitive *bri1* mutants in *Arabidopsis thaliana* (marked in *green*) are deficient for activity of the BRI gene; the genes mutated in other BR-insensitive mutants identified in tomato (marked in *red*) and pea (marked in *yellow*) have not yet been identified at the molecular level

for other essential components (presence of multiple gene copies) or deleterious pleiotropic effects (e.g. gametophyte or embryo lethality) may have prevented their identification through loss-of-function mutations. Plants carrying two mutant *bri1* alleles, *bri1-1* and *cbb2* (*bri1-2*) have been subjected to detailed phenotypic analysis (Clouse et al. 1996; Kauschmann et al. 1996). The *bri1-1* mutant was isolated on the basis of resistance to BRs with respect to root growth which is inhibited in *Arabidopsis* seedlings grown on BR-containing synthetic medium (Clouse et al. 1993). Root growth of *bri1-1* seedlings was not inhibited by a wide range of BR concentrations applied through the medium but was as sensitive as that of the WT upon treatments with auxin, cytokinin, and ethylene and showed only a very minor decrease in sensitivity towards gibberellins (Clouse et al. 1996). A hypersensitivity of the *bri1-1* roots was observed upon abscisic acid treatment. Further evidence for a specific insensitivity towards BRs has been provided by the analysis of the *cbb2* (*bri1-2*) mutant which was studied in parallel to the BR-deficient *dwf1-6* and *cbb3* mutants mentioned above (Kauschmann et al. 1996). While morphologically indistinguishable from untreated, severely dwarfed *cbb3* seedlings, *cbb2* (*bri1-2*) plants were dramatically different with respect to their response to exogenously applied BRs that rescued the phenotypic defects of *cbb3* seedlings but did not affect the growth of *cbb2* (*bri1-2*). As for the BR-deficient *cbb3* and *dwf1-6* mutants, the growth defect of *cbb2* (*bri1-2*) could not be rescued by any other phytohormone or phytohormone inhibitor treatment (Kauschmann et al. 1996). Furthermore, the steady-state mRNA levels of the *TCH4* and *MERI5* genes were reduced to the same extent in *cbb3* and *cbb2* (*bri1-2*), but in contrast to the situation in *cbb3* (and *dwf1-6*) they could not be induced in *cbb2* (*bri1-2*) through BR feeding. The specificity of this defect towards BRs was demonstrated by gibberellin-feeding experiments that resulted in induced expression of *MERI5* in the BR-insensitive *cbb2* (*bri1-2*) mutant, the BR-deficient *cbb3* and *dwf1-6* mutants, and the WT (Kauschmann et al. 1996). A set of additional 18 *bri1* alleles has been identified, one of which carried a mutation that resulted in a detectable restriction fragment length polymorphism located within the *BRI1* gene and thus strongly supported its map-based cloning (Li and Chory 1997). The gene *BRI1* encodes a putative leucine-rich repeat (LRR) receptor-like kinase (RLK) showing striking similarities to other plant LRR-RLK genes such as *CLAVATA1*, *ERECTA*, or *Xa21* (Song et al. 1995; Torii et al. 1996; Clark et al. 1997). The proposed function of these genes is the transmission of signals responsible for the regulation of developmental processes that probably involve cell-cell communication or plant pathogen recognition. Consistent with the pleiotropic effect of a loss of *BRI1* activity, it is constitutively expressed throughout WT plants, both in the light and in darkness. The structural features of the predicted BRI1 protein, with a (probably extracellular) LRR domain that may comprise a ligand-binding domain and an intracellular kinase domain potentially involved in the signal transduction to intracellular targets, strongly indicate a role for BRI1 as a receptor molecule. While the function of the intracellular domain of BRI1 as a kinase has been demonstrated in vitro (J. Li, personal communication; S.D. Clouse, personal communication), and by analysis of several mutant alleles was shown to be essential for BRI1 activity (Li and Chory 1997), the role of the extracellular domain of the protein is less clear. A ligand that would bind to the LRR domain is not yet known. Two models have been proposed for the function of BRI1 as a component of the BR-signal transduction pathway (Li and Chory 1997). An unusual interruption of the LRRs by a stretch of 70 amino acids may form a BR-binding site for direct interaction with BRs. Alternatively, BR perception may be mediated via potential steroid-binding proteins that bind to the LRR domain. The latter possibility would be consistent with the role of LRR domains in protein-protein interactions, with which they have hitherto been implicated (Kobe and Deisenhofer 1994). A leucine zipper motif, located in the extracellular domain at the N-terminus of the mature protein indicates the potential of BRI to form homo- or heterodimers upon ligand binding. Such a potential interaction with other LRR-RLKs (discussed in Koncz 1998) is a particularly attractive possible explanation of the BRI1 mode of action. The possible involvement of BRI1 with several different proteins may help to explain the diverse developmental changes caused by loss of BRI1 function and to link the numerous reports of increased resistance of BR-treated plants to pathogen attack (e.g. Kamuro et al. 1997; Khripach et al. 1997), and the modulation of pathogenesis-related (PR) protein expression by BRs (Szekeres et al. 1996) to BR action.

Clearly, further work will be required to elucidate the precise function of BRI1 (Fig. 3) and to exclude the possibility of an indirect role of BRI1 in BR signalling: involvement of BRI1 in the establishment of the cells competence to react to BRs (e.g. by stimulation of the expression of components of the BR signalling pathway or by modulation of their activation state) rather than a function in the mediation of the BR signal itself could also explain the *bri1* mutant phenotype and would be consistent with the currently available data. Like the putative steroid-binding proteins, the native substrates of the BRI1 kinase await molecular identification and characterisation.

Another putative component involved in the control of the expression of BR-regulated genes, TCH4-BF1, has recently been identified (C. Müssig, A. Kauschmann, and T. Altmann, unpublished results). TCH4-BF1 contains a zinc-finger-like motif, the PHD finger, which has been identified in a variety of (putative) transcription factors, most notably in members of the *Drosophila Polycomb* and *trithorax* group genes and in a subset of transcription factor co-activators and co-repressors (Aasland et al. 1995; LeDouarin et al. 1995; Friedman et al. 1996). The PHD finger may mediate binding to DNA (or RNA) or protein-protein interactions. The TCH4-BF1 protein expressed in *Escherichia coli* was shown through gel mobility-shift assays to bind to a specific fragment of the BR-inducible *TCH4* promoter

and to be phosphorylated in vitro by recombinant BRI1 kinase domain (M.-H. Oh and S.D. Clouse, personal communication).

In summary, the identification of BRI1 is an important step towards a molecular understanding of BR signal transduction. This provides access to highly valuable tools for identifying other components involved, and the data already collected will serve as an excellent entry point to start answering the large number of open questions related to the possible existence of BRI1-independent BR signal transduction pathways (e.g. mediated via nuclear receptors similar to animal steroid receptors; Beato et al. 1995; Thummel 1995, 1996), the potential existence of branches within and cross-talk between these pathway(s), the potential interaction between BR signalling and other cellular signal transduction pathways, the identification of a full set of genes (pen-)ultimately regulated by BRs, and the potential non-genomic effects (direct alterations of cellular activities) triggered by BRs.

Future prospects

It can be expected that a further advancement in the knowledge of BR biosynthesis and mode of action will rapidly be achieved from approaches that integrate molecular/genetic, biochemical, and biophysical techniques. The current limited set of known BR-deficient mutants indicates that more genes encoding biosynthetic factors can probably be identified genetically. In addition, more BR-related mutants such as second-site suppressors and enhancers of the known mutations should be isolated that will uncover additional factors of regulatory or enzymic function. The analysis of these novel mutants, the further characterisation of the already known biosynthetic mutants, and the study of the enzymatic and regulatory functions of the corresponding gene products will modify our view of the BR-biosynthetic pathway or network. Mutants identified or created in other plant species will provide further evidence for a general function of BRs as regulatory factors in plant growth, development and metabolism. (It is interesting to note that BR-deficient or insensitive monocot mutants have not yet been identified.) Increased knowledge of the genes and factors regulated by BRs, the components that mediate the cellular response(s) triggered by BRs, and their (potential) interaction with compounds involved in other signalling pathways, will allow elucidation of the precise role that BRs play in the various processes which are indicated by the broad spectrum of phenotypic changes observed in the BR-deficient or insensitive mutants and the responses elicited through exogenous BR application. Important tools that will permit a rapid progress towards this goal are the isolated genes which are involved in BR biosynthesis or signal transduction. These provide the means to generate modified plants with spatially or temporally controlled (subtle) changes in BR contents or sensitivity; such plants will be an important complement to the currently available collection of mutants.

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