The importance of brassinosteroids (BRs, a specific class of ecdysone-like plant steroids) as essential endogenous regulators of growth and development is demonstrated through a growing number of well characterised Arabidopsis, pea, and tomato mutants deficient in BR biosynthesis or BR response. Thus, a rapid advancement in understanding the molecular genetics of BR biosynthesis and mode of action can be witnessed, which will be further enhanced through the availability of a set of extremely valuable molecular tools for the analysis of the biological function of BRs.

**Introduction**

For many years, research on Brassinosteroids (BRs) was mainly focused on the analysis of naturally occurring members of this class of substances, the analysis of the responses of explants or intact plants to exogenous applications of BRs, and the elucidation of their biosynthesis and metabolic conversions (for extensive reviews on these topics, see [1–7,8•,9•]). Thus, over 40 BRs have been identified in as many as 36 plants species which, like animal steroids such as oestrogen, testosterone, or ecdysone, are composed of a typical steroidal skeleton with specific substitutions required for biological activity (see Figure 1). Furthermore, a wealth of information was accumulated on the developmental and physiological changes elicited upon BR application to either explants such as hypocotyl or epicotyl segments or to intact plants [2–4,7,10]. Information on the biosynthesis (Figure 2) [8•,9•] and the metabolism of BRs [6] was obtained more recently and it can be regarded as an ideal coincidence that the full elucidation of the biosynthetic pathway [8•] was achieved almost exactly at the time that the first mutants impaired in BR biosynthesis or response were identified and the corresponding genes isolated [11,12,13]. Since that time, the spectrum of known mutants/genes has been broadened and the identified mutants have been analysed in more detail. The set of described mutants and the current status of their characterisation is summarised in this review. Emphasis is given to the knowledge gained since January 1997 and also to the consequences of this information on the conclusions about the biological role(s) of BRs and their mode of action that can be drawn from these data are discussed. This is by no means meant to discredit other work but was done to limit the contents of this review to the most recent advances in the field which is shifting towards the application of more integrated approaches using genetic, molecular and biochemical tools.

**Mutants impaired in BR metabolism**

Careful biochemical work resulted in the establishment of a branched biosynthetic pathway from the phytosterol campesterol to brassinolide (Figure 2) [8•], and provided an excellent framework for the biochemical characterisation of mutants impaired in BR biosynthesis.

Four different genes have been isolated from Arabidopsis thaliana [11–14,15•] and a further gene from tomato [16], which upon mutation result in BR deficiency in the corresponding mutant plants. Furthermore, two pea mutants have been shown to be BR deficient ([17•]; Yokota Y, Nomura T, Kitasaka Y, Takasuto S, Reid JB, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p94) and an additional four candidates for BR-responsive Arabidopsis mutants ([15•]; KA Feldmann, personal communication) and four BR-responsive tomato mutants have been identified (SD Clouse, personal communication; GJ Bishop, personal communication). All of these mutants display a characteristic dwarf
phenotype that can only be normalised through BR feeding, and not by treatment with any other phytohormone or phytohormone antagonist ([11–13,16,17•,18]; Klahre U, Fujioka S, Yokota T, Chua N-H, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p99; Yokota Y, Nomura T, Kitasaka Y, Takesuto S, Reid JB, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p94; SD Clouse, personal communication; GJ Bishop, personal communication).

Mutations in the DWF1 (DWF1)/DIMINUTO (DIM) gene of Arabidopsis [13,14,19] encoding a protein which contains a domain conserved in several FAD-dependent oxidases [20], result in BR-deficiency due to a lack of the formation of the phytosterol precursor to the BR biosynthetic pathway, campesterol. Thus, 24-methylenecholesterol has been shown to accumulate in the mutant and, in contrast to the wild-type, mutant seedlings were unable to convert deuterium labelled 24-methylenecholesterol or the intermediate 24-methyl desmosterol to campesterol (Klahre U, Fujioka S, Yokota T, Chua N-H, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p99). The lkb mutant of pea was shown to be deficient for the same reaction and to be furthermore affected in the synthesis of other phytosterols such as sitosterol, sitostanol, and stigmasterol, the levels of which are also drastically reduced (Yokota Y, Nomura T, Kitasaka Y, Takesuto S, Reid JB, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p94). As a result of the increased content of the corresponding precursor, isofucosterol, the sterol composition of its membranes is strongly altered (Yokota Y, Nomura T, Kitasaka Y, Takesuto S, Reid JB, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p94) potentially causing detrimental effects on membrane function. This observation needs attention when conclusions about the role of BRs are drawn from experiments with these BR-deficient phytosterol mutants.

The first biosynthetic reaction specific to the BR biosynthetic pathway, the conversion of campesterol to campestanol, that proceeds via a recently identified intermediate pathway, the conversion of campesterol to stigmasterol, the sterol composition of its membranes is strongly impaired in the det2 and lk mutants of Arabidopsis and pea, respectively ([11]; Yokota Y, Nomura T, Kitasaka Y, Takesuto S, Reid JB, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p94). The DET2 protein shares very high amino acid sequence similarity with mammalian steroid 5α-reductases and was shown to convert mammalian steroids including testosterone and progesterone to the corresponding dihydro-derivatives [22•]. Furthermore, expression of human steroid 5α-reductase in transgenic Arabidopsis plants resulted in complementation of the det2 mutation. The level of the natural substrate of the DET2 protein in plants, (24R)-24-methylenecholesterol-4-en-3-one, is increased 3-fold in det2 mutants, while the campestanol contents are reduced to 8–15% of the wild-type levels and the contents of all other BRs analysed are below 10% of the wild-type [21••]. Similarly, reduced levels of endogenous castasterone and 6-deoxocastasterone were observed in the lk mutant (Yokota Y, Nomura T, Kitasaka Y, Takesuto S, Reid JB, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p94).

Two Arabidopsis loci, dwarf4 (dwarf4) and constitutive photomorphogenesis and dwarfism (cpd)/cabbage3 (cbb3)/dwarf3 (dwarf3), have been identified ([12,13,15•,18]; KA Feldmann, personal communication) that encode cytochrome P450 monoxygenases responsible for the introduction of hydroxyl groups into the BR side chain. The DWF4 gene product (CYP90B) has been shown to mediate the hydroxylation of the C22 atom in the early and the late C6 oxidation pathways (Figure 2) [15•], a reaction that has been proposed to be a rate-limiting step in BR-biosynthesis [23]. Interestingly, feeding of the synthetic C22 hydroxylated compounds 22-hydroxy-campesterol and 6-hydroxy-cathasterone to dwarf4 also rescued this mutant [15•], indicating a relaxed substrate specificity of the early enzymes. This might suggest that the various modifications of the BR biosynthetic intermediates do not occur in a particular fixed order and that the BR-biosynthetic pathway could be viewed as a network of reactions rather than a (branched) linear arrangement of conversions. Like other BR-deficient mutants, the dwarf4 mutant displays the prominent feature of a ‘de-etiolated’ or ‘constitutive photomorphogenic’ morphology [24,25], also exhibited by seedlings grown in darkness, with the development of a short hypocotyl, lack of an apical hook, opened cotyledons and the emergence of primary leaves. In contrast to previous interpretations [11,12] this alteration in the morphogenesis of the mutant plants has been proposed [18] to be a (secondary) consequence of the dwarfism, rather than a result of the loss of a direct regulatory function of BRs in this process [18]. It was speculated [18] that a close proximity between the growth medium and the apical meristem may mimic, to some extent, conditions established through submerged culture which have earlier been shown to trigger leaf development, derepression of light inducible genes and even flowering in wild-type Arabidopsis plants [26]. This plausible interpretation argues against a direct role for BRs in light regulated plant development. A further difference of the dwarf4 mutant, to the cbb3/cpd mutants [12,13] for example, seemingly is the development of slightly attenuated symptoms with an apparently larger height of dwarf4 plants at maturity, and the reduced fertility of dwarf4 was due to shorter stamen filaments [18] rather than the pollen defects observed for the cpd mutant [12]. In the light of the close similarity of the gene functions of DWF4 and CBB3/CPD, these differences are not immediately obvious and would foster a further detailed comparative analysis. Differences in the phenotypic expression of these various mutants may be due to different degrees of (partial) genetic redundancy of
the corresponding genes, including the potential existence of parallel (but differentially regulated) biosynthetic pathways, or may point to specific individual functions of the different BRs (here primarily discussed as biosynthetic...
Brassinosteroid (BR) biosynthetic pathway as exemplified by the route(s) from 24-methylene-cholesterol via the phytosterol campesterol towards brassinolide. Campesterol is one of the major phytosterols which are constituents of membranes. The conversion of campesterol to campestanol via a recently identified intermediate, (24R)-24-methylcholesten-4-en-3-one, and another proposed intermediate, (24R)-24-methyl-5x,8,8a-trien-3-one [21], provides the first step into the BR-specific pathway. Two hydroxylation reactions in the side chain may either occur after (early C6 oxidation pathway) or before (late C6 oxidation pathway) the hydroxylation and oxidation of the C6 position in the B ring of the sterol. The side chain hydroxylations that lead to the formation of cathasterone or 6-deoxo-cathasterone, respectively, are followed by epimerization of the hydroxy group at position 3 in the A ring and another hydroxylation at position 2 to form castasterone, or 6-deoxo-castasterone in the case of the late C6 oxidation pathway. The latter is oxidised to castasterone which is finally converted to brassinolide through the formation of a lactone structure in the B ring. The sites of the blocks in the metabolism caused by genetic lesions in the various BR deficient mutants are marked with the corresponding gene/allele symbols. Arabidopsis thaliana mutants are printed in green, pea mutants in yellow, and tomato mutants in red. At the bottom, BRI 1, a/the putative brassinosteroid receptor is schematically drawn with an extracellular leucine rich repeat domain and an intracellular kinase domain separated by a transmembrane region. BR-binding to the putative extracellular receptor domain (potentially mediated via steroid binding proteins) has not been demonstrated yet; the native substrates (labelled with question marks) of the intracellular kinase domain seek identification and characterization and the possible existence of BRI1 independent BR-signalling pathway(s) can not yet be excluded

intermediates) formed through the action of the affected gene products, and thus may provide important information on BR function.

The second hydroxylation reaction in the BR side chain, at C23, is mediated by the CPD gene product (CYP90A) in Arabidopsis thaliana [12], that was also recently shown to be involved in the late C6 oxidation pathway (C Koncz, personal communication). Similarly, BR-feeding experiments with the tomato dumpy (dpy) mutant indicated a defect in C23-hydroxylation, which, therefore, indicates that dpy is most probably deficient for the corresponding CYP90A ortholog (SD Clouse, personal communication).

Four further BR-responsive Arabidopsis dwarf mutants, dwf5, dwf7, dwf8, and dwf9, have been identified ([15•]; KA Feldmann, personal communication). For dwf5 and dwf7 the (potential) block in BR metabolism appears to reside prior to the formation of 24-methylenecholesterol and dwf8 seems to be affected late in the BR-pathway (KA Feldmann, personal communication).

The tomato dwarf (d) mutant, which is impaired in another cytochrome P450, CYP85 [16], has recently been shown to be BR-responsive and to be deficient for endogenous castasterone (GJ Bishop, personal communication). Overexpression of the DWARF gene under the control of the CaMV 35S promoter in transgenic tomato plants resulted in 3-fold increased levels of Castasterone and a 20% increased plant height as compared to wild-type plants (GJ Bishop, personal communication). Although the nature of the biochemical reaction catalysed by the D encoded CYP85 is not yet known, these data indicate a function of CYP85 in BR biosynthesis. Further dwarfed tomato mutants such as Crk (crinkled), cb-2 (cabbage-2), and thr (tomato brassinosteroid responsive) have been shown to be strongly BR responsive with respect to hypocotyl elongation (GJ Bishop, personal communication) and await further detailed physiological and molecular/genetic analysis.

Studies on the promoter activity of biosynthetic genes provided the first insight into the site of BR biosynthesis and its regulation. Thus, it was demonstrated that the CPD promoter is active in etiolated seedlings only in cotyledons, whereas in light-grown plants a transient activity was observed in cotyledons, which at later stages of development shifted to developing leaves and in ageing leaves gradually decayed to become restricted to the leaf margins [27•]. These findings implicate a necessity of BR-transport to organs such as the hypocotyl, shoot axis and floral organs which, according to the phenotypes of the mutants, clearly require BRs for normal development but may not be able to synthesise BRs. Furthermore, a striking analogy to the end-product regulation of steriodogenic P450 genes in animals was observed for the CPD gene, whose expression was specifically repressed by BRs in a protein synthesis dependent manner but was not influenced by other plant growth factors such as auxin, ethylene, gibberellin, cytokinin, jasmonic acid, and salicylic acid [27••]. The promoter of the tomato D gene was shown to be expressed in submeristematic elongating regions of tomato seedlings (Bishop GJ, Harrison K, Jones J, Kamiya Y, J Exp Bot 1998, 49:66, supplement). These observations indicate that only a subset of plant organs/tissues are competent to BR-biosynthesis and that at least part of the control of BR levels is exerted by a negative feed-back loop on biosynthetic genes.

**BR response mutant(s)**

The second class of BR-related mutants are impaired in BR sensing and/or response. These insensitive mutants are expected to be blocked 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. BR-insensitive mutants have been identified for Arabidopsis, pea, and tomato with the largest collection, again, comprised by the Arabidopsis mutants. Surprising, however, was the observation that all BR-insensitive Arabidopsis mutants hitherto described are allelic and are affected in the BRI1 locus ([28,13,29••,15•]; SD Clouse, personal communication). Thus, apparently, only one factor central to the mediation of all BR responses could be identified genetically. This
may either be due to genetic redundancy of the genes coding for other essential components (presence of multiple gene copies), or due to deleterious pleiotropic effects (e.g. gametophyte or embryo lethality) caused by a loss of function of these genes or due to the fairly limited set of criteria (root growth inhibition by BRs and dwarfism) hitherto used to identify BR-insensitive mutants.

A large screen for ethyl methanesulfonate EMS-induced, BR-insensitive (bri) Arabidopsis mutants resulted in the identification of 18 new bri1 alleles, and provided a means to rapidly isolate the BRIIgene through the identification of a mutation in the gene that resulted in a detectable RFLP [29••]. The BRII gene was shown to encode a putative leucine rich repeat (LRR) receptor like kinase (RLK) which appeared to be constitutively expressed throughout the plant, both, in the light and in darkness. The predicted protein shows striking similarities to other plant LRR-RLK gene products such as those of CLAVATA1, ERECTA, or Xa21 which are involved in as divergent processes as the mediation of developmental signal pathways (probably involving cell–cell communication) and pathogen recognition [30,31,32]. The presence of the (probably extracellular) LRR-domain and an intracellular kinase domain strongly indicate a role for BRII as a receptor, with the LRR comprising a ligand binding domain and the kinase domain being involved in the signal transduction to intracellular targets. The nature of the proposed ligand which would bind to the LRR-domain has not been elucidated. Based on the assumption that BRII indeed functions as a receptor directly involved in BR signalling, two equally attractive possibilities have been proposed; the extracellular domain may (a) bind BRs directly (potentially at a stretch of 70 amino acids which interrupt the LRRs), or it may (b) interact with BRs via a potential steroid binding protein [29••]. Another sequence motif located N-terminally in the putative extracellular domain of the mature protein, a leucine zipper motif, would indicate that BRI may form homo- or heterodimers.

By analogy to animal tyrosine receptor kinases, BRII may homo- or heterodimerise upon ligand binding. This is particularly attractive in the light of the similarity between BRII and the Xa21 disease resistance gene of rice, and the reports of increased resistance of BR-treated plants to pathogen attack (e.g. Kamuro Y, Takasuto S, Watanabe T; Noguchi T, Kuriyama H, Suganuma H, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p.111; Khripach VA, Zhabinskii VA, Malevannaya NN, Proceedings of the Plant Growth Regulation Society of America 8–12 August 1997, Atlanta p101), as well as the modulation of pathogenesis-related (PR) protein expression by BRs [12]. Further exciting possible functions of BRII may involve the interaction with other LRR-RLKs involved in the regulation of several developmental processes, e.g. CLAVATA1 or ERECTA (discussed in [33]). The function of the BRII kinase domain has been demonstrated in vitro (J Li, personal communication; SD Clouse, personal communication) and the importance of its intactness was shown by analysis of several mutant alleles [29••].

Although the possibility of BRII acting in a signalling pathway which is not triggered by BRs but would be necessary to provide competence to the cells 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) cannot yet be excluded, the function of BRII probably is that of a receptor for BRs (potentially bound to a steroid binding protein) which initiates a protein phosphorylation cascade. Like the putative steroid binding proteins, the native substrates of the BRII kinase await molecular identification and characterisation.

Conclusions

The identification and characterisation of additional BR-deficient mutants (especially in species other than Arabidopsis) and the further biochemical analysis of the previously identified mutants substantially strengthened the conclusions drawn from the phenotypic alteration observed in the mutants on the biological role of BRs and corroborated previously/independently obtained biochemical data on BR biosynthesis. The availability of well defined biosynthetic mutants and of the corresponding genes provide the means to proceed to another level of biochemical analysis (including the enzymic characterisation of biosynthetic factors and their temporal and spatial regulation) and to identify and understand regulatory pathways/networks operating in the control of BR levels and in the (potential) cross-regulation of the various phytohormones/signalling molecules that ultimately determine the physiological status of cells and tissues.

The phenotypic analysis of the BR-deficient mutants demonstrated a major role of BRs in the regulation of cell wall expansion growth and provided indications for their involvement in such a divergent set of processes in plants as photo-/skotomorphogenesis (morphogenesis in the light in darkness), fertilisation, cell/tissue specification, and stress responses. The future of BR research will surely involve in-depth analysis of the function of BRs in the associated reactions and will be strongly supported by the ability to modulate BR-levels in planta through antisense inhibition and overexpression of the identified biosynthetic genes. The understanding of the role(s) of BRs will also involve the identification of the full set of BR-responsive genes and the analysis of their expression patterns which, again, will be strongly aided by the availability of BR-deficient and BR-insensitive mutants. Finally, the identification and characterisation of the first component involved in BR signalling provides an excellent entry point to elucidate the signal transduction pathway(s) triggered or modulated by BRs. The analysis of the function of BRs will furthermore be stimulated by the characterisation of novel mutants (most notably suppressors or enhancers of the hitherto known mutations) and will require the
integration of a broad spectrum of genetic/genomic, molecular biological, biochemical, and biophysical approaches.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest