ReseaRch papeR

Brassinosteroid biosynthesis and signalling in Petunia hybrida

Nathalie Verhoef1,*, Takao Yokota2, Kyomi Shibata3, Gert-Jan de Boer2, Tom Gerats4, Michiel Vandenbussche5, Ronald Koes1 and Erik Souer1,†

1 Department of Molecular Cell Biology, section Genetics, VU-University, de Boelelaan 1085, 1081 HV Amsterdam, The Netherlands
2 Department of Biosciences, Teikyo University, 1-1 Toyoateodai, Utsunomiya, Japan
3 Enza Zaden, Postbus 7, 1600 AA Enkhuizen, The Netherlands
4 Department of Plant Genetics, Institute for Water and Wetland Research, Radboud University Nijmegen, 6525 ED, Nijmegen, The Netherlands
5 Laboratoire de Reproduction et Développement des Plantes, Université de Lyon, Centre National de la Recherche Scientifique, Institut National de la Recherche Agronomique/Ecole Normale Supérieure, F-69347 Lyon cedex 07, France

* Present address: NSure, Postbus 14, 6700 AA Wageningen, The Netherlands.
† To whom correspondence should be addressed. E-mail: erik.souer@vu.nl

Received 18 December 2012; Revised 18 February 2013; Accepted 11 March 2013

Abstract

Brassinosteroids (BRs) are steroidal plant hormones that play an important role in the growth and development of plants. The biosynthesis of sterols and BRs as well as the signalling cascade they induce in plants have been elucidated largely through metabolic studies and the analysis of mutants in Arabidopsis and rice. Only fragmentary details about BR signalling in other plant species are known. Here a forward genetics strategy was used in Petunia hybrida, by which 19 families with phenotypic alterations typical for BR deficiency mutants were identified. In all mutants, the endogenous BR levels were severely reduced. In seven families, the tagged genes were revealed as the petunia BR biosynthesis genes CYP90A1 and CYP85A1 and the BR receptor gene BRI1. In addition, several homologues of key regulators of the BR signalling pathway were cloned from petunia based on homology with their Arabidopsis counterparts, including the BRI1 receptor, a member of the BES1/BZR1 transcription factor family (PhBEH2), and two GSK3-like kinases (PSK8 and PSK9). PhBEH2 was shown to interact with PSK8 and 14-3-3 proteins in yeast, revealing similar interactions to those during BR signalling in Arabidopsis. Interestingly, PhBEH2 also interacted with proteins implicated in other signalling pathways. This suggests that PhBEH2 might function as an important hub in the cross-talk between diverse signalling pathways.

Key words: BES1, BIN2, brassinosteroids, BRI1, BZR1, compact disc, dwarf, Petunia hybrida, SEC.

Introduction

The importance of steroidal hormones in mammalian development has already been known for almost eight decades. The idea that plant steroids have hormonal functions arose when scientists reported the isolation of brassinolide (BL), a steroid with strong plant growth-promoting activity, from bee-collected pollen of Brassica napus L. (Grove et al., 1979). Brassinosteroids (BRs) are widely distributed among the plant kingdom and so far they have been found in gymnosperms, monocots, dicots, a pteridophyte, a bryophyte, and a chlorophyte (Bajguz and Tretyn, 2003). The subsequent identification of several BR biosynthesis and signalling mutants further supported the importance of these compounds for growth and development, and nowadays BRs are accepted as phytohormones. BRs are involved in various growth and...
developmental processes such as cell division and elongation, vascular differentiation, abiotic and biotic stresses, photomorphogenesis, germination, senescence, and fertility (Clouse and Sasse, 1998; Bajguz and Hayat, 2009).

The sterol biosynthesis pathway can be divided into three domains (Fig. 1). In the first part, the mevalonate-derived squalene is converted via multiple steps into 24-methylene lophenol. Subsequently, the pathway diverges into two downstream branches. One branch produces sitosterol and stigmas- terol, while the other produces campesterol. These sterols are integrated into cellular membrane components. In addition, the biosynthetic precursor campesterol is utilized to synthesize BRs (Lindsey et al., 2003). Plants have multiple ways to synthesize BL from campesterol. Detailed studies on the BR biosynthesis route in Catharanthus roseus and Arabidopsis thaliana led to the identification of two parallel branched routes, better known as the early and late C-6 oxidation pathways that converge at clasterone (Fig. 1). The two pathways are linked to each other, implying that they are not totally autonomous (Shimada et al., 2001). Although the early and late C-6 oxidation pathways are found throughout the plant kingdom, it seems that in species such as tomato and tobacco the late C-6 oxidation pathway is more prevalent (Nomura et al., 2001). Fujioka et al. (2002) identified an early C-22 oxidation pathway, also named the CN-independent pathway, that merges into the late C-6 oxidation pathway, constituting the major route to produce biologically active BRs as shown in Fig. 1 (Onishi et al., 2012; Zhao and Li, 2012). In addition, two shortcuts were reported that convert 22-OH-3-one and 3-epi-6-deoxoCT via C-23 hydroxylation to 6-deoxy3DT and 6-deoxyTY, respectively (Ohnishi et al., 2006).

A number of sterol and BL biosynthesis mutants have been described, predominantly in Arabidopsis, and many of the genes have been cloned and studied (reviewed by Clouse, 2002). BR biosynthesis mutants and some sterol mutants such as dwarf7 (dvw7), diminuto (dim), and dwarf5 (dvw5) can be rescued by exogenous application of BR. However, mutants that are affected in genes functioning in early steps of the sterol biosynthesis pathway such as FACKEL (FK), STERO METHYL TRANSFERASE 1 and 2 (SMT1, SMT2), and HYDRA1 (HYD1) are embryo lethal and cannot be rescued by application of exogenous BR. Presumably, the absence of these sterols interferes with membrane integrity.

BR signalling has primarily been elaborated by studies in Arabidopsis. The currently known BR signalling pathway in Arabidopsis runs from membrane to nucleus (reviewed by Clouse, 2011). A key event is the degradation of a negative regulator of BR signalling, the Arabidopsis GSK3/SHAGGY-like kinase BRASSINOSTEROID INSENSITIVE 2 (BIN2; Kim et al., 2009). The negative effect of BIN2 on BR signalling is performed via phosphorylation of the closely related plant-specific transcription factors BR11-EMS-SUPPRESSOR 1 (BES1) and BRASSINOZOLE RESISTANT 1 (BZR1; Wang et al., 2002; Yin et al., 2002). It was shown that other Arabidopsis GSK3-like protein kinases (ASKs) act redundantly with BIN2 in the BR signalling pathway (Vert and Chory, 2006; Kim et al., 2009; Yan et al., 2009; Rozhon et al., 2010). Phosphorylation of BES1/ BZR1 results in inhibition of their activity through proteasome-mediated degradation, cytoplasmic retention by 14-3-3 proteins, and inhibition of their DNA binding and transcription activation activities (Li et al., 2001; He et al., 2002; Yin et al., 2002; Vert and Chory, 2006; Gampala et al., 2007; Ryu et al., 2007).

Here an extensive analysis of BR biosynthesis and signalling in petunia is presented. The goal of the study is to examine to what extent the biosynthesis of steroids and BRs and the signalling cascades downstream in plants are conserved. The authors' petunia collection was browsed for mutants that exhibit a typical BR deficiency phenotype; five BR biosynthesis or signalling mutants were identified and their BR content was determined. In three cases, the tagged genes could be identified. Furthermore, several petunia homologues of key components of the BR signalling pathway in Arabidopsis were isolated and it was determined if a similar network of interactions between these factors can occur. It was also found that several components of other hormone pathways could interact with the petunia homologue of a member of the BES1/BZR1 family of transcription factors. These interactions reveal new mechanisms, not previously recognized, that suggest extensive cross-talk between BR signalling and other hormone signalling pathways.

Materials and methods

Brassinolide application

Young plantlets were each sprayed each 2 d or 3 d until run-off with 1 μM 24-epibrassinolide (Wako Biochemistry) or with a solvent control (0.01% ethanol and 0.1% Tween-20) for a couple of weeks.

Purification and GC-MS analysis of sterols and BRs in petunia tissue

Petunia plantlets were freeze-dried and subjected to extraction and chromatography according to Nomura et al. (1999). In high-performance liquid chromatography, fractions 48/49 (22-OH-CR and 28-nor-6-deox-oCT), 50/51 (22-OH-3-one, 6-deoxoCT), and 52/53 (3-epi-6-deoxoCT) were collected in addition to the fractions cited in Nomura et al. (1999). The respective BRs were derivatized and analysed by gas chromatography–mass spectrometry (GC-MS) as described (Nomura et al., 2001) except that a Shimadzu GC-MS2010 was used. Sterols in petunia were analysed according to Nomura et al. (2004).

Genomic DNA isolation and PCR

Petunia DNA was isolated by grinding one young leaf in liquid nitrogen. To this 400 μl of DNA extraction buffer was added (0.1 M TRIS pH 8.0, 0.5 M NaCl, 50 mM EDTA, and 10 mM β-mercaptoethanol) and samples were incubated for 15–30 min at 65 °C. Next 250 μl of phenol/chloroform was added, and samples were shaken and subsequently centrifuged for 5 min at maximum speed in a microcentrifuge. The supernatant was transferred to a clean tube and DNA was precipitated using 2-propanol and washed with 70% ethanol. The pellet was dissolved in 100 μl of H2O containing DNase-free RNase (10 mg ml−1) followed by an incubation step for 15–30 min at 65 °C. PCRs were performed by using 2 μl of genomic DNA in a standard PCR. The nucleotide sequences of the primers used are listed in Supplementary Table S1 available at JXB online.

Isolation of 3’ gene fragments by rapid amplification of cDNA ends (RACE) PCR

In order to isolate cDNA fragments, total RNA was isolated from leaves followed by first-strand cDNA synthesis using the RACE1 primer as described previously (Quattrocchio et al., 2006). PCR
amplification was carried out using gene-specific primers or, to isolate 3’ ends of cDNAs, by using one gene-specific primer in combination with the RACE2 primer under standard PCR conditions (annealing temperature 55 °C). All primer sequences used are listed in Supplementary Table S1 at JXB online. PCR fragments were directly sequenced or cloned in pGEM-Teasy (Promega) and sequenced.

Isolation of 5’ gene fragments by SOTI-PCR

To complete sequences at the 5’ end, for some genes a somatic insertion-mediated PCR was performed (Rebocho et al., 2008). Here, somatic $dTph1$ insertions in the target gene that may occur in some cells of petunia W138 plants were used. W138 genomic DNA was subjected to nested PCR using gene-specific primers and $dTph1$ transposon primers (see Supplementary Table S1 at JXB online). In the first PCR, the $dTph1$ transposon primers out11 or out12 were used in a standard PCR with annealing temperature 55 °C. In the second, nested PCR, 1 μl of the out11 reaction was re-amplified with out15 and a nested gene-specific primer, while the out12 reaction was re-amplified with out6 and a nested gene-specific primer. The second reaction was a touch-down PCR where annealing started at 70 °C and decreased by 1 °C every cycle until reaching 55 °C, followed by 25 additional cycles at 55 °C. PCR fragments were directly sequenced or cloned in pGEM-Teasy (Promega) and sequenced.

Phylogenetic analysis

Phylogenetic trees were generated using the Neighbor-Joining method of ClustalX (ftp://ftp.ebi.ac.uk/pub/software/clustalw2/). Bootstrap mode (1000 replications) was used for estimating the level of similarity.
of confidence assigned to the particular nodes in the tree. The tree was visualized with the help of Treeviewer 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and rooted using ClustalW (http://www.bioinformatics.nl/tools/clustalw.html). Alignments were coloured using Boxshade (http://www.ch.embnet.org).

**Yeast two-hybrid screen**

The full-length coding sequence of *PhBEH2* was amplified by reverse transcription–PCR (RT–PCR) on total RNA isolated from leaves using Phusion polymerase (Finnzymes) and primers M736 and M737. The amplification product was cloned in the *EcoRI/SalI* sites in pBD-GAL4 and pAD-GAL4 (Stratagene). The PSK8-GAL4BD fusion was made by cutting *PhBEH2* yeast two-hybrid interactant 78 with *EcoRI/SalI* and ligation of the fragment in pBD-GAL4. The plasmids were introduced into the yeast strain PJ69 (James et al., 1996), which carries HIS3, ADE2, and LACZ reporter genes driven by distinct GAL4-responsive promoters. For the yeast two-hybrid screen, PhBEH2/PSK8 was used as bait against a cDNA library from young petunia inflorescences (Souer et al., 2008). The library screen was performed as described previously (Quattrocchio et al., 2006). A total of 100,000 and 300,000 transformed yeast cells were screened for PhBEH2 and PSK8, respectively. Plasmid DNA was isolated from positive (HIS, ADE) colonies, transformed to *Escherichia coli*, sequenced, and subsequently reintroduced in PJ69 together with the bait plasmid expressing PhBEH2/PSK8 to confirm interactions.

For the yeast two-hybrid assay between members of the *BES1/BZR1* family and GSK3/SHAGGY-like family, members from *Arabidopsis* and petunia were amplified by RT–PCR using Phusion polymerase (Finnzymes) on Petunia leaf total RNA or from existing plasmid clones. The amplification products were cloned in *EcoRI/SalI* sites in pBD-GAL4 and/or pAD-GAL4 (Stratagene).

**BIFC (bimolecular fluorescence complementation) analysis**

For expression in protoplasts, full-length coding sequences and the N- and C-terminal halves of yellow fluorescent protein (YFP) were amplified with primers containing attB sites facilitating (Multisite) Gateway cloning (Invitrogen). After recombination in pDONR vectors, the obtained entry clones were recombined with the destination vectors V141 (pK7FWG2) to generate PhBEH2-GFP (green fluorescent protein) or V137 (pK2GW7.0) to generate the split YFP versions. The Gateway vector V154 (pB7WGY2.0) expressing free YFP was used as a control.

**Accession numbers**

Sequence data from this article can be found in the GenBank data library under accession numbers KC633285 (PhDWARF7), KC633286, (PhCPD), KC633287 (PhCYP85), KC633288 (PhDWARF5), KC633289 (PhDET3), KC633290 (PhSMT2), KC633291 (PhROT3), KC633292 (PhDIM), KC633293 (PhDWARF4), KC633294 (PhDET2), KC633295 (PhBRI1), KC633296 (PhPSK9), KC633297 (PhPSK8), KC633298 (PhBEH2), KC633299 (PROTEIN INTERACTING with BEH2 1 (PIB1), KC633300 (PIB8), KC633301 (PIB16), KC633302 (PIB16), KC633303 (PIB25), KC633304 (PIB41), KC633305 (PIB60), KC633306 (PIB67), KC633307 (PIB81), KC633308 [PROTEIN INTERACTING with GSK3 1 (PIG1)], KC633309 (PIG3), KC633310 (PIG4), KC633311 (PIG16), KC633312 (PIG21), KC633313 (PIG28) and KC633314 (PIG93).

**Results**

**Identification of petunia sterol/BR biosynthesis and signalling mutants**

In order to study BR biosynthesis and signalling in petunia, populations of the mutagenic *Petunia hybrida* line W138 were visually screened (Gerats et al., 1990; van Houwelingen et al., 1998). Nineteen mutant families (Table 1) with similar phenotypes were identified that phenocopy sterol/BR biosynthesis or signalling mutants from other species. All mutants exhibited the reduced stature and dark round shiny leaves typical for plants with BR deficiency (Fig. 2A, B). Due to their appearance, these mutants were named *compact disc* (cd).

In order to identify whether these mutants are BR biosynthesis or signalling mutants, individual mutant plants were sprayed 2–3 times a week with 1 μM 24-epibrassinolide. Of 19 mutant families, 18 clearly responded to the steroid, resulting in elongation of internodes, petals, and leaves (Table 1, Fig. 2C). Complementation of the mutant phenotype in these plants indicated that these are sterol/BR biosynthesis mutants. Only one mutant family, *cd10* (cd10), did not respond to exogenous application of 24-epibrassinolide, suggesting that it is mutated in a BR signalling gene.

Cross-polulation between the mutant families revealed that the 19 mutants could be assigned to five different complementation groups (see Table 1). Ten families identified at Enza Zaden harbour a *dTph1* insertion in the same BR biosynthesis gene, *CD9*. As some of these families are derived from a common ancestor (see Table 1), it is likely that they represent only three different alleles. Taken together, the total number of sterol/BR biosynthesis gene hits is four, while the remaining mutant *cd10* represents a signalling mutant. All mutants have a short robust stature with thick, small, round, and dark-green leaves, with only some slight differences in the severity of the dwarfish phenotype between the complementation groups. The *cd10* mutants remained extremely small and, even after growing for months in the greenhouse, did not flower. The biosynthesis mutants clearly grew slowly and flowered later, and fertility seemed to be reduced in the *cd2* and especially in the *cd1* mutants.

**BR and steroid levels in cd mutants**

To characterize the mutations further, quantitative GC-MS analysis was used to measure endogenous steroid and BR levels in the wild type and *cd* mutants (Tables 2, 3). Wild-type petunia, like tomato, does not accumulate BL, but rather castasterone (CS), as the bioactive compound (Table 2). In the *cd1* mutant, the levels of CS and 6-deoxoCS were greatly reduced. The amounts of sterols were similar to those of the wild type, indicating no lesion in sterol biosynthesis. Therefore, *cd1* seems to be mutated in an unknown brassinosteroid enzyme. Concomitantly with a large decrease in the CS and 6-deoxoCS levels, the *cd2* mutant overaccumulated 22-OH-CR, indicating a mutation in the petunia *CYP90A1 (CPD)* gene. In the *cd3* mutant, massive accumulation of 6-deoxoCS was observed, suggesting that this mutant has a defect in the conversion of 6-deoxoCS to castasterone, a reaction that in tomato is catalysed by the CYP85A1 enzyme (Bishop et al., 1999). In the *cd9* mutant, sterols are abundant but the pathway end-products CS and 6-deoxoCS were severely reduced. In *cd9*, 22-OH-CR, 22-OH-3-one, and 6-deoxoCT accumulated, suggesting a mutation in a CYP90C1- or CYP90D1-like
Brassinosteroids in *Petunia hybrida* | 2439

Table 1. Overview of cd alleles and results of complementation experiment by brassinolide treatment.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Allele</th>
<th>Origin</th>
<th>Complemented by BR&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>cd&lt;sup&gt;1&lt;/sup&gt;</td>
<td>cd1-W503</td>
<td>VU</td>
<td>Yes</td>
</tr>
<tr>
<td>cd1-K2078</td>
<td>Nijmegen</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>cd2-W2254</td>
<td>VU</td>
<td>Yes</td>
</tr>
<tr>
<td>cd2-P2013</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd2-P2015</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd2-P2018</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd3</td>
<td>cd3-D2213</td>
<td>VU</td>
<td>Yes</td>
</tr>
<tr>
<td>cd3-P2014</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cd9-P2016</td>
<td>ENZA</td>
<td>Yes</td>
</tr>
<tr>
<td>cd9-P2019</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2021</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2022</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2023</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2024</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2025</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2026</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2027</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd9-P2028</td>
<td>ENZA</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>cd10</td>
<td>cd10-P2020</td>
<td>ENZA</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> Complementation was tested by spraying with 1 μM 24-epibrassinolide.
<sup>b</sup> Due to reduced fertility, cd1 alleleism was confirmed molecularly as independent insertions in PhCYP90A1 (see text).
<sup>c</sup> cd9<sup>p2016</sup>, cd9<sup>p2019</sup>, cd9<sup>p2022</sup>, cd9<sup>p2024</sup>, cd9<sup>p2025</sup>, cd9<sup>p2026</sup>, cd9<sup>p2027</sup>, and cd9<sup>p2028</sup> are derived from a common ancestor and thus might represent identical alleles.

Table 2. Endogenous BR levels of wild-type petunia (W138), cd1, cd2, cd3, and cd9 plants (ng kg<sup>-1</sup> FW).

<table>
<thead>
<tr>
<th>BR/steroid</th>
<th>Wild type</th>
<th>cd1</th>
<th>cd2</th>
<th>cd3</th>
<th>cd9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue FW (g)</td>
<td>95.0</td>
<td>88.0</td>
<td>82.1</td>
<td>102.0</td>
<td>26.6</td>
</tr>
<tr>
<td>BL</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CS</td>
<td>943</td>
<td>24</td>
<td>92</td>
<td>59</td>
<td>68</td>
</tr>
<tr>
<td>6-DeoxoCS</td>
<td>7621</td>
<td>157</td>
<td>86</td>
<td>59</td>
<td>68</td>
</tr>
<tr>
<td>6-DeoxoTY</td>
<td>566</td>
<td>610</td>
<td>488</td>
<td>1008</td>
<td>512</td>
</tr>
<tr>
<td>6-Deoxo3DT</td>
<td>116</td>
<td>135</td>
<td>202</td>
<td>230</td>
<td>257</td>
</tr>
<tr>
<td>6-DeoxoTE</td>
<td>191</td>
<td>142</td>
<td>170</td>
<td>109</td>
<td>ND</td>
</tr>
<tr>
<td>6-DeoxoCT</td>
<td>4471</td>
<td>7770</td>
<td>4055</td>
<td>1668</td>
<td>12 260</td>
</tr>
<tr>
<td>TY</td>
<td>Lost</td>
<td>Lost</td>
<td>Lost</td>
<td>Lost</td>
<td>ND</td>
</tr>
<tr>
<td>3DT</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TE</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>22-OH-CR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>754</td>
<td>1151</td>
<td>4337</td>
<td>392</td>
<td>4956</td>
</tr>
<tr>
<td>22-OH-3-one&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2858</td>
<td>5275</td>
<td>1208</td>
<td>1471</td>
<td>16 739</td>
</tr>
<tr>
<td>3-Epi-6-deoxoCT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6571</td>
<td>3891</td>
<td>1531</td>
<td>5715</td>
<td>10 777</td>
</tr>
</tbody>
</table>

FW, fresh weight; ND, not detectable; lost, internal standards not recovered.
<sup>a</sup> Quantified by peak areas because internal standards are unavailable.

Abbreviations of BRs: BL (brassinolide), CS (castasterone), 6-deoxoCS (6-deoxocastasterone), 6-deoxoTY (6-deoxotyphasterol), 6-deoxoTE (6-deoxoteasterone), 6-deoxoCT (6-deoxocathasterone), TY (typhasterol), 3DT (3-dehydroteasterone), TE (teasterone), 22-OH-CR [(22S)-22-hydroxycampsterol], 22-OH-3-one [(22S,24R)-22-hydroxy-5-ergostan-3-one], 3-epi-6-deoxoCT (3-epi-6-deoxocathasterone).
enzyme. In conclusion, it was found that cd1, cd2, cd3, and cd9 are all BR deficient.

cd2 and cd3 encode the petunia CYP90A1 and CYP85A1 homologues, respectively.

The biochemical analysis of BR content in cd mutants in some cases provided clues on the identity of the affected gene. Using a combination of database mining and primer design based on sequences of Solanaceae species (e.g. tomato, tobacco, potato, etc.; Supplementary Table S1 at JXB online), it was possible to clone gene and/or cDNA fragments of petunia homologues of the Arabidopsis BR biosynthesis genes CYP90A1, DWF5, CYP85A1, DIM, DWF7, SMT2, DETIOLATED2 (DET2), DWARF4 (DWF4), and CYP90C1 (ROT3), and a number of signalling genes (see below).

The obtained sequence data of petunia BR biosynthesis genes were used to determine in each cd mutant if any of these known genes is disrupted by a dTph1 insertion. When DNA isolated from cd2 plants (Fig. 3A) was used as a template, PCRs identified dTph1 insertions at different sites in the petunia homologue of the BR biosynthesis gene CYP90A1 (Szekeres et al., 1996) (Fig. 3D, F). The dTph1 transposon insertion in allele cd2W2254 was found in the first exon, ~80 nucleotides after the ATG. The other three mutants (cd2P2013, cd2P2015, and cd2P2018) carry a dTph1 insertion at different sites in the second exon (Fig. 3D). Plants in progeny of cd2W2254 occasionally gave rise to revertant shoots (Fig. 3B).

Table 3. Endogenous steroid levels of wild-type petunia (W138), cd1, and cd9 plants (g kg⁻¹ FW).

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Wild type</th>
<th>cd1</th>
<th>cd9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>3.4</td>
<td>4.7</td>
<td>4.5</td>
</tr>
<tr>
<td>24-Methylenecholesterol</td>
<td>24.0</td>
<td>19.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Campesterol</td>
<td>30.2</td>
<td>42.0</td>
<td>71.5</td>
</tr>
<tr>
<td>Isofucosterol</td>
<td>140.2</td>
<td>392.0</td>
<td>376.0</td>
</tr>
<tr>
<td>Stigmastanol</td>
<td>19.3</td>
<td>14.0</td>
<td>31.9</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>36.0</td>
<td>79.9</td>
<td>92.3</td>
</tr>
</tbody>
</table>

Fig. 3. Molecular analysis of two BR biosynthesis mutant families. (A) Mutant phenotype of a cd2W2254 plant. (B) A cd2W2254 plant that develops a revertant shoot (cd2W2254-4). (C) PCR with CYP90A1-specific primers on genomic DNA extracted from plants carrying various CYP90A1 alleles. (D) Schematic drawing of the petunia CYP90A1 gene and mutant alleles. (E) Schematic drawing of the petunia CYP85A1 gene and mutant alleles. In D and E, boxes represent exons and the thin lines represent introns. The triangles and line illustrate the dTph1 transposon insertions and footprint in the indicated alleles. (F) Sequence analysis of the dTph1 insertions in various CYP90A1 and CYP85A1 alleles. The red sequence indicates the target site duplication.
PCR and sequence analysis on genomic DNA extracted from a revertant shoot (cd2M2194-4) revealed excision of dTph1 from CYP90A1, leaving a 6 bp footprint (Fig. 3F). Furthermore, a stable cd2 mutant (cd2M2194-1) was identified in which excision of dTph1 created an 8 bp footprint (Fig. 3F).

The identity of cd3P2014 was revealed by mining a 3D indexed petunia insertion database generated from the ENZA population (Vandenbussche et al., 2008). A transposon-flanking sequence unique for family P2014 showed homology to CYP85A1 (Bishop et al., 1999). The dTph1 element was identified in exon 6 of the petunia CYP85A1 homologue (Fig. 3E). In progeny of cd3P2014, a stable mutant was shown to carry a mutant allele containing a 7bp footprint (cd3S2007). In plants carrying the cd3P22213 allele, a transposable element could not be identified in the petunia CYP85A1 homologue. However, sequencing of the CYP85A1 coding sequence from one of the progeny (family cd3-K2066) showed an 8 bp footprint in exon 1, indicating the previous existence of a transposable element at this site (Fig. 3E, F).

Analysis of cd1 and cd9 mutants did not reveal transposon insertions in the putative petunia homologues of DWARF5, DET3, DIM, DWARF7, SMT2, DET2, DWARF4, or ROT3. It must be said that the complete sequences of these genes are currently unknown. As 3’ sequences (DIM), 5’ sequences (DWARF5, CYP85, DWARF7, SMT2), or both (DET3, DET2, DWARF4, ROT3) are unknown, it cannot be excluded that there are dTph1 insertions present in these parts of the gene. In the 3D indexed petunia insertion database (Vandenbussche et al., 2008), transposon insertions were identified in petunia homologues of DIM and DET2, both without phenotypic consequences, suggesting that these genes are redundant in petunia (see the Supplementary data at JXB online).

cd10 is mutated in the petunia BRI homologue

In Arabidopsis, BRs are perceived by the leucine-rich repeat (LRR) receptor-like kinase BRI1 (Li and Chory, 1997). From the P. hybrida expressed sequence tag (EST) collection, a sequence that showed a high degree of similarity to the 3’ end of BRI1 (GenBank accession no. CV294320) was retrieved. The full-length mRNA sequence of W138 PhBRI1 was obtained via RACE-PCR and SOTI-PCR (see the Materials and methods). Comparison of the derived amino acid sequence of PhBRI1 with AtBRI1, OsBRI1, LeBRI1, and NbBRI1 revealed 63.3, 53.4, 84.1, and 91.3% identity, respectively (Fig. 4A; Supplementary Fig. S1 at JXB online). Similar to the other identified BRIs, PhBRI1 has an LRR domain, a putative leucine-zipper motif, a transmembrane domain to anchor the protein in the plasma membrane, and a cytoplasmatic kinase domain (Fig. 4B; Supplementary Fig. S1). The BRI1 extracellular domain of petunia contains 25 tandem N-terminal LRRs, which is interrupted by a 68 amino acid non-repetitive island between the 21st and 22nd LRR.

Because cd10P2020 failed to respond to application of 24-epibrassinolide, cd10 was assumed to be mutated in a BR signalling gene (Fig. 2C). One of the prime candidates for being hit in cd10P2020 is the homologue of the Arabidopsis BR receptor, BRI1. PCR analysis on genomic DNA with PhBRI1-specific primers revealed that cd10 plants indeed contain an insertion in the 5’ part of the gene (Fig. 4C, D). Sequence analysis of the 5’ part of PhBRI1 in cd10 revealed that the dTph1

![Fig. 4. Characterization of the BRI1 receptor from petunia.](image)
transposable element was inserted in the third LRR sequence motif (Fig. 4B).

Identification of a BEH2 orthologue in petunia

To explore the BR signalling cascade in petunia further, the aim was to identify signalling components downstream of PhBR1. In Arabidopsis, the BES1/BZR1 transcription factor family plays an important role in the regulation of BR-responsive genes (Zhao et al., 2002). A PCR-based approach was performed to identify potential petunia homologues. Primers were developed based on the sequences of all six BES1/BZR1 family members of Arabidopsis and a nested 3’ RACE-PCR on petunia leaf cDNA yielded a PCR fragment of the desired size. Sequence analysis revealed high sequence identity with members of the BES1/BZR1 family. The 5’ end was isolated by SOTI-PCR (see the Materials and methods). The derived amino acid sequence has the highest sequence identity to the BES1/BZR1 family member AtBEH2 (64.2%) and therefore the clone was named PhBEH2 (Fig. 5A; Supplementary Fig. S2 at JXB online). Like the previously characterized BES1/BZR1 family members from Arabidopsis, PhBEH2 has a putative bipartite nuclear localization signal (NLS) at its N-terminus. The central part of PhBEH2 is rich in serine/threonine residues that match with the phosphorylation sites for GSK3 kinases (Wang et al., 2002). In addition a PEST motif was identified that seems to play an important role in protein degradation as BES1 and BZR1 proteins were stabilized in bes1-D and b3r1-D mutants carrying a mutation in this motif (Wang et al., 2002; Yin et al., 2002).

PhBEH2 binds to a GSK3/SHAGGY-like kinase and 14–3–3 proteins

To identify proteins that interact with PhBEH2, a yeast two-hybrid screen was performed. As many transcription factors carry activation domains, auto-activation of the full-length cDNA of PhBEH2 on yeast reporters was tested first. Indeed, introduction of the PhBEH2–GAL4-binding domain fusion resulted in some activation of the HIS reporter, but selection on histidine and adenine resulted in no growth (Supplementary Fig. S4 at JXB online). Subsequently, the full-length cDNA of PhBEH2 was used as bait to screen a two-hybrid cDNA library made from young petunia inflorescences, and 12 interacting proteins were found (Fig. 5B, C). Sequence analysis showed that five of these proteins were 14–3–3 proteins. Another interesting interacting partner, which was named PSK8, had high similarity to the GSK3-like kinase BIN2 from Arabidopsis (Fig. 5C). Other interaction partners include proteins homologous to SECRET AGENT (SEC), which is an O-linked N-acetylglucosamine transferase (OGT), ARIA (ARMADILLO Repeat protein Interacting with AFB2), TPL (TOPLESS), and two RRM (RNA recognition motif) proteins of which one is a homologue of FPA (Flowering Protein A; Fig. 5C).

To verify some of the interactions found in yeast in plant cells, BiFC was used in petunia leaf protoplasts (Hu et al., 2002). As numerous interactions with PhBEH2 were detected, the search was restricted to interactions known from Arabidopsis research. In addition, the aim was to verify the interaction with the potential OGT PhSEC. As positive controls, free YFP as well as a fusion between PhBEH2 and GFP were used (Fig. 5D, E). When fused to GFP, BEH2 was mainly detected in the nucleus, but clearly in the cytoplasm too. For BiFC, translational fusions of PhBEH2, Ph14-3–3k, PSK8, and PhSEC with the N- and C-terminal halves of YFP were generated. Interaction was detected between PhBEH2 and 14-3-3k (Fig. 5G), PSK8 (Fig. 5F, H), and PhSEC (Fig. 5I). The interaction between PhBEH2 and 14-3-3k occurred in the cytoplasm and nucleus, whereas interaction with PSK8 and PhSEC appeared almost exclusively in the nucleus. It thus seems that at least in the case of 14-3-3k, PSK8, and PhSEC, the interaction with PhBEH2 can also occur in plant cells.

Isolation and characterization of two group II members of the GSK3/SHAGGY-like family from petunia

One of the interacting partners of PhBEH2 was PSK8, a protein that has strong homology to the GSK3-like kinase BIN2 from Arabidopsis (Fig. 6A; Supplementary Fig. S3 at JXB online). From a previous yeast two-hybrid screen, performed in the authors’ laboratory, using one of the transcription factors involved in flower pigmentation as bait, another GSK3-like kinase was identified as an artefact. As this clone lacked the 5’ half of the cDNA, SOTI-PCRs (see the Materials and methods) were performed to isolate the remaining part. This clone was renamed PSK9. Both PSK8 and PSK9 are related to BIN2 as they group together with the other group II ASKs (Fig. 6A). Kim et al. (2009) showed that the phosphatase BSU1 inactivates BIN2 by dephosphorylation at pTyr200. This tyrosine residue is also conserved in PSK8 and PSK9 (Supplementary Fig. S3).

To gain more insight into the function of PSK8, a yeast two-hybrid screen was performed, where full-length PSK8 was used as bait to screen a cDNA library made from young petunia inflorescences. Eight different cDNAs that interacted with PSK8 were identified. The interacting partners included two different LRR-extensins, REMORIN, and several unknown proteins. (Fig. 6B, C).

Key regulators of the petunia/Arabidopsis BR pathway are functionally homologous

PSK8 and PSK9 are highly homologous to the group II GSK3/SHAGGY-like kinases from Arabidopsis, and this prompted the examination of whether they also have functional homology. In order to investigate this, yeast two-hybrid assays were performed with members of the BES1/BZR1 family and the group II GSK3/SHAGGY-like kinases from Arabidopsis and petunia. AtBIN2 interacted with AtBES1, AtBZR1, and also with BEH2 from petunia (Fig. 7). A similar behaviour was found for the petunia BIN2 homologue PSK8. However, PSK9 did not bind to PhBEH2 or to the Arabidopsis members. As the interaction with PSK8/
BIN2 showed that the fusions of BES/BZR proteins with GAL4-AD are functional in yeast, it seems that either PSK9 does not interact with the BES1/BZR1 family or the fusion between PSK9 and the GAL4-binding domain is somehow not functional. The yeast-two hybrid results suggest that group II GSK3/SHAGGY-like kinases and members of the BES/BZR1 family from *Arabidopsis* and petunia are structurally highly homologous.

**Discussion**

The synthesis of BRs and the signalling cascade downstream of the bioactive compounds have been largely elucidated over the past 10 years (Fujioka and Yokota, 2003; Clouse, 2011). As in many plant research areas, these findings basically originate from *Arabidopsis*. Partial confirmation of evolutionary conservation as well as additions came from numerous reports on BR biosynthesis and signalling in other species (e.g. Nomura et al., 2001; Montoya et al., 2002; Bai et al., 2007; Koh et al., 2007). The available resources in petunia have been used to identify large parts of the BR biosynthesis and signalling route. The initial results point to conserved BR biosynthesis genes and interacting signalling proteins, as well as to potential new relationships between hormone signalling pathways.

**Novel and known BR mutants**

Generally, a mutation in a BR biosynthesis gene causes the accumulation of a precursor(s) that should have been metabolized and a reduction of the (end)product(s). In the *cd2* mutant, accumulation of 22-OH-CR, which has been recognized as the first metabolite of campesterol in the early C-22 oxidation branch of BR biosynthesis, was observed (Fujita et al., 2006; Fig. 1). A high level of accumulation of 22-OH-CR was also observed by Ohnishi et al. (2012) when
they researched the cdp/cyp90a1 mutant. The biochemical evidence strongly suggested that CD2 is homologous to CPD/CYP90A1, although the product, 22-OH-4-en-3-one, has not been analysed. Indeed, molecular analysis confirmed CD2 as the petunia homologue of CYP90A1 (Fig. 3; Szekeres et al., 1996). In cd3, the build-up of 6-deoxoCS and the concomitant reduction of CS was observed, indicating a lesion in CYP85A1. Once more the biochemical data were confirmed as cd3 was found to be mutated in the petunia homologue of CYP85A1 (Fig. 3; Bishop et al., 1996).

Endogenous levels of upstream BRs in cd1 are quite similar to those of the wild type, and sterol levels are normal. However, the levels of both 6-deoxoCS and CS, which appear in the last steps of biosynthesis, are reduced. No accumulation of other measured BR biosynthesis intermediates could be identified. Thus, the nature of the cd1 mutation is currently unknown and it might represent an enzyme that has not been identified yet in any other plant species. In cd9, the levels of 22-OH-3-one, 3-epi-6-deoxoCT, and 6-deoxoCT that are substrates of CYP90C1 (ROT3)/CYP90D1 were higher as compared with the wild type, indicating a potential deficiency in a CYP90C1/CYP90D1-like activity (Fig. 1). However, the Arabidopsis CYP90C1/CYP90D1 double mutant does not accumulate 22-OH-3-one, 3-epi-6-deoxoCT, and 6-deoxoCT (Ohnishi et al., 2006). It thus seems that, in contrast to the situation in Arabidopsis, the CYP90C1 (ROT3)/CYP90D1 activity in petunia is encoded by a single enzyme. dTph1 insertions could not be found in the ROT3 homologue of cd9, but it cannot be ruled out that the insertion might be located in the 5’ or 3’ part that currently is lacking. Altogether, the evidence available points to unknown BR biosynthesis enzymes or perhaps regulators of BR biosynthesis enzymes mutated in cd1 or cd9. Cloning of the responsible genes can be accomplished in the future by using amplified fragment length polymorphism (AFLP)-based transposon display or high throughput sequencing of dTph1-flanking sequences (Van den Broeck et al., 1998; Vandenbussche et al., 2008).

**BR signalling mutants**

The single signalling mutant that was identified, cd10, was shown to harbour an insertion in the petunia homologue of the BR receptor, BRI1 (Li and Chory et al., 1997). The cd10 mutant exhibits an extremely dwarfed stature and did not flower at all. All known bril mutants do flower, except the
osbril mutant from rice (Nakamura et al., 2006). The strong mutants from rice and petunia might reflect complete loss of BRI1 function, whereas in other species either only partial loss-of-function alleles were identified, or partial redundancy in BRI1 function might exist, and/or another non BR-dependent flowering pathway plays a dominant role.

In an amino acid alignment of BRI1 from different species, the highest similarity was observed in the crucial kinase domain (Supplementary Fig. S1 at JXB online; Tang et al., 2008; X. Wang et al., 2008). The extracellular domain of PhBRRI1 contains 25 tandem N-terminal LRRs and a 68 amino acid non-repetitive island between the 21st and 22nd LRR. This island together with LRR22 has proved to be important for BR binding by BRI1 in Arabidopsis (Kinoshiba et al., 2005). Thus it seems that the perception of the BR hormone is evolutionarily well conserved.

Apart from PhBRRI1, petunia homologues belonging to the BES1/BZR1 family as well as GSK3-like kinases, key components of the BR signalling pathway in Arabidopsis, were cloned. The BES1/BZR1 family homologue that was isolated from petunia (PhBEH2) showed the highest sequence identity to the BES1/BZR1 family member BEH2 (64.2%). In Arabidopsis, the brz1/bes1 double mutant only exhibits a semi-dwarf phenotype. It thus seems likely that BEH2 and perhaps other members of the BES1/BZR1 family are redundant to BES1 and BZR1.

Interactions between BR signalling components

The interaction spectrum of PhBEH2 in yeast supports a role in BR signalling. First, PhBEH2 is capable of interacting with Arabidopsis BIN2 (Fig. 7), a proven negative regulator of BR signalling (Li et al., 2001). Secondly, the PhBEH2 protein interacted with several 14-3-3 proteins (Fig. 5C). Previous research in Arabidopsis showed that 14-3-3 proteins increase the cytoplasmic retention of phosphorylated BES1/BZR1 (Gampala et al., 2007). Mutation of a BIN2 phosphorylation site in BZR1 was shown to abolish 14-3-3 binding, leading to increased nuclear localization of the BES1/BZR1 protein (Bai et al., 2007; Gampala et al., 2007). Like AtBZR1 and AtBES1, PhBEH2 also possesses the conserved putative 14-3-3-binding site, suggesting that the regulation of the BZR1/BES1 family by 14-3-3 proteins might be conserved among plants. Thirdly, PhBEH2 interacted with a petunia GSK3-like kinase (PSK8) that belongs to the group II GSK3-like kinases (Figs 5, 7). Arabidopsis group II GSK3-like kinases have been shown to regulate BR signalling negatively by phosphorylating the BZR1/BES1 family (Vert and Chory, 2006; Yan et al., 2009; Rhozon et al., 2010). To reveal the involvement of PSK8 in BR signalling, phosphorylation of PhBEH2 by PSK8 should be demonstrated and knock-outs might ultimately reveal their function. In addition to PSK8, another group II member (PSK9) was also identified in petunia. Based solely on similarity, it seems likely that PSK9 is also involved in BR signalling. The lack of interaction with PhBEH2 and Arabidopsis BES1 and BZR1 might be due to aberrant processing of the PSK9–GAL4 fusion RNA and/or protein in yeast.

Identification of new interactions

The present yeast two-hybrid screen with PhBEH2 revealed several interacting proteins that function in diverse signal transduction pathways. This suggests that PhBEH2 could be an important hub between BR and other signalling pathways. Recently, a number of reports highlighted interactions between BR signalling and other hormone pathways (reviewed by Choudhary et al., 2012).

One of the most interesting partners of PhBEH2 is a protein homologous to the Arabidopsis gene SECRET AGENT (SEC) encoding an OGT (Hartweck et al., 2002, 2006). For SEC, the interaction was confirmed in yeast using BiFC in petunia protoplasts (Fig. 5I). SEC catalyses the transfer of a single O-linked-N-acetylglucosamine to specific serine/threonine residues. SEC is partly redundant with another OGT in Arabidopsis called SPINDLY (SPY). Shimada et al. (2006) reported that mutation of the rice SPINDLY gene (OsSPY) reduced the expression of several BR biosynthesis genes and OsBRI1. As expected, the levels of sterol and BR compounds were slightly up-regulated in the OsSPY knock-out mutant.

O-GlCNacylation of target proteins might affect their activity, localization, stability, and interactions with other proteins. The fact that OGTs add acetylglucosamine to serine/threonine residues of proteins suggests a model where OGTs and GSKs compete for modification of these residues in the BES/BZR family of proteins. Such a scenario, where an OGT competes with a kinase, is a widely recognized mechanism in animal research (Kamemura et al., 2002; Z. Wang et al., 2008; Butkinaare et al., 2009). Most strikingly, there are specific examples where GSK3 phosphorylation and O-GlCNacylation occur on the same site of proteins (Wang et al., 2007). It would be interesting to see whether PhSEC adds acetylglucosamine to PhBEH2 and, if so, what the consequences are for the function of PhBEH2.

Multiple links between petunia BR signalling components and other pathways were identified. For instance, PhBEH2 interacts with a protein that has high sequence homology to ARIA, a positive regulator of the ABA signalling cascade. PhBEH2 also interacts with two proteins containing RRMs. One of the RRM proteins is a homologue of FPA, a gene that regulates flowering time in Arabidopsis via a pathway that is independent of daylength (Schomburg et al., 2001). Various connections between flowering and BRs have been revealed (Domagalska et al., 2007; Lee et al., 2008a, b; Yu et al., 2008). This is consistent with the finding that BR biosynthesis mutants as well as signalling mutants are late flowering or, like the cd10 (petunia bril1) mutant, non-flowering. Although interesting, further research should confirm these findings.

Remarkably, no known homologues of the BR signalling pathway were found in the yeast two-hybrid screen performed with PSK8. Using PhBEH2 as bait, PSK8 was isolated, but using the same cDNA library the reverse interaction was not identified. It might be that none of the BES1/BZR1 family members present in the library is in-frame with the GAL4-binding domain. Nonetheless, the yeast two-hybrid screen with PSK8 might have revealed other roles of group II GSK kinases that have not been recognized yet.
PSK8 interacted with a number of proteins that are poorly characterized. Among these are a member of the LLR-
extensin (LRX) and of the REMORIN family. So far, the action of GSK kinases in plants was found to occur via phosphorylation of transcription factors such as those belonging to the BES1/BZR family. Here, the identified interactions infer a direct role for GSK3-like proteins in the regulation of enzyme-like proteins, a mechanism that has interfaces with some of the roles of GSK3 in animals.

Supplementary data
Supplementary data are available at JXB online.

Figure S1. Alignment of the deduced amino acid sequences of BR11 and homologues.

Figure S2. Alignment of the deduced amino acid sequences of BES/BZR and homologues.

Figure S3. Alignment of the deduced amino acid sequences of class II GSK3/SHAGGY-like kinases from Arabidopsis and petunia.

Figure S4. Yeast one-hybrid to test for autoactivation of baits.

Table S1. Primers used for PCR.

Table S2. GenBank accession numbers

Acknowledgements
We thank Allan Awanian and Longinus Igbojionu for their assistance during some of the cloning experiments, Iris Bijker and Serena Della Pina for their help with the BiFC experiment, and Maartje Kuijpers for plant care. NV was supported by a grant from the Netherlands Organisation for Scientific Research (NWO). TY was supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2008–2012.

References


Butkinaree C, Park K, Hart GW. 2009. O-linked beta-


