Identification of brassinosteroid-related genes by means of transcript co-response analyses

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ABSTRACT

The comprehensive systems-biology database (CSB.DB) was used to reveal brassinosteroid (BR)-related genes from expression profiles based on co-response analyses. Genes exhibiting simultaneous changes in transcript levels are candidates of common transcriptional regulation. Combining numerous different experiments in data matrices allows ruling out outliers and conditional changes of transcript levels. CSB.DB was queried for transcriptional co-responses with the BR-signalling components BRI1 and BAK1: 301 out of 9694 genes represented in the nasc0271 database showed co-responses with both genes. As expected, these genes comprised pathway-involved genes (e.g. 72 BR-induced genes), because the BRI1 and BAK1 proteins are required for BR-responses. But transcript co-response takes the analysis a step further compared with direct approaches because BR-related non BR-responsive genes were identified. Insights into networks and the functional context of genes are provided, because factors determining expression patterns are reflected in correlations. Our findings demonstrate that transcript co-response analysis presents a valuable resource to uncover common regulatory patterns of genes. Different data matrices in CSB.DB allow examination of specific biological questions. All matrices are publicly available through CSB.DB. This work presents one possible roadmap to use the CSB.DB resources.

INTRODUCTION

Brassinosteroids (BRs) are highly potent growth-promoting sterol derivatives. BR-deficient and BR-insensitive mutants in Arabidopsis, pea, tomato, barley and rice show dwarfism (1). The growth-promoting effect of BR was assigned to changes in transcript levels of genes involved in cell wall modifications such as xyloglucan endotransglucosylase/hydrolases and expansins. Other BR-regulated genes point to further mechanisms contributing to growth. BR apparently coordinates diverse processes, partly through interactions with other phytohormones. Enhanced resistance of BR-treated plants to temperature, salt, water, phytopathogens and other environmental stresses has been reported (2–4). However, underlying molecular mechanisms are unknown. The growth effects of exogenous BR are light-dependent. Arabidopsis mutants such as det2, cpd and bri1 display short hypocotyls, opened cotyledons, and emergence of primary leaves in darkness. These findings suggest a cross-talk between photomorphogenesis and steroid signal transduction (5).

In several studies, BR-responsive gene expression in Arabidopsis was analysed (6–10). Comparisons of expression profiling experiments revealed that the majority of identified genes do not show consistent BR-dependent expression in different genotypes, environmental conditions, developmental stages and tissues, and upon BR-treatment (11,12). Thus, gene expression patterns are conditional, and the identified genes probably present only a subset of genes involved in BR-responses. Another reason for the incomplete discovery of genomic effects is the hitherto limited number of experiments, as gene expression varies even under highly controlled conditions. Many genes fail to meet the stringent selection criteria routinely applied in expression profiling experiments.

Cross-experiment co-response analysis provides an alternative approach which is based on the assumption that common transcriptional control of genes should be reflected in synchronous changes in transcript levels. Co-response analysis describes common changes of transcript levels among gene pairs. Publicly available expression profiles represent a rich resource for cross-experiment investigations. CSB.DB [http://csbdb.mpimp-golm.mpg.de/ (13)] provides access...
to co-response analysis based on numerous independent expression profiling experiments.

Here, we describe a strategy to identify BR-associated genes using the **BRI1** and **BAK1** genes. In the following, these anchors for subsequent co-response analyses will be termed ‘guide genes’ and correlated genes will be termed ‘associated genes’. We demonstrate means of *in silico* cross-checking and confirmation using the publicly available Affymetrix expression profiles provided by the AtGenExpress consortium. In addition, 44 cell wall and growth-related genes were selected for wet-lab experimental validation and subsequent real-time RT–PCR.

**MATERIALS AND METHODS**

Co-response analyses

Transcript co-responses were retrieved from CSB DB [a comprehensive systems-biology database; http://csdb.mpimp-golm.mpg.de (13)] for the data matrices nasc0271, nasc0272 and nasc0273. A total of 123 expression profiles from 22 experiments were obtained from NASC [http://

affymetrix.arabidopsis.info/ (14)] and used for generation of the data matrices [supplement.XLS sheet 11 (nasc profiles)]. All profiles were based on 22k Affymetrix ATH1 GeneChips (Affymetrix, La Jolla, CA). The number of Present and Marginal calls was calculated for each profile. In most cases, 2 or 3 profiles per experiment with the highest numbers of Present and Marginal calls were selected for nasc0271. Nasc0271 covers the expression of 9694 genes that were well measured in at least 85% of the underlying expression profiles. The nasc0272 and nasc0273 matrices were generated from profiles which ranked 2nd and 3rd according to the numbers of Present and Marginal calls. The nasc0272 matrix was based on 51 profiles and represents 8927 genes. The nasc0273 matrix was based on 49 profiles and represents 8691 genes. Correlations were calculated for each gene per category.

**Real-time RT–PCR analysis**

Total RNA was isolated using the Invisorb Spin Plant RNA kit (Invitek, Berlin, Germany). One microgram of total RNA was reverse-transcribed with Superscript II reverse transcriptase (Invitrogen) in a reaction volume of 28.5 µl to generate first-strand cDNA. Real-time RT–PCR was performed with 1 µl of the 1:3.5 diluted first-strand cDNA reaction and the SYBR Green reagent (Applied Biosystems, Foster City, CA) in a 25 µl volume. Primer sequences are given in Table 1. Data were normalized to the *eIF1a* gene (At5g60390) and then compared according to the formula (considering as example the *KCS1* gene):

\[ nC_T = C_{T_{KCS1}} - C_{T_{eIF1a}} \]

\[ \Delta C_T = \text{signal log ratio} = nC_{T_{mutant}} - nC_{T_{WT}} \]

Amplification efficiency (*E*) was checked for all primer pairs. Briefly, *E*-values were derived from the log slope of fluorescence versus cycle number curve for a particular primer pair, using the Equation \( (1 + E) = 10^{10slope} \). *E*-values for all primer pairs are summarized in Table 1 and used to calculate normalized fold change values, using the Equation \((1 + E)^{\Delta C_T}\). Control experiments showed that use of different control genes [either *eIF1a* or *eIF4A1* (At3g13920, primers: ACAATGT-GGTTGTCAAGAGCTG and GCAGAGCAACACAGC-AACAGAA)] did not bias results.

**Analysis of Affymetrix expression profiles**

Normalization and expression analysis were performed with the MAS 5.0 and GCOS software (Affymetrix). Output of all experiments was multiplied by a scaling factor to adjust its average intensity to a target intensity of 100. Results of Absolute and Comparison expression analysis were imported into MS Access2003 and screened for significant changes. The Detection algorithm calculates detection p-values and assigns Present, Marginal or Absent calls. Standard parameters were applied to remove genes with Absent and Marginal calls. Induced genes were expected to be Present in experiments representing higher phytohormone levels (‘experimental’ profiles). Repressed genes were expected to be Present in experiments representing lower phytohormone levels. The distribution of retrieved genes into functional categories was computed by adding-up the relative assignment coefficient for each gene per category.
levels (‘baseline’ profiles). Simultaneously, Affymetrix Change and Signal Log Ratio algorithms were used in order to identify changes with high reliability. The Change algorithm is based on Wilcoxon’s signed rank test and produces a final change p-value. This p-value ranges from 0.0 to 1.0. The signal log ratio estimates the magnitude and direction of change of a transcript. Significant increases of transcript levels in comparisons of two profiles are indicated by change p-values of >0.99 and ≥0.99 and ≤0.01, respectively (for details see www.affymetrix.com). Table 2 specifies AtGenExpress CEL files used for this study. Plant material was grown in liquid MS medium for 7 days at 23°C prior to the BL-, CS-, BRZ-, GA- and PAC-treatments. Two additional profiles were used [7 h EBL and control treatment of wild-type (Col-0) plants grown in half-concentrated MS medium supplemented with 1% sucrose and solidified with 0.7% agar (17)].

RESULTS

Transcript co-response analysis: a novel approach to identify BR-related genes

Central part of CSB.DB is a set of co-response databases (CoR.DBs) which are based on publicly available transcript profiles. The basic assumption is that common transcriptional control of genes is accompanied by synchronous changes of transcript levels. Scanning for best co-responses among changing transcript levels allows the deduction of hypotheses about common regulation of genes (18). We propose a general strategy to exploit CSB.DB. The strategy is illustrated in Figure 1. The biological question determines the choice of the type of
data matrix. Complex data matrices identify genes which are ‘constitutively’ associated with the guide genes. Specific matrices (e.g. representing certain tissues or stress conditions) identify genes within a specific context. After the selection of a suitable data matrix, useful guide genes have to be identified. The expression pattern and function determine the suitability of guide genes. Therefore, biological knowledge is required. The guide genes are used to screen for genes which show similar expression patterns in the profiles underlying a data matrix. Statistical methods and experiments allow testing of the identified genes. In the following section, the strategy is demonstrated using BR-related genes.

Assembly and selection of data matrices

Complex data matrices can be assembled by selecting transcript profiles representing many different experimental conditions. Complex data matrices include different genotypes, environmental conditions and developmental stages. To identify BR-related genes, the complex nasc0271 database was used. The matrix comprised 51 expression profiles [http://csbdb.mpimp-golm.mpg.de/csbdb/home/matrices/ath_nasc0271.html; supplement.XLS sheet 11 (nasc profiles)] representing a wide range of experimental conditions with a minimal overlap of identical experiments. All expression profiles were normalized using the MAS 5.0 software. Transcript measurements were required to have high quality, i.e. a detection call of Marginal or Present (according to standard parameters of the MAS 5.0 Detection algorithm) in at least 85% of the experiments. Thus, the nasc0271 matrix contained only 9694 accessible genes. Two other complex matrices (nasc0272 and nasc0273) were established using additional expression profiles [supplement.XLS sheet 11 (nasc profiles)]. These matrices were used to test and confirm the results obtained with the nasc0271 matrix. The nasc0272 and nasc0273 matrices consisted of 51 and 49 expression profiles and represented 8927 and 8691 genes, respectively.

Selection of guide genes and identification of transcript co-responses

Biological knowledge is required for the selection of useful guide genes. The screen for BR-related genes shows that different options emerge. Three different classes of genes could be used: either known BR-responsive genes or BR-biosynthesis genes, or genes involved in BR-signalling.

(i) Use of known BR-responsive genes for co-response analyses reveals further BR-responsive genes (Table 3; statistical parameters below). However, BR-responsive genes are also likely to be regulated by other factors, and the functional context is unclear. For example, several BR-regulated such as BEE1, DRT100, IAA3, MSS3, SAUR-AC1, SAUR16 and TCH4 are auxin-regulated (9,19), and the use of these genes will result in the identification of further auxin-related genes.

(ii) The use of BR-biosynthesis genes, such as CPD, DWFA and CYP85A1, should result in higher specificity. Transcript co-responses with the CPD gene recovered several known BR-regulated genes (Table 3). However, transcripts of other BR-biosynthesis genes were excluded from the data matrices because of quality concerns and thus could not be tested.

(iii) Use of BR-signalling components presents a third alternative. BR responses depend on signalling components such as BRI1, BAK1, BIN2, BZR1 and BES1. BRI1 is an essential receptor component for BR-responses. BR-insensitivity of bri1 mutants (20–22) indicates that major BR-responses depend on BRI1. BAK1 is a receptor-like
kinase which forms a heterodimer with BRI1 (23,24). BAK1 was identified independently by a yeast two-hybrid screen for BRI1-interacting proteins and as suppressor of a weak bri1 allele. A null allele of BAK1 results in reduced (but not abolished) sensitivity to BR (23). In the presence of BR, the BRI1 and BAK1 proteins initiate a phosphorylation cascade which regulates BR-responsive genes. Downstream components such as BZR1 and BES1 regulate subsets of BR-responsive genes (10). However, BZR1 and BES1 could mediate responses to other stimuli as well, because the complex phosphorylation cascade between the BRI1/BAK1 and BZR/BES1 proteins is likely to receive additional input signals and thereby modulate BR-responses. In fact, BRI1 and BAK1 identified more known BR-regulated genes in the matrices than other signalling components (Table 3).

The BRI1 protein is the major BR-receptor component in Arabidopsis. However, Arabidopsis BRI1 may bind other ligands. For example, tomato BRI1 perceives both BR and the peptide hormone systemin (25,26). Therefore, BAK1 was included as a second guide gene. BRI1 and BAK1 showed variable and correlated transcript levels throughout the set of expression profiles (Figure 2). Thus, the requirements for subsequent intersection analysis were met. Transcript co-response calculations are based on changes in mRNA levels within the underlying expression profiles. Spearman’s non-parametric rank correlation coefficient ($r_s$), p-value and power were used to retrieve transcript co-responses. Exclusion criteria for the statistical parameters were $r_s > 0.35$, $p < 0.01$ and power >0.7, respectively.

The BRI1 gene was associated with 1179 genes in the nasc0271 matrix (see below). These transcript co-responses identified 11 of 23 known BR-induced genes present in the nasc0271 matrix [Table 3 and supplement.XLS sheet 8 (nasc0271)]. We define this observation as a co-response recovery of 47.8% and a specificity of 0.9% (for definitions and calculations, see Table 3). A total of 720 genes showed
Table 3. Recovery of known BR-regulated genes by transcript co-response analyses

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>References</th>
<th>nasc0271</th>
<th>Present</th>
<th>%rec</th>
<th>%spec</th>
<th>nasc0272</th>
<th>Present</th>
<th>%rec</th>
<th>%spec</th>
<th>nasc0273</th>
<th>Present</th>
<th>%rec</th>
<th>%spec</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g19350</td>
<td>BES1</td>
<td>BR-signalling positive regulator</td>
<td>(10)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>At1g55610</td>
<td>BRL1</td>
<td>Protein kinase</td>
<td>(35)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>At1g75080</td>
<td>BZR1</td>
<td>BR-signalling positive regulator</td>
<td>(36)</td>
<td>x</td>
<td>0.0</td>
<td>0.0</td>
<td>x</td>
<td>4.5</td>
<td>0.1</td>
<td>x</td>
<td>9.1</td>
<td>0.2</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>At1g13380</td>
<td>BRL2</td>
<td>Protein kinase</td>
<td>(n.a.)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>At4g18710</td>
<td>BIN2</td>
<td>Shaggy-like kinase</td>
<td>(37)</td>
<td>x</td>
<td>30.4</td>
<td>0.6</td>
<td>x</td>
<td>22.7</td>
<td>0.8</td>
<td>x</td>
<td>18.2</td>
<td>0.4</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>At4g3330</td>
<td>BAK1</td>
<td>Receptor kinase</td>
<td>(23, 24)</td>
<td>x</td>
<td>47.8</td>
<td>1.5</td>
<td>x</td>
<td>31.8</td>
<td>1.8</td>
<td>x</td>
<td>36.4</td>
<td>1.7</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>At4g39400</td>
<td>BRI1</td>
<td>Receptor kinase</td>
<td>(20)</td>
<td>x</td>
<td>47.8</td>
<td>0.9</td>
<td>x</td>
<td>45.5</td>
<td>1.7</td>
<td>x</td>
<td>45.5</td>
<td>1.0</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

In the nasc0271, nasc0272 and nasc0273 matrices, 23, 22 and 22 published BR-regulated genes were present, respectively. BR-signalling genes, BR-biosynthesis genes and BR-regulated genes were used for co-response analyses. BR-regulated genes were termed ‘recovered’ in case significant correlations that were calculated (criteria as mentioned in the text). ‘%spec’ gives the percentage of identified known BR-regulated genes relating to the total number of genes showing significant co-responses. Underlying data are provided as Supplementary Material [supplement.XLS sheet 8 (nasc0271), sheet 9 (nasc0272) and sheet 10 (nasc0273)]. *n.a.: Gene not analysable. ’x’: Gene present in data matrix.
co-responses with the \textit{BAK1} gene. The test for co-responses with known BR-induced genes resulted in 47.8\% recovery and 1.5\% specificity [Table 3 and supplemenXLS sheet 8 (nasc0271)].

The intersection gene query with \textit{BRI1} and \textit{BAK1} resulted in the identification of 301 co-responding genes. Public databases contained information that allowed functional categorization of >50\% of these genes [supplemenXLS sheet 1 (301 genes); supplementary data (Figure S1 and Table SI)]. In agreement with high correlation of \textit{BRI1} and \textit{BAK1} expression, 8 of 23 known BR-regulated genes were still present after intersection analysis in the nasc0271 matrix resulting in 34.8\% recovery and 2.7\% specificity (Table 4).

Two other data matrices were used for an intersection gene query with the \textit{BRI1} and \textit{BAK1} genes. Intersection gene queries with the nasc0272 and nasc0273 matrices resulted in the identification of 144 and 161 genes, respectively: 69\% and 71\% of these genes were also present in the \textit{BRI1}/\textit{BAK1} intersection of the nasc0271 matrix [including several known BR-regulated genes; Table 3 and supplemenXLS sheet 9 (nasc0272) and sheet 10 (nasc0273)]. Therefore, the nasc0271, nasc0272 and nasc0273 data matrices produced similar results.

EVALUATION OF IDENTIFIED GENES

Statistical evaluation of co-responses

Non-parametric bootstrap analysis with 2000 bootstrap samples was exemplarily performed with 135 associated genes, which could be functionally classified (including \textit{BRI1} and \textit{BAK1}). The resulting Spearman correlation coefficients (\(r_s\)), \(p\)-values and power values confirmed correlated behaviour of both the \textit{BAK1} gene and the \textit{BRI1} gene with the respective other 134 genes. A complete matrix of all gene correlations is provided [supplement.XLS sheet 2 (Spearman rs), sheet 3 (\(p\)-value) and sheet 4 (power)].

Affymetrix expression profiling experiments

The \textit{BRI1}/\textit{BAK1} intersection gene query was expected to identify associated genes which we call ‘BR-related’ genes. The BR-related genes comprise different classes of genes, namely BR-responsive genes and genes which mirror the functional context of \textit{BRI1} and \textit{BAK1}.

Altered transcript levels in plants with altered BR-levels or altered BR-sensitivity confirm the BR-responsiveness of genes. Publicly available expression profiles were used for \textit{in silico} cross-checking of the 301 associated genes identified with the nasc0271 matrix. AtGenExpress is a multinational coordinated effort to uncover the transcriptome of \textit{Arabidopsis} (http://www.uni-frankfurt.de/fb15/botanik/mcb/AFGN/atgenex.htm). For this study, expression profiles established by Hideki Goda (Plant Science Center and Plant Functions Laboratory, RIKEN, Japan) and two own profiles (17) were used. Expression profiles were analyzed using the stringent settings of the statistical algorithms of the MAS5.0 and GCOS software (see Materials and Methods).

Thirty expression profiles of brassinolide (BL)-, 24-epibrassinolide (EBL)-, castasterone (CS)- or control-treated wild-type and BR-deficient \textit{det}2 plants were used for 15 comparisons. Results were screened for the overlap with the 301 associated genes: 55 genes showed stronger expression upon BR-treatment in at least two independent situations [supplement.XLS sheet 6 (Affx results BRs, BRZ)]; 14 genes including \textit{BRI1} and a \textit{BRI1} homolog (At1g72180) revealed weaker expression in at least two independent experiments. These up- or down-regulated genes did not show conflicting expression patterns (i.e. were not inversely regulated in other situations).

Exogenously applied BR strongly promotes growth. The observed transcript co-responses could be related to growth rather than to specific BR action. In this case, expression may be induced by other growth-promoting compounds such as GA. Therefore, 24 expression profiles of GA3- or control-treated wild-type and \textit{gal}–5 plants were used for 12 comparisons. Genes with significantly altered transcript levels were compared with the 301 candidate genes. Only one gene showed stronger expression upon GA-treatment [supplement.XLS sheet 7 (Affx results GA3, PAC)].

Reduced BR-levels should result in weaker expression of BR-induced genes. Brassinazole (BRZ) is a specific BR biosynthesis inhibitor: 12 expression profiles of BRZ- or control-treated wild-type plants were used for 8 comparisons; 17 (of 301) genes showed reduced expression in the presence of BRZ in at least two independent situations [supplement.XLS sheet 6 (Affx results BRs, BRZ)]. In order to test the effects of
reduced GA-levels, eight expression profiles of paclobutrazol (PAC)- or control-treated wild-type plants were used for four comparisons. Only two genes (of 301) showed weaker expression in presence of PAC [supplement.XLS sheet 7 (Affx results GA3, PAC)]. Thus, transcript levels of the identified genes were marginally affected by GA. This implies that the genes are not secondarily regulated by growth, and dissection of the genomic basis of BR-promoted growth is feasible.

Real-time RT–PCR analysis of growth-related genes

Transcript co-responses reveal the functional context of guide genes. The most obvious BR-effect is promotion of growth, and the identified associated genes should mirror this BR-function. The associated genes were manually screened and 44 genes with predicted or known functions in growth processes were identified and selected for real-time RT–PCR analysis. The correlated behaviour of these genes in the nasc0271 data matrix is illustrated in Figure 3.

BR-induced genes were expected to have reduced transcript levels in BR-mutants and increased transcript levels in BR-treated plants. Three sets of experiments were performed: (i) Wild-type, *dof1*–6, *cbb3* and *cbb2* plants (all C24 background, mutants allelic to the *dim, cpd* and *bri1* mutants) (21) were grown in half-concentrated Murashige and Skoog medium. Plant material was harvested 14 or 19 days after sowing. (ii) Wild-type, *dof1*–6 and *CPD*-antisense plants (27) were grown in soil. Plant material was harvested 28 days after sowing. (iii) Wild-type and *dof1*–6 plants were grown in half-concentrated Murashige and Skoog medium and either treated with a control solution or 300 nM EBL.

Transcript levels were determined using real-time RT–PCR. Eight-fold change values were established (Tables 5 and 6). Fold change values >1.0 indicate a positive BR-effect on transcript levels. *C*<sub>T</sub> values are provided in Table SII (Supplementary Material).

Twenty-three genes showed reduced transcript levels in at least two BR-mutants (Table 5). These genes include known BR-responsive genes such as *KCS1* and *TIP2.1* (Δ-*TIP*) (17), four further aquaporins [TIP1.1 (*At2g36830*), TIP1.2 (*At2g45960*), TIP2.1 (*At3g16240*) and TIP1.2 (*At3g26520*), a KCS1 homologue (*At5g43760*), and genes presumably involved in cell wall modifications [*At1g27600*, AGP21 (*At1g55330*), At2g06850, AGP9 (*At2g14890*), At3g05910, At3g24480, At3g28180, At3g57790, AGP21 (*At4g12730*), At4g13340 and At4g18670]. Fold changes of
growth-related genes were more pronounced in 14- and 19-day-old plants in comparison to 28-day-old plants, probably due to reduced growth rates in older plants (Table 5). Six (of 23) genes also displayed higher mRNA levels in BR-treated plants (Table 5). Three genes displayed stronger expression in BR-mutants or weaker expression upon BR-application (Table 6). Eighteen genes did not exhibit BR-responsive expression or showed variable transcript levels (Table 6).

**Transcript co-responses point to additional BR functions**

Positive effects of BR on plant growth upon cold stress and salt stress have been reported (28). The *ERD4 (EARLY RESPONSIVE TO DEHYDRATION 4), ERD6, ERD15* and *At1g29470* (similar to *ERD3*) genes showed co-responses with *BRI1* and *BAK1* [Supplementary EXCEL file sheet 1 (301 genes)]. Genes involved in auxin responses [*IAA7, IAA14, IAA16, TIR1* homologs (*At3g26810* and *At4g03190*) and *GRH1* (*At4g03190*)], auxin transport (*PIN3* and *AUX1*) and ethylene responses (*EIL1*, *At3g11930*, *ERF7* and *EIN2*) were identified, thus providing further evidence for phytohormone interactions. Expression of the *IAA14* and *At3g11930* genes was reported to be weaker in roots of the *dwarf1–6* mutant in comparison to roots of wild-type plants (8). Transcription factors such as *BEE1, GTL1* and *MYC1* showed co-responses. These genes are induced upon BR-treatment (6,29). Co-responses of genes encoding ubiquitin-conjugating enzymes (*At1g63800* and *At1g64230*), the ubiquitin-ligase RMA1 and F-box proteins (*At1g30200*, *At1g67480*, *At2g18280*, *At3g06380*, *At3g61060*, *At5g27920* and *At5g60570*) may indicate that BR promotes specific protein degradation and thus modifies signalling pathways.

Interestingly, several genes showing high sequence similarity to BR-signalling components also showed co-responses [genes similar to *BRI1*: *At1g28440, At1g72180*, and *At2g18280*, *At3g61060*, *At5g27920* and *At5g60570*] may indicate that BR promotes specific protein degradation and thus modifies signalling pathways.

**DISCUSSION**

**Transcript co-response analyses: a novel approach**

Transcript co-response analysis differs from direct approaches in several ways.

(i) Transcript co-response analysis allows ruling out outliers and conditional changes of transcript levels. The genotype, developmental stage, environmental conditions and tissue...
No consistent BR-dependent or variable expression

and reduced fertility. BR-mutants show altered light and stress responses, delayed physiological events, because physiological networks are disrupted. Expression profiling experiments of BR-mutants do not necessarily reflect normal physiological levels (i.e. in the experiments used for the nasc0271 matrix). Antagonistic events probably serve to limit responses and counter toxic BR-effects. BR-treatments repress BR-biosynthetic genes such as CPD and DWF4 (30,31) and BR-signalling genes such as BRI1, and induce genes involved in the inactivation of BR such as BAS1 (7,32). Thus, BR-repressed genes partly represent components required for BR-action.

Due to conditional disturbances of networks, previous expression profiling approaches identified widely different sets of BR-regulated genes (11,12). Transcript co-response analysis allows to analyse BR-related genes in plants with intact BR-biosynthesis and BR-signalling pathways. In fact, the analysis of BR-related genes is barely possible in a data matrix based on expression profiles of BR-mutants: BR-mutants do not show BR-dependent expression patterns, correlations of BR-related genes are disrupted. Similarly, correlations of BR-related genes are at least partly disrupted in BR-treated plants because of antagonistic events.

The combination of many different experiments accounts for the fact that only 23 or 22 genes were present in the complex matrices used in this study. It also explains that the BRI1 and BAK1 guide genes identified only a subset of

table 6. Real-time RT–PCR analysis of cell wall- and growth-related genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Sterile culture</th>
<th>Soil</th>
<th>WT vs dwf1-6: (28 days)</th>
<th>WT vs aCPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLA9</td>
<td>At5g03870 Fasciclin-like arabinogalactan-protein</td>
<td>1.6 1.1 0.8 1.0 1.5 1.5 1.1</td>
<td>1.0</td>
<td>1.7 1.4 1.3 0.7 0.5 0.7 1.2</td>
<td></td>
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<tr>
<td>CYCD1.1</td>
<td>At5g70210 Cyclin</td>
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<td>At4g02500</td>
<td>Galactosyl transferase</td>
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<td>At5g01210</td>
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<td>DINO10</td>
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<td>FLA1</td>
<td>At5g95730 Fasciclin-like arabinogalactan-protein</td>
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</table>

Stronger expression in mutants or weaker expression upon BR-application

Details given in legend of Table 5.
these genes. Different context-specific data matrices probably would raise the recovery.

Identification of BR-related genes: BR-regulated genes and the functional context

Our statistical approach was based on a wide range of experiments. A total of 51 expression profiles were included in the nasc0271 matrix. The BRI1 and BAK1 genes were selected to screen for BR-related genes. One group of genes was identified which showed positively correlated expression in the nasc0271 matrix [Supplementary EXCEL file sheet 1 (301 genes)]. Experimental evaluation using BR-mutants, BR- and BRZ-treated plants divided this group into three subgroups: BR-induced genes [in total 72 genes (24%)], BR-repressed genes [in total 16 genes (5%)] and genes which were not affected by altered BR-levels or showed variable expression [213 genes (71%)]. The identification of BR-repressed genes and the large number of genes which were not affected by BR-treatments appear to contradict BR-induction inferred from positive correlations with BRI1 and BAK1. However, as mentioned above the identification of common expression patterns in correlation matrices constitutes a context analysis.

On one hand, transcript co-response analysis identifies pathway-involved genes, because BRI1 and BAK1 are required for BR-responses. The specificity of the guide genes was demonstrated by the finding that the identified genes were virtually unaffected by GA. On the other hand, transcript co-responses provide insights into networks. Gene expression patterns are determined by various factors, and these factors are mirrored in correlations. In theory, the observed transcript co-responses could (also) represent co-responses with such unknown factors. Co-response analyses in different matrices could hold the potential to identify upstream factors which determine BRI1 and BAK1 expression.

The functional context comprises physiological pathways, which are (also) regulated by other signalling pathways. Growth-related genes exemplify one aspect of the functional context of the BRI1 and BAK1 genes. BR promotes growth in all plant organs at early developmental stages. There is increasing evidence for involvement of several molecular mechanisms such as cell wall loosening, acidification of wall space, carbohydrate allocation, carbon assimilation and control of aquaporin activity (33). BR apparently coordinates and integrates diverse processes required for growth. Transcript levels of growth-related genes were analysed in BR-mutants and BR- or BRZ-treated plants. A subset of these genes could not be identified as BR-responsive despite the fact that a large set of experiments was performed.

Another example is the identification of auxin-signalling components (e.g. IAA genes), which are not BR-responsive. The BR–auxin interaction is an important aspect in growth processes (19,33). Thus, the transcript co-responses provided additional information about the growth processes in which BR is involved that could not be derived from direct approaches.

Potentials and limitations of transcript co-response analyses

Transcript co-responses can help to learn more about physiological pathways. The results critically depend on the guide genes and the data matrix. Biological knowledge is required for selection of suitable guide genes, and co-responses for a given gene cannot be expected in all data matrices. In this study, the BR-signalling components BRI1 and BAK1 were used as guide genes in combination with the complex nasc0271 matrix. However, BRI1 and BAK1 may not represent suitable guide genes in other data matrices. For example, BRI1 or BAK1 homologs could perform better in tissue or stress-specific matrices.

This implies that specific data matrices are necessary to identify co-responses for genes with context-specific expression. For example, numerous stress-related genes are only expressed under stress. Therefore, co-response analysis with stress-signalling components results in incomplete coverage of target genes in a complex matrix. Tissue-specific matrices hold the potential to analyse expression patterns which are hidden in whole plant profiles.

Statistical approaches have limitations. Correlations do not allow discrimination between primary and secondary events and are not equal to causal relationships. Thus, direct approaches are still necessary for the functional characterization of genes. Other limitations are shared with direct approaches. Reliable measurements of all transcripts are not feasible, and there is an ongoing debate about optimal normalization procedures. In this study, the Affymetrix MAS5.0 and GCOS software were used to normalize the expression profiles. For specific data matrices, alternative normalization procedures will be implemented (e.g. RMA for tissue-specific matrices).

CSB.DB: a valuable public resource to uncover genomic effects

In this study, we focused on genes showing positive transcript co-responses. CSB.DB also allows screening for genes which display negative correlation coefficients. Following the presented approach, 404 genes would have had negative transcript co-responses with both BRI1 and BAK1 [r < (−0.35), p-value < 0.01 and power > 0.7]. Future work shall address the analysis and experimental verification of negative co-responses. We currently construct subsets of expression experiments which will allow screening for co-responses under specific experimental conditions. All data matrices will be publicly available.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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