Identification of brassinosteroid responsive genes in *Arabidopsis* by cDNA array

HU Yuxin (胡玉欣)¹, WANG Zhengke (汪政科)², WANG Yonghong (王永红)¹, BAO Fang (包方)¹, LI Ning (李凝)³, PENG Zhenhua (彭镇华)² & LI Jiayang (李家洋)¹

¹. Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China;  
². Institute of Forest, Chinese Academy of Forestry, Beijing 100091, China;  
³. Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China

Correspondence should be addressed to Li Jiayang (email: jyli@genetics.ac.cn)

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**Abstract** We have systematically monitored brassinosteroid (BR) responsive genes in a BR-deficient mutant *det2* suspension culture of *Arabidopsis* by using a cDNA array approach. Among 13000 cDNA clones arrayed on filters, 53 BR responsive clones were identified and designated BRR1—BRR53. Sequence analysis of 43 clones showed that 19 clones are novel genes, 3 clones are genes involved in the control of cell division, 4 clones are genes related to plant stress responses, 4 clones are transcriptional factor or signal transduction component genes, and 3 clones are genes involved in RNA splicing or structure forming. In addition, we also found that BR regulated the transcription of genes related to many physiological processes, such as photoreaction, ion transportation and some metabolic processes. These findings present molecular evidence that BR plays an essential role in plant growth and development.

Keywords: cDNA array, brassinosteroid responsive gene (BRR), *Arabidopsis thaliana*.

Brassinosteroids (BRs) are the steroid hormones found in plants[1]. Previous studies have shown that BR, like other plant hormones such as auxin and cytokinin, is the hormone essential for normal plant growth and development. BR regulates plant cell elongation and division, vascular differentiation, senescence, pollen fertility and stress responses[2,3]. Recent studies on BR deficient mutant have elucidated the biosynthesis pathway of BR in higher plants[3,4]. However, the research on its molecular mechanism and signal transduction is less fruitful, at least in part because little has been known about its regulated genes. Although a few BR-regulated genes were identified recently, the molecular mechanism of BR action remains unclear. Recently, an effective technique called cDNA microarray has been developed and used to monitor gene expression in plants[5,6]. However, its application to identification of BR-regulated genes has not been reported so far.

To identify BR responsive genes, we have developed a simple but effective cDNA array method to monitor gene expression based on filter hybridization[7]. With this method, BR responsive genes in *Arabidopsis thaliana* were identified and analyzed among 13000 arrayed cDNA
clones and a part of BR responsive genes have been cloned. This paved a way for further investigation and understanding of mechanism of BR function in plant growth and development.

1 Materials and methods

1.1 Plant material

*Arabidopsis thaliana* BR-deficient mutant *det2*\(^8\) was used to produce cultured suspension cells.

1.2 Suspension culture and hormone treatment

Seeds of *det2* were soaked in 70% ethanol for 3–5 min, and then in 10% Bleach for 10–15 min for surface sterilization, washed 3–4 times with sterilized water. For callus induction, the sterilized seeds were cultured on B5 medium containing 2% glucose, 4.5 \(\text{mol/L} 2,4\text{-dichlorophenoxyacetic acid}\) (2,4-D), 0.45 \(\text{mol/L}\) kinetin (KT) and 0.8% agar in the dark at 25\(^\circ\)C. The suspension culture was established by suspending well-grown calli in the liquid medium as described above and maintained in constant low light with orbital shaking at 130 rpm. The medium was replaced weekly.

Before being treated with 24-epi-brassinolide (BL), suspension cultures were washed three times with B5 medium and maintained in B5 medium for 48 h for hormone starvation. Suspension cultures were treated for 2 h with 5 \(\text{mol/L} \text{BL or equal volume of DMSO (control), respectively.}\) RNA was extracted and used for preparation of cDNA array probe. For Northern blot analysis, suspension cultures were treated with 5 \(\text{mol/L} \text{BL for 0, 1, 2 and 4 h.}\)

1.3 cDNA array

The cDNA array procedures were basically carried out as previously described\(^7\). The high density filters were prepared using the Biomek 2000 HDRT system, and were probed with \(\alpha\)-\(^32\)P-dCTP-labelled first strand cDNA. The signal was analyzed with a phosphoimager (Molecular Dynamics, CA, USA).

1.4 RNA extraction and RNA gel-blot analysis

Total RNA was isolated according to the guanidinium-thiocyanate-chloroform extraction procedure\(^9\). Suspension cells were powdered in liquid nitrogen, extracted with guanidinium thiocyanate-phenol-chloroform, precipitated by ethanol, purified with LiCl and chloroform each time. Total RNA was suspended in nuclease-free water and stored at \(−20\)\(^\circ\)C after quantification.

The total RNA of 15 \(\mu\)g of each sample was fractionated in a formaldehyde agarose gel, blotted onto nylon filters and immobilized in a vacuum baker at 80\(^\circ\)C for 2 h. Hybridization was performed in Church buffer\(^10\) at 65\(^\circ\)C for 16–20 h and followed by washing of the filter with mild stringency.

1.5 Sequence analysis

Differentially expressed cDNA clones were subcloned into a pBluescript II SK(+) vector and
sequenced with ABI373A DNA Sequencer (Perkin Elmer, USA). DNA or protein homology search against GeneBank database was performed using the program Blast.

2 Results

2.1 Identification of BR responsive clones

To understand the molecular mechanism of BR function, a cDNA array approach was used to identify BR responsive genes. A set of 12 high density filters containing triplicate 13000 cDNA clones were prepared with Biomek 2000 and BR responsive clones were identified with det2 suspension culture of Arabidopsis. The det2 mutant was chosen because it is defective in an early step of the BR biosynthetic pathway and has very low level of endogenous BR [8]. Fig. 1 shows the gene expression patterns of det2 cells in one of the filters. Although the expression pattern was similar, there were some clones whose expression levels were distinctly different between the BL-treated and the control. Some were induced by BL, while some were suppressed (fig. 1).

Out of the 13000 arrayed clones, 53 were found to be BR responsive and designated BRR1—BRR53.

To confirm the BR responsive clones identified, RNA gel-blot analysis was performed with 25 clones and the results of 10 clones were shown in fig. 2. Most of these clones demonstrated the same responsiveness as those shown in the cDNA array. However, the Northern results of seven clones (BRR19, BRR25, BRR2, BRR9, BRR33, BRR35, BRR43) were not well reproducible in the Northern blot analyses (see below).

2.2 BR responsive genes

A total of 43 differentially expressed cDNA clones were subcloned into a pBluescript II SK (+) vector. Sequencing and homology analyses revealed that 19 clones have their corresponding genomic DNA sequence stored in GeneBank, whereas the other 24 clones have homologous cDNAs or protein sequences instead. BR responsive genes were classified according to their functions as shown in table 1.
As shown in table 1, BR responsive genes are involved in many aspects of plant growth and development. Three genes are related to cell division or differentiation control, including a D-type cyclin gene \( \text{CycD3} \), which proves that BR is involved in cell division. Four genes are involved in plant stress responses, including two heat-shock protein genes and a cyclophilin gene. In addition four genes are involved in transcriptional regulation or signal transduction of BR, including three transcription factors and a putative protein kinase gene. Another three genes are related to RNA splicing and structure. BR was found to play a role in plant photoreaction, ion transportation and other metabolic processes. Sequencing analysis also demonstrated that the unreproducible genes in Northern blot analysis were genes mainly related to photosynthesis (see section 3).

3 Discussion

Differential gene expression is associated with the plant growth and development and physiological responses to the internal/external environment cues. The plant hormones play many roles in growth and development as well as all metabolic processes including affecting tissue- and stage-specific gene expression. In order to understand the mechanism of their actions, we have systematically studied the BR responsive genes by cDNA array. Furthermore, because we employed the BR-deficient mutant cell line as the material to deprive the interference of the endogenous BR to some extent, it is possible that the BR-responsive genes that we identified may have the genes that are difficult to be found in normal plants. Moreover, the identification of these BR responsive genes or the BR-regulatory genes paves a way for further study of the BR signal transduction and regulatory mechanism.

Three genes involved in controlling of cell division were found to be BR responsive. Among them, \( \text{CycD3} \) is a D-type cyclin which functions as a mediator of environmental stimuli to drive cell division\(^{[11]}\), and the activation of cell division by cytokinin has been proved to be through the transcriptional induction of \( \text{CycD3} \)^{[12]}. Previously we also presented the evidence that the promo-
tive effect of BR on cell division involves a distinct \textit{CycD3}-dependent pathway\cite{13}. Although the \textit{in vivo} function of Myb-like protein remains unknown, it has been suggested to be involved in differentiation or other cellular processes\cite{14}. \textit{MLO2} is another gene related to cell division and found in yeast. Its overexpression leads to the suppression of yeast cell division\cite{15}.

Table 1  Genes (clones) responsive to BR treatment

<table>
<thead>
<tr>
<th>Function</th>
<th>Clone</th>
<th>Identity (D/P)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unknown</td>
<td>19</td>
<td>+/−</td>
<td></td>
</tr>
<tr>
<td>Related cell division</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{BRR36}</td>
<td>+</td>
<td>CycD3</td>
<td>D97</td>
</tr>
<tr>
<td>\textit{BRR53}</td>
<td>−</td>
<td>Myb-like protein</td>
<td>D91</td>
</tr>
<tr>
<td>\textit{BRR21}</td>
<td>−</td>
<td>MLO2(Yeast)</td>
<td>P43</td>
</tr>
<tr>
<td>Related to stress response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{BRR32}</td>
<td>+</td>
<td>Cyclophilin</td>
<td>D93</td>
</tr>
<tr>
<td>\textit{BRR11}</td>
<td>+</td>
<td>Hsp90a(81)</td>
<td>D93</td>
</tr>
<tr>
<td>\textit{BRR52}</td>
<td>+</td>
<td>Hsp90b(82)</td>
<td>D98</td>
</tr>
<tr>
<td>\textit{BRR6}</td>
<td>+</td>
<td>Putative stress protein</td>
<td>P40</td>
</tr>
<tr>
<td>Related to signal transduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{BRR8}</td>
<td>−</td>
<td>RAV1 DNA binding protein</td>
<td>D92</td>
</tr>
<tr>
<td>\textit{BRR23}</td>
<td>−</td>
<td>CCAAT binding factor</td>
<td>P45</td>
</tr>
<tr>
<td>\textit{BRR26}</td>
<td>−</td>
<td>Putative kinase(Yeast)</td>
<td>P45</td>
</tr>
<tr>
<td>\textit{BRR27}</td>
<td>−</td>
<td>GAGA transcription factor</td>
<td>P47</td>
</tr>
<tr>
<td>Related to RNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{BRR22}</td>
<td>−</td>
<td>RNA helicase</td>
<td>D98</td>
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<tr>
<td>\textit{BRR40}</td>
<td>−</td>
<td>U2snRNP auxiliary factor</td>
<td>P74</td>
</tr>
<tr>
<td>\textit{BRR51}</td>
<td>−</td>
<td>PolyA binding protein</td>
<td>D97</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{BRR1}</td>
<td>−</td>
<td>Ferritin</td>
<td>D92</td>
</tr>
<tr>
<td>\textit{BRR3}</td>
<td>+</td>
<td>CHLH(Mg\textsuperscript{2+} chelatase)</td>
<td>D98</td>
</tr>
<tr>
<td>\textit{BRR14}</td>
<td>+</td>
<td>PEAL1-4 mRNA</td>
<td>D85</td>
</tr>
<tr>
<td>\textit{BRR25}</td>
<td>?\textsuperscript{a}</td>
<td>\textit{AtsIA}</td>
<td>D85</td>
</tr>
<tr>
<td>\textit{BRR19}</td>
<td>?</td>
<td>\textit{Ats1B}</td>
<td>D93</td>
</tr>
<tr>
<td>\textit{BRR9}</td>
<td>?</td>
<td>\textit{AtsIA}</td>
<td>D91</td>
</tr>
<tr>
<td>\textit{BRR43}</td>
<td>?</td>
<td>oxygen-evolving complex protein 3(photosystem II)</td>
<td>D99</td>
</tr>
<tr>
<td>\textit{BRR49}</td>
<td>+</td>
<td>1-acyl-sn-glycerol-3-phosphate acyltransferase</td>
<td>D88</td>
</tr>
<tr>
<td>\textit{BRR13}</td>
<td>+</td>
<td>Prolylcarboxypeptidase</td>
<td>P69</td>
</tr>
<tr>
<td>\textit{BRR2}</td>
<td>?</td>
<td>ATP synthase delta subunit</td>
<td>D83</td>
</tr>
</tbody>
</table>

\textsuperscript{a}  +, genes induced by BL treatment; −, genes suppressed by BL treatment. b) D, DNA; P, protein. c) ?, BR responsive clones that were not well reproducible in Northern blot analyses.

BR can also enhance plant stress tolerance, especially to heat, chilling and salt\cite{3}. We found that 4 genes involved in stress response are BR-inducible. The finding that two members of \textit{Hsp90} gene family, \textit{Hsp81} and \textit{Hsp82}, were induced by BL suggests that BR may enhance the plant heat tolerance by regulating the expression of \textit{Hsp90}\cite{16}. In human and animals, the intracellular receptors of steroid hormones, such as estrogen, progesterin, androgen and glucocorticoid, are large complexes. Not only is Hsp90 a component of the complex that makes steroid receptor maintain its active conformation, but it is also involved in the signal transduction of this steroid hormone\cite{17}.

Cyclophilin, another conserved molecular chaperone that normally exists in animal and plant cells, plays a role in protein folding. Seven \textit{cyclophilin} genes have been found in \textit{Arabidopsis thaliana}\cite{18}. Yeast cyclophilin-deficient mutant ceases to grow under heat shock, but grow well under the normal condition\cite{19}. The \textit{cyclophilin} was also found to be inducible upon heat shock in
maize and soybean\textsuperscript{[20]}. These results suggest that cyclophilin is an important protein involved in plant stress tolerance.

We also found that BR regulates the transcription of 4 genes related to BR signal transduction or transcriptional and post-transcriptional regulation. RAV1 is a DNA binding protein with two DNA binding domains whose cellular functions remain unknown\textsuperscript{[21]}. BRR26, a putative protein kinase, contains a highly conserved protein kinase domain, indicating that this protein kinase is involved in BR signal transduction. The finding that BL regulates CCAAT binding factor and GATA transcription factor suggests that BR regulates expression of some of the genes through transcription factor. Moreover, transcriptional alterations of RNA helicase, U2 snRNP auxiliary factor and polyA binding protein imply that BR regulates the gene expression either at the post-transcriptional or at the translational level.

BRs have also been shown to play a role in photoreaction\textsuperscript{[22]} and several photosynthesis-related genes, such as \textit{Ats}, have been shown to have an altered gene expression in the BR-deficient mutant\textsuperscript{[23]}. In our experiments, we also observed that the transcription of four photosynthesis-related genes are affected by the BR treatment. However, they were not well reproducible in Northern blot analysis. It may be due to the materials that we used were cell cultures, in which photoreaction is known to be unessential for the growth and proliferation. Mg\textsuperscript{2+} chelatase, a key enzyme that chelates Mg\textsuperscript{2+} to porphyrin in chlorophyll biosynthesis, is composed of three subunits, CHLI, CHLD and CHLH\textsuperscript{[24]}. Induction of the \textit{CHLH} by BR shows that BR is involved in the photoreaction regulation. The identification of BR responsive genes also suggests that BR regulates ion transportation and many other metabolic processes (metabolic enzyme genes, ATP synthase gene as shown in table 1).

Although the functions of many BR-responsive genes are still not clear, the investigation of these novel BR-related genes will greatly broaden our knowledge on the molecular mechanism of BR action in higher plants.

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作者: 胡玉欣, 汪政科, 王永红, 包方, 李凝, 彭镇华, 李家洋
作者单位: 胡玉欣, 王永红, 包方, 李家洋(Institute of Genetics, Chinese Academy of Sciences, Beijing 100101, China), 汪政科, 彭镇华(Institute of Forest, Chinese Academy of Forestry, Beijing 100091, China), 李凝(Department of Biology, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China)

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