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G.-X. Yang · A. Jan · S.-H. Shen · J. Yazaki
M. Ishikawa · Z. Shimatani · N. Kishimoto · S. Kikuchi
H. Matsumoto · S. Komatsu

Microarray analysis of brassinosteroids- and gibberellin-regulated gene expression in rice seedlings

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Abstract In order to understand the molecular mechanisms by which two types of phytohormones, brassinosteroids (BRs) and gibberellins (GAs), regulate plant growth and development, a cDNA microarray was made containing 4000 clones randomly selected from a rice cDNA library constructed from RNA isolated from seedlings treated with GA_3 and brassinolide (BL). The array was then probed to identify differences in expression using labelled cDNAs prepared from treated and control seedlings. Nine and 29 unique cDNA clones were up-regulated, while 32 and 42 unique cDNA clones were down-regulated by BL and GA_3, respectively. The predicted products of these BL- and GA-regulated genes fall into such functional categories as signal transduction, transcription, metabolism, cellular organization, and defense or anti-stress responses. Northern analysis of the selected BL- and GA_3-regulated genes not only confirmed their BL or GA_3 dose dependent expression, but also revealed significant differences in the degree of expression in different organs and in response to other phytohormones. These results demonstrate that BR and GA influence growth and development by coordinately regulating the expression of specific groups of genes.

Keywords Brassinolide · Gene expression · Gibberellin · Microarray analysis · Rice

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G.-X. Yang · A. Jan · S.-H. Shen · J. Yazaki · M. Ishikawa
N. Kishimoto · S. Kikuchi · S. Komatsu (✉)
Department of Molecular Genetics, National Institute of Agrobiological Sciences, 305-8602 Tsukuba, Japan
E-mail: skomatsu@affrc.go.jp
Tel.: +81-29-8387446
Fax: +81-29-8387464

A. Jan · H. Matsumoto
Institute of Applied Biochemistry, University of Tsukuba, 305-8572 Tsukuba, Japan

Z. Shimatani
STAFF-Institute, 305-0854 Tsukuba, Japan

Introduction

Brassinosteroids (BRs) and gibberellins (GAs) are two groups of plant growth regulators that are essential for normal growth and development (Mandava 1988; Swain and Olszewski 1996). While rapid progress has been made in the study of the biosynthesis and metabolism of BRs (Schumacher and Chory 2000) and GAs (Hedden and Kamiya 1997) using biochemical techniques, as well as by the characterization of their biosynthetic mutants, not much is known about how they regulate a wide variety of physiological processes at the molecular level.

Mutations in at least eight loci in *Arabidopsis*, and several additional loci in tomato and pea, have been found to lead to phenotypes that show characteristics of BR deficiency, and feeding with BRs rescues these mutants fully (Schumacher and Chory 2000). Recently, a rice gene that is mutated in a BR-deficient dwarf mutant, and which encodes a C-6 oxidase, was isolated and characterized, suggesting that endogenous BRs are also important for normal growth and development in monocot plants (Hong et al. 2002). The genes for most of the enzymes involved in GA biosynthesis have been isolated and characterized (Olszewski et al. 2002). The *SD1* gene, mutations in which are responsible for the short stature of the semi-dwarf, high-yield rice variety IR8, has recently been cloned. It was found to encode a GA_{20} oxidase (GA20ox-2), a key enzyme in the GA biosynthesis pathway, and its characterization provided useful information for regulating the heights of other crop plants by manipulating GA biosynthesis (Sasaki et al. 2002).

Analyses of BR-insensitive dwarf mutants of *Arabidopsis* have led to the identification of an important component of BR signal transduction, the putative receptor BRI1 (for BR-Insensitive) (Li and Chory 1997). The counterpart of BRI1 has also been cloned from a dwarf mutant of rice (Yamamuro et al. 2000). *BIN2* (*Arabidopsis Brassinosteroid-Insensitive2*), which

complements the BR-insensitive *Arabidopsis* mutant *bin2*, codes for a negative regulator of BR signaling, a GSK3/SHAGGY-like kinase (Li and Nam 2002). Recently, another receptor-like protein kinase BAK1 (*bri1-associated receptor kinase 1-1Dominant*) was identified. The evidence indicates that BAK1 and BRI1 function together to mediate BR signaling (Li et al. 2002).

Although the molecular mechanism by which plants respond to GA is still largely unknown, several important components of the GA signal transduction pathway have been identified. The *dwarf1* (*d1*) mutant in rice is characterized by a GA insensitive semi-dwarf phenotype, and cloning of the *D1* locus revealed that it encodes the putative α-subunit of the heterotrimeric G protein (Ashikari et al. 1990). Recent studies have identified *SPY, RGA* and *GAI* as negative regulators of GA signaling in *Arabidopsis* (Sun 2000). SPY is an O-GlcNAc transferase that post-translationally modifies cytosolic and nuclear proteins (Thornton et al. 1999). The two putative transcript factors GAI (Peng et al. 1997) and RGA (Silverstone et al. 1998), which are very similar to each other, were shown to mediate responses to GA, and their functions appear to overlap.

The major effects of BR and GA on plant growth and development are mediated via the modulation of gene expression, because inhibitors of RNA and protein synthesis interfere with these processes. Very few BR-regulated genes have been identified so far, and most, including *TCH4* (Kauschmann et al. 1996), *LeBR1* (Koka et al. 2000) and *BRU1* (Oh et al. 1998), encode cell wall-modifying enzymes such as xyloglucan endotransglycosylases (XETs). These results provide a direct link between BRs and an induced growth response. The induction of *cycD3* transcription by epibrassinolide may represent a mechanism by which BR can promote cell division (Hu et al. 2000). Significant progress has been made towards an understanding of the mechanism of GA action in the cereal aleurone, where GAs induce the synthesis and secretion of a number of hydrolytic enzymes (Cercos et al. 1999). Although some other GA-regulated genes have been identified in shoot (Shi et al. 1992), leaf (Speulman and Salamini 1995), flower (Van den Heuvel et al. 2002) and stem (Van der Knaap et al. 2000) in various plants, how GAs regulate the growth and development of these organs is still not clear.

To further our understanding of the molecular mechanisms by which these two important classes of phytohormone regulate the growth and development of plants, it is necessary to identify and analyze more genes that are controlled by them. In this study, a cDNA microarray containing 4000 clones randomly selected from a rice cDNA library prepared from seedlings treated with GA_3 and brassinolide (BL), the most active form of BR, was analyzed, with the aim of identifying new genes that exhibit transcriptional regulation by BR and GA.

Materials and methods

Plant material

Rice (*Oryza sativa* L. cv Nipponbare) was grown under white fluorescent light (about 600 μmol m^{-2} s^{-1}, 12 h light period/day) at 25°C and 75% relative humidity in a growth chamber. The shoot (above ground parts) was prepared from 1-week-old seedlings. Callus was cultured in N_6 liquid medium and subcultured every other week. Samples were floated on 10 ml of distilled water in 60×15 mm Petri dishes containing BL (Fuji Chemicals, Toyama, Japan), GA_3 (Wako Pure Chemicals, Osaka, Japan), indole-acetic acid (IAA; Wako) or abscisic acid (ABA; Wako) or 0.1% (v/v) dimethyl sulfoxide as a control.

cDNA library construction

A cDNA library was constructed from shoots of 1-week-old seedlings treated with 1 μM BL and 5 μM GA_3 for 24 h with the goal of enriching for BR- and GA-regulated genes. Double-stranded cDNA was synthesized using λZAPII cDNA synthesis kit (Stratagene, Cedar Creek, Tex., USA). cDNAs longer than 500 bp were collected by passage over a Sepharose CL-2B column, and ligated to the Uni-ZAP XR vector (Stratagene), and the ligated cDNA was packaged in vitro using a Gigapack extract (Stratagene). The phage library was converted into plasmid form by mass excision according to the procedure described by Stratagene.

Amplification of cDNAs and preparation of the cDNA microarrays

Four thousand clones were picked at random from the cDNA library. Insert cDNAs were amplified by PCR in a 96-well format using pBluescript T3 (5′-GAAATTAACCCTCACTAA AGG-3′) and T7 (5′-TGTAATACGACTCACTATAGGGC-3′) primers in 50-μl reactions. Reactions were performed for 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 3 min at 72°C, followed by a final 7-min extension at 72°C. The PCR products were first purified using 96-well Multiscreen PCR plates (Millipore, Bedford, Mass., USA), and then 3 μl of each reaction was fractionated in agarose gels to confirm amplification quality and quantity. The purified cDNA inserts were mixed with reagent D (Amersham Biosciences, Little Chalfont, Bucks., UK), and arrayed in duplicate from 384-well plates onto aluminum-coated and DMSO-optimized glass slides using the Array Spotter Generation III (Amersham Biosciences).

Preparation of fluorescent probes and hybridization

Fluorescent probes were prepared as follows. Aliquots (1 μg) of mRNAs isolated from shoots treated with either 1 μM BL or 5 μM GA_3 for 24 h, or water as a control, were reverse transcribed in 20-μl volumes containing 1 mM Cy5 or Cy3 dCTP (Amersham Pharmacia Biotech), anchored oligo$(dT)_{25}$, random nonamers, dithiothreitol, dNTPs and SuperScript II (Invitrogen, Carlsbad, Calif., USA). After incubation at 42°C for 2 h, the reaction was stopped, and the RNA was degraded by first heating at 94°C for 3 min and then treating with NaOH at 37°C for 15 min. Fluorescently labeled probes were purified using the QIAquick PCR purification kit (Qiagen). Hybridization was performed as previously described (Yazaki et al. 2000). Microarray slides were scanned in both Cy5 and Cy3 channels with an Array Scanner Generation III (Amersham Biosciences).

Data analysis

The fluorescence intensity for each fluor and each element on the array was captured by using ArrayGauge version 1.21 (Fujifilm, Tokyo, Japan). The local background was subtracted from the value for each spot on the array. Normalization of Cy3 and Cy5 signal intensity was performed by adjusting the total signal intensities of the two images. Signal data for each element in Cy3 and Cy5 channels were calculated based on the formula: signal = (signal intensity of each element)/(total signal intensities) $\times 10^6$.

Sequencing analysis and gene annotation

cDNA clones representing genes whose expression was found to be influenced by BL and GA_3, based on the microarray analysis, were partially sequenced from the 3′-end using dye-labeled terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Warrington, UK) and an automated DNA sequencer (Model 377, Applied Biosystems). Gene annotation was done first by comparing the 3′-end sequences with the full-length rice cDNA database (Kikuchi et al. 2003) and then with the GenBank database using the BLAST programs. Similarity was considered to be significant when the optimized BLAST similarity score at the amino acid level was higher than 100.

RNA extraction and Northern analysis

Tissue samples were quick-frozen in liquid nitrogen. Samples were ground to a powder using a mortar and pestle, and total RNAs were isolated according to the procedure described by Chomcyznski and Sacchi (1987). The mRNAs for cDNA library construction and microarray probes preparation were purified using an Oligotex-dT-30 mRNA purification kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. For Northern analyses, 20-μg aliquots of total RNA were fractionated on 1.2% agarose gels containing 6% formaldehyde, and transferred to Hybond-N^+ nylon membranes (Amersham Biosciences). The relative amounts of total RNA present in the different lanes on Northern blots were determined by visualization of ethidium bromide-stained rRNA bands. Probes for Northern analysis were full-length insert cDNAs, which were amplified from pBluescript plasmids by PCR using T3 and T7 primers, purified from agarose gels using the QIAEX II Gel Extraction Kit (Qiagen), and radiolabeled using [α-^{32}P]dCTP (Amersham Biosciences) and the random prime labeling system (Rediprime II, Amersham Biosciences). Hybridization was performed at 42°C in an ultrasensitive hybridization buffer (ULTRAhyb, Ambion, Austin, Tex., USA) overnight. The blots were washed twice first in 2×SSC, 0.1% SDS at 42°C for 5 min, then in 0.1×SSC, 0.1% SDS at 68°C for 15 min, they were finally detected with X-ray film (Kodak, Rochester, N.Y., USA) or analyzed with a phosphor image program using the Typhoon 8600k variable imager (Amersham Biosciences).

Results

Microarray construction and hybridization

To enrich for GA- and BR-induced genes, a cDNA library was first constructed from mRNAs isolated from seedlings that had been treated with BL and GA_3. Inserts that were longer than 500 bp were amplified by PCR from randomly selected cDNA clones, using T3/T7 primers. The resulting microarray comprised cDNAs representing 3936 transcripts. To increase the reliability of signal detection, each amplified sample was spotted twice, in two subarrays, resulting in a total array of 7872 data points per slide. Hybridization was done twice and only those clones that showed the same changes in both experiments were included in subsequent analyses.

To monitor BL- and GA_3-regulated gene expression, mRNA samples were isolated from shoots of 1-week-old rice seedlings that had been treated for 24 h with either 1 μM BL or 5 μM GA_3. The RNAs were reverse transcribed into cDNA in the presence of Cy5 or Cy3 fluorescent dye, respectively. Fluorescence-labeled control and BL- or GA_3-treated sample probes were then mixed and hybridized simultaneously to the cDNA microarray. After scanning Cy5 and Cy3 channels and global normalization, the Cy3/Cy5 ratio was calculated for each element using the globally normalized data. Since about 98% of the clones display less than ±50% variation, fluorescent signal differences greater than two-fold between the control and hormone-treated samples were considered to be significant. After data analysis, a total of 139 clones were found to show significant differences in expression between control and hormone treatments (Table 1). All these clones were partially sequenced from the 3′-end, and annotation was done first by comparing the 3′-end sequences with our database of full-length cDNAs from rice (Kikuchi et al. 2003), then with the GenBank database, using BLAST programs. In total, nine clones representing nine unique genes showed more than two-fold stronger signals in the BL-treated sample than in the control, while 34 clones, representing 32 unique genes, were found to be down regulated by BL. Forty clones, derived from 29 unique genes, showed up regulated expression in the presence of GA_3, while 56 clones, representing 42 unique genes, were down-regulated by GA_3.

Identification of genes that are up regulated by BL

Among the nine unique genes up regulated by BL (Table 2), six were found to be present in our database of full-length rice cDNAs, while clone 2994 showed no significant homology to any known sequences. Clone 3904 was highly homologous to a sequence in rice genomic DNA. One clone (No. 592) showed homology to a human gene product of unknown function. Clone 1352 encodes a NAM (non-meristem mutant) protein. In

Table 1 Summary of the numbers of clones up- or down-regulated by BL and GA_3 treatment in rice seedlings

Treatments	Number of up-regulated clones	Number of unique clones	Number of down-regulated clones	Number of unique clones
BL	9	9	34	32
GA_3	40	29	56	42
Total	49	38	90	74

Table 2 List of clones up regulated by BL, as detected by cDNA microarray analysis

Element No.[a]	Putative ID[b]	Best BLAST hit[c]	Hit in full-length cDNA database[d]	Induction ratio[e]
1352	*Oryza sativa* putative NAM protein	AAL86494	No	2.79
1719	*O. sativa* phosphoenolpyruvate carboxykinase	AJ250829	Yes	4.24
1952	*O. sativa* transcription factor Myb	AY026332	Yes	4.91
3741	*O. sativa* phosphatidylinositol synthase	AJ238318	Yes	2.56
3559	*O. sativa* GTP-binding protein	AF327517	Yes	3.45
4276	*O. sativa* for PR1a protein	AJ278436	Yes	7.67
592	Unnamed protein	AX027925	No	2.85
3904	*O. sativa* genomic sequence	AC096688	Yes	2.95
2994	Unknown	-	No	2.51

[a]Serial numbers of the clones used in the microarray
[b]Based on BLAST analysis; the gene with the highest score was selected
[c]Accession No. of BLAST hit
[d]Presence or absence in the rice full-length cDNA database
[e]Fluorescence intensity ratios (treatment/control)

loss-of-function *nam* mutants of *Petunia*, embryos fail to elaborate shoot apical meristem, and the seedlings do not develop shoots or leaves (Duval et al. 2002). Clone 1719 encodes phosphoenolpyruvate carboxykinase, an enzyme that catalyzes the reversible reaction: oxaloacetate + ATP ↔ PEP + ADP + CO_2. This reaction is important in plant metabolism because it lies at the interface between organic acid, amino acid, and sugar metabolism (Chen et al. 2002). Clone 3741 encodes a phosphatidylinositol synthase, a key enzyme responsible for the production of phosphatidylinositol, one of the abundant phospholipids in cell membranes (Collin et al. 1999). The product of clone 1952 belongs to the Myb family of transcription factors. MYB proteins constitute a diverse class of DNA-binding proteins, and function in regulating cell cycle, cellular morphogenesis and controlling secondary metabolism (Jin and Martin 1999). Clone 3559 codes for a GTP-binding protein, and GTP-binding proteins affect many different signaling pathways in plants (Jones 2002). One clone that is up-regulated by BL encodes a rice PR1 protein, indicating a role for BL in protection against attack by pathogens.

Identification of genes that are down regulated by BL

Among the clones representing rice genes down regulated by BL, 25 were found to have homologs in the full-length cDNA database. This accounts for 76% of down regulated clones. Nine clones were annotated to known functional genes; four clones encode putative rice proteins. While 11 clones showed homologies to rice or *Arabidopsis* genomic DNA sequences, the remaining eight clones did not show homology to any sequences in the databases (Table 3). Five clones, showing homology to or encoding a putative glucan 1,3 beta-glucosidase precursor (clone 3), salT (clone 11), a metallothionein-like protein (clone 599), NADPH-dependent HC-toxin reductase (clone 1347) and wali7 (wheat aluminum induced, clone 1623) respectively, are associated with responses to stresses. Clone 3911 encodes ribosomal protein S7, one of the ubiquitous components of the small subunit of the ribosome. Clones 438 and 439 code for protein kinases, which are important signaling components. Clone 401 is homologous to an mRNA for H^+/Ca^{2+} exchanger 2 from *Ipomoea nil*. Regulation of H^+/Ca^{2+} exchange is important in determining the duration and amplitude of cytosolic Ca^{2+} oscillations (Pittman et al. 2002). Clone 70 encodes a homologue of a Photosystem I protein from barley, indicating the possible involvement of BL in the process of photosynthesis.

Identification of genes that are up-regulated by GA_3

Of the clones up regulated by GA_3, 72% found hits in the full-length cDNA database (Table 4). Three clones showed no homology at all in a BLAST search. Nine clones, representing six unique genes, encoded products that were found to be highly homologous to proteins of known function in database searches, while the other 28 clones, representing 20 unique genes, showed homologies to genomic DNA sequences of either rice or *Arabidopsis*.

Four clones encoding the same XET were found to be up regulated by GA_3. XETs, the cell wall-loosening enzymes essential for cell elongation, are encoded by a multigene family, and some isoforms have been found to be up-regulated by GA_3 (Uozu et al. 2000). This result also confirms the reliability of the microarray experiment. Three genes involved in responses to various stresses in plants were identified as being up-regulated by GA_3. Clone 4528 encodes a PR1 homolog. Clone 2398 is homologous to a barley ORF for a glutathione peroxidase-like protein (GPX-like). Uroporphyrinogen decarboxylase (UROD), encoded by clone 3102, is a key enzyme in the tetrapyrrole biosynthetic pathway. Transgenic tobacco plants with reduced activity of UROD are characterized by the accumulation of photosensitizing tetrapyrrole intermediates, antioxidative responses, and necrotic leaf lesions (Kruse et al. 1995). Clone 2045 encodes a chloroplast precursor of an inorganic pyrophosphatase (PPase). A gene for a

Table 3 List of the 34 clones down regulated by BL, as detected by cDNA microarray analysis

Element No.	Putative ID	Best BLAST hit	Hit in full-length cDNA database	Repression ratio[a]
438	*O. sativa* protein kinase	AF004947	No	2.28
439	*O. sativa* protein kinase	AF004947	No	2.26
401	*Ipomoea nil* H^+/Ca^{2+} exchanger 2	AB018526	Yes	2.96
3911	*Secale cereale* ribosomal protein S7	AF118149	Yes	2.51
70	*Hordeum vulgare* photosystem I protein (SPI-L)	M61146	Yes	2.23
3	*O. sativa* putative glucan 1,3 beta-glucosidase	AAM08821	Yes	2.04
11	*O. sativa* salT protein	AF001395	Yes	2.03
599	*O. sativa* metallothionein-like protein	U57638	No	2.92
1347	*H. vulgare* NADPH-dependent HC-toxin reductase	U77463	Yes	2.79
1623	*Triticum aestivum* wali7	L28008	Yes	6.25
290	*O. sativa* putative CGi-203 homolog	AC021893	No	2.21
393	*O. sativa* putative protein	BAB86519	Yes	2.99
602	*O. sativa* putative protein	BAB90785	No	2.51
41	*O. sativa* putative protein	AAL84286	Yes	2.30
427	*O. sativa* genomic sequence	AP002844	Yes	2.93
584	*O. sativa* genomic sequence	AC118675	Yes	2.40
1348	*O. sativa* genomic sequence	AP002071	Yes	4.07
1357	*O. sativa* genomic sequence	AP002071	Yes	4.39
426	*O. sativa* genomic sequence	AL606652	Yes	2.38
430	*O. sativa* genomic sequence	AP003286	Yes	2.32
389	*O. sativa* genomic sequence	AL663984	Yes	2.21
586	*O. sativa* genomic sequence	AL731599	Yes	2.59
1286	*O. sativa* genomic sequence	AP006049	Yes	2.34
596	*A. thaliana* genomic sequence	AL035539	Yes	3.13
834	*A. thaliana* genomic sequence	AC007019	Yes	2.39
1308	*A. thaliana* genomic sequence	AL079344.1	Yes	5.05
18	Unknown	-	Yes	2.14
44	Unknown	-	Yes	2.03
185	Unknown	-	No	2.36
385	Unknown	-	No	2.75
421	Unknown	-	Yes	2.66
582	Unknown	-	Yes	3.82
826	Unknown	-	No	2.53
1358	Unknown	-	No	6.25

[a]Fluorescence intensity ratios (control/treatment)

pyruvate dehydrogenase kinase I, represented by clone 975, was identified as another gene that is up-regulated by GA_3. Pyruvate dehydrogenase kinase is a negative regulator of the mitochondrial pyruvate dehydrogenase (PDH) complex (mtPDC), and plays a pivotal role in controlling mtPDC activity, and hence, in the TCA cycle and cell respiration (Zou et al. 1999).

Identification of genes that are down regulated by GA_3

Of the clones derived from genes that are down-regulated by GA_3, 43 (77%) found hits in our cDNA database (Table 5). Thirteen unique clones were annotated to known functional genes, while 15 clones showed homology to genomic DNA sequences from either rice or *Arabidopsis*. One clone (No. 3534) is homologous to a sequence in the chloroplast genome of rice. Twelve clones did not show homology to any sequence in the databases.

Clone 487 codes for a putative Myb transcription factor, which belongs to R2R3-MYB family. One clone (No. 514) encodes a phosphatidylinositol synthase. Clone 2477 codes for a subunit of the 60S ribosomal particle. Two oxidase genes were also found to be down regulated by GA_3. One of them is a plastid oxidase (clone 673), and the other is a peroxisome glycolate oxidase encoded by clone 103, an enzyme of the glycolate pathway that functions in photorespiration. Clone 4314 is homologous to a *Prunus serotina* gene for an amygdalin hydrolase isoform (AH I), a β-glucosidase that is responsible for the hydrolysis of glucoside-amygdalin. The remaining GA_3 down-regulated genes that have putative identities are all related to stresses. They include heat shock protein HSP82; a probenazole-inducible gene (PBZ1), the induced expression of which increased resistance to rice blast disease (Nakashita et al. 2001); beta 1,3-glucanase; a chitinase is encoded by two clones; S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT), an enzyme involved in plant defense responses mediated by salicylic acid (SA; Ross et al. 1999) is encoded by four clones; the salT protein, which is induced in response to salt stress, is encoded by six clones; two types of metallothionein (MT)-like proteins are encoded by one and six clones respectively; these genes could be induced by heavy metals, as well as other stresses such as heat shock, wounding and virus infection (Ma et al. 2003).

Table 4 List of the 40 clones up regulated by GA_3, as detected by cDNA microarray analysis

Element No.	Putative ID	Best BLAST hit	Hit in full-length cDNA database	Induction ratio[a]
3102	*O. sativa* uroporhyrinogen decarboxylase UROD	AF119232	Yes	2.25
975	*O. sativa* pyruvate dehydrogensase kinase 1	AY026039	Yes	2.32
2045	*A thaliana* inorganic pyrophosphatase	AJ252210	Yes	2.13
46	*H. vulgare* xyloglucan endotransglycosylase-like protein	X93173	Yes	2.38
647	*H. vulgare* xyloglucan endotransglycosylase-like protein	X93173	Yes	2.38
1646	*H. vulgare* xyloglucan endotransglycosylase-like protein	X93173	Yes	2.40
3990	*H. vulgare* xyloglucan endotransglycosylase-like protein	X93173	Yes	2.51
2398	*H. vulgare* GPX12Hv	AJ238697	Yes	2.26
4528	*Zea mays* pathogenesis-related protein	U82200	Yes	2.17
230	*O. sativa* genomic sequence	AL606633	Yes	2.05
1571	*O. sativa* genomic sequence	AP003105	Yes	2.16
23	*O. sativa* genomic sequence	AP003434	Yes	2.05
1270	*O. sativa* genomic sequence	AP003434	Yes	2.04
50	*O. sativa* genomic sequence	AC079853	No	2.08
144	*O. sativa* genomic sequence	AP001383	Yes	2.01
1056	*O. sativa* genomic sequence	AL512546	Yes	2.05
1262	*O. sativa* genomic sequence	AC026758	Yes	2.13
4296	*O. sativa* genomic sequence	AC026758	Yes	2.13
1064	*O. sativa* genomic sequence	AP003249	Yes	2.17
1168	*O. sativa* genomic sequence	AP003249	Yes	2.16
1402	*O. sativa* genomic sequence	AP003249	Yes	2.09
2178	*O. sativa* genomic sequence	AP003249	Yes	2.11
3206	*O. sativa* genomic sequence	AP003263	Yes	2.77
3377	*O. sativa* genomic sequence	AC037197	Yes	2.71
3938	*O. sativa* genomic sequence	AP003248	No	2.09
4455	*O. sativa* genomic sequence	AC137074	No	2.01
341	*A. thaliana* genomic sequence	AC013483	Yes	2.00
1080	*A. thaliana* genomic sequence	AL161571	Yes	2.11
2435	*A. thaliana* genomic sequence	AL161571	Yes	2.01
2424	*A. thaliana* genomic sequence	AL162651	Yes	2.05
2620	*A. thaliana* genomic sequence	AL0793411	Yes	3.02
4402	*A. thaliana* genomic sequence	AC011437	Yes	7.38
4516	*A. thaliana* genomic sequence	AC027656	Yes	2.16
4324	Sequence from Patent EP1002865	AX027925	No	2.22
4335	Sequence from Patent EP1002865	AX027925	No	2.18
4460	Sequence from Patent EP1002865	AX027925	No	2.19
4491	Sequence from Patent EP1002865	AX027925	No	2.09
2647	Unknown	-	No	2.06
2655	Unknown	-	No	2.16
4543	Unknown	-	No	2.01

[a]Fluorescence intensity ratios (treatment/control)

Expression analysis of selected genes

To evaluate the results obtained by microarray analysis, two BL up-regulated genes (clone 2994, unknown function; clone 4276, PR1 protein) and two GA_3 up-regulated genes (clone 1571, unknown function; clone 1646, XET) were selected for Northern analysis. Total RNAs were extracted from 1-week-old seedlings that had been treated with 1 μM BL or 5 μM GA_3 for 24 h. The results showed that levels of transcripts of all these four genes were indeed increased by BL and GA_3, respectively (Fig. 1).

Next, in order to examine their expression levels in different organs or tissues, total RNAs were extracted from callus, and from root, leaf blade and leaf sheath of 1-week-old seedlings. Figure 2 shows that these four genes displayed different expression levels and patterns. No. 2994, a gene of unknown function that is positively regulated by BL, is expressed at moderate levels in root and leaf sheath, while the signal in callus tissue was very weak and no signal was detected in the leaf blade. The PR1 transcript was found to be expressed at about the same levels in all organs and tissues examined, except in the leaf blade, where no signal at all was observed. No. 1517, a gene of unknown function that is up-regulated by GA_3, is expressed highly in callus and at a moderate level in leaf sheath, but not at all in leaf blade and root. The XET gene was expressed in all the organs and tissues examined, but at relatively higher levels in leaf sheath and root (Fig. 2).

The dose-dependency of their response to hormone was also examined for these four genes. Leaf sheath from 1-week-old seedlings was treated with 0, 0.1, 1 and 10 μM BL, or 0, 1, 5, 10 and 50 μM GA_3, respectively, for 24 h. All these four genes showed dose-dependent expression (Fig. 3). It was found that 1 μM BL is optimal for induction of gene No. 2994 and PR1 (Fig. 3A), while 5 μM GA_3 is the best concentration for the induction of gene No. 1517 and XET (Fig. 3B).

Table 5 List of the 56 clones down-regulated by GA_3, as detected by cDNA microarray analysis

Element No.	Putative ID	Best BLAST hit	Hit in full-length cDNA database	Repression ratio[a]
103	*O. sativa* glycolate oxidase (GOX)	AF022740	Yes	2.28
196	*O. sativa* heat shock protein (HSP82)	Z15018	Yes	2.18
487	*O. sativa* heat shock protein (MYB124)	AF371982	Yes	2.78
514	*O. sativa* phosphatidylinositol synthase	AJ238318	Yes	2.29
589	*O. sativa* beta 1,3 glucanase	AB027428	Yes	2.04
673	*O. sativa* oxidase (IM1)	AF085174	Yes	2.19
1277	*O. sativa* gene for PBZ1	D82066	Yes	2.16
2477	*O. sativa* 60S ribosomal protein	AF140494	Yes	2.27
1434	*O. sativa* chitinase	D55708	Yes	2.09
1581	*O. sativa* chitinase	D55708	Yes	2.23
126	*O. sativa* S-adenosyl-L-methionine:salicyclic acid carboxyl methyltransferase	D55708	Yes	2.16
440	*O. sativa* S-adenosyl-L-methionine:salicyclic acid carboxyl methyltransferase	D55708	Yes	2.40
4175	*O. sativa* S-adenosyl-L-methionine:salicyclic acid carboxyl methyltransferase	D55708	Yes	2.29
111	*O. sativa* salT protein	AF001395	Yes	2.79
303	*O. sativa* salT protein	AF001395	Yes	2.79
495	*O. sativa* salT protein	AF001395	Yes	2.40
611	*O. sativa* salT protein	AF001395	Yes	2.51
2154	*O. sativa* salT protein	AF001395	Yes	2.59
2510	*O. sativa* salT protein	AF001395	Yes	2.40
4225	*O. sativa* salT protein	AF001395	Yes	2.54
2069	*O. sativa* metallothionein-like protein	AF017366	Yes	2.00
5	*O. sativa* metallothionein-like protein	AF017396	Yes	2.33
699	*O. sativa* metallothionein-like protein	AF017396	Yes	2.03
782	*O. sativa* metallothionein-like protein	AF017396	Yes	2.33
1846	*O. sativa* metallothionein-like protein	AF017396	Yes	2.14
2993	*O. sativa* metallothionein-like protein	AF017396	Yes	2.29
3073	*O. sativa* metallothionein-like protein	AF017396	Yes	2.16
4314	*Prunus serotina* amygdalin hydrolase isoform AH I	U26025	Yes	2.41
602	*O. sativa* genomic sequence	AP004368	Yes	2.15
1261	*O. sativa* genomic sequence	AP002071	Yes	2.00
1292	*O. sativa* genomic sequence	AP002971	Yes	2.98
1711	*O. sativa* genomic sequence	AC078839	Yes	2.40
2507	*O. sativa* genomic sequence	AL713964	Yes	2.47
3546	*O. sativa* genomic sequence	AY022841	Yes	2.39
538	*O. sativa* genomic sequence	AC118675	Yes	2.78
586	*O. sativa* genomic sequence	AL731599	Yes	2.60
1721	*O. sativa* genomic sequence	AL606625	Yes	2.49
3534	*O. sativa* genomic sequence	X15901	Yes	2.27
419	*A. thaliana* genomic sequence	AL049660	Yes	2.05
887	*A. thaliana* genomic sequence	AP000381	Yes	2.10
1312	*A. thaliana* genomic sequence	AC006931	Yes	2.78
1354	*A. thaliana* genomic sequence	AC004005	Yes	3.91
1671	*A. thaliana* genomic sequence	AL132954	Yes	2.53
2204	*A. thaliana* genomic sequence	AP004127	Yes	2.21
1812	*H. vulgare* partial mRNA; clone cMWG0699	AJ234426	Yes	2.67
37	Unknown	-	No	2.00
187	Unknown	-	No	2.09
492	Unknown	-	No	2.24
683	Unknown	-	No	2.41
1393	Unknown	-	No	2.55
1823	Unknown	-	No	2.00
2535	Unknown	-	No	2.16
3322	Unknown	-	No	2.46
3365	Unknown	-	No	2.16
3765	Unknown	-	No	2.21
3935	Unknown	-	No	2.59
4133	Unknown	-	No	2.57

[a]Fluorescence intensity ratios (control/treatment)

Cross-talk between phytohormones plays an important role in regulating plant growth and development (Davies 1995). To examine if the expression of these BL or GA_3 regulated genes is also affect by other phytohormones, samples of leaf sheath from 1-week-old seedlings were treated with either 1 μM BL or 5 μM

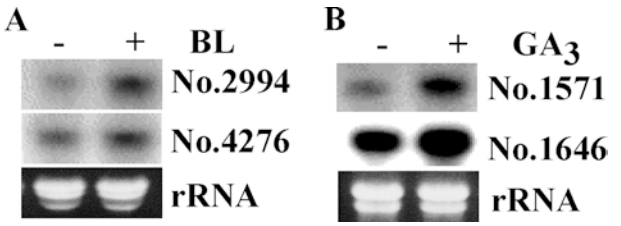
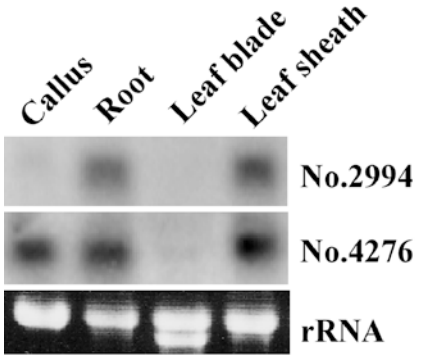


Fig. 1A, B Northern analyses of selected genes up-regulated by BL or GA_3. Total RNAs were extracted from 1-week-old seedlings treated with 1 μM BL (**A**) or 5 μM GA_3 (**B**) for 24 h, and probed with PCR-amplified full-length inserts from cDNA clones. rRNA stained with ethidium bromide was used as a loading control



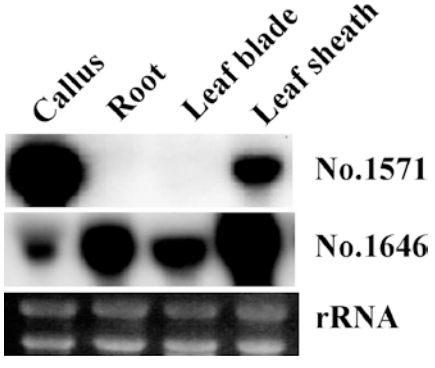


Fig. 2 Organ-specific expression of selected genes up-regulated by BL or GA_3. Total RNAs were extracted from callus or from root, leaf blade and leaf sheath of 1-week-old seedlings, and probed with PCR-amplified full-length inserts from cDNA clones. rRNA stained with ethidium bromide was used as a loading control

IAA, GA_3, or ABA for 24 h. Of the four kinds of hormones used, only BL boosted expression of the PR1 transcript, while auxin (IAA) had an inhibitory effect. The expression of gene No. 2994 was enhanced by BL and by ABA (Fig. 4). Expression of the two GA_3-responsive genes was increased specifically by GA_3, and not affected by BL or IAA, while ABA had an antagonistic effect (Fig. 4).

Finally, the induction kinetics of gene No. 2994 by BL, and XET by GA_3 was analyzed. Leaf sheath from 1-week-old seedlings was treated either with 1 μM BL or with 5 μM GA_3 for 1, 3, 6, 12 and 24 h. Figure 5 shows that induction of the two genes by BL and GA_3,

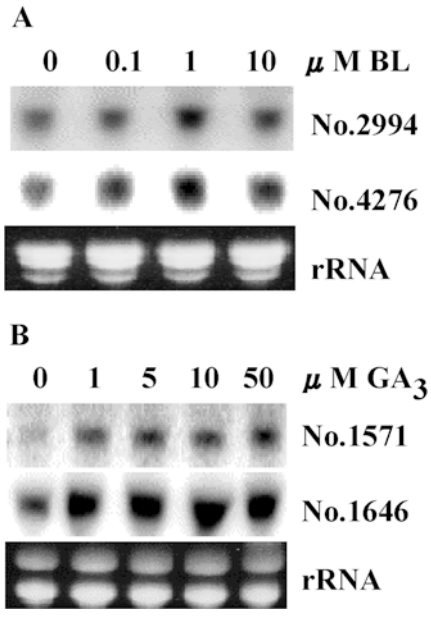


Fig. 3A, B Effect of different concentrations of BL and GA_3 on selected genes that are up-regulated by BL or GA_3 up-regulated genes expression in leaf sheath. Leaf sheath from 1-week-old seedlings was treated with 0, 0.1, 1 and 10 μM BL (**A**) or with 0, 1, 5, 10 and 50 μM GA_3 (**B**), respectively, for 24 h. Total RNAs were extracted and probed with PCR amplified full-length inserts from selected cDNA clones. rRNA stained with ethidium bromide was used as a loading control

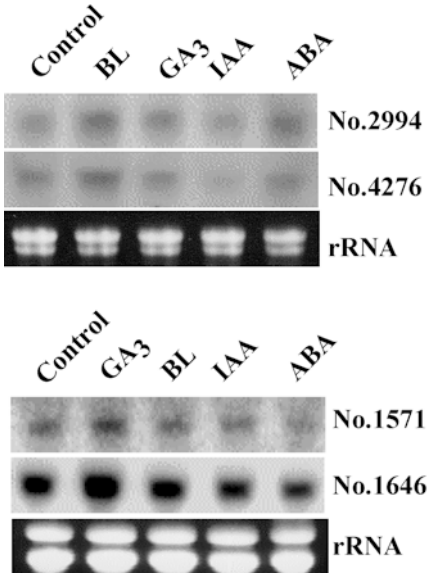


Fig. 4 Effect of different phytohormones on selected genes up-regulated by BL or GA_3 in leaf sheath. Leaf sheath tissue from 1-week-old seedlings was treated with either 1 μM BL, 5 μM GA_3, 5 μM IAA or 5 μM ABA, respectively, for 24 h. Total RNAs were extracted and probed with PCR-amplified full-length inserts from the selected cDNA clones. rRNA stained with ethidium bromide served as a loading control

respectively, occurred very fast, being detectable within 1 h after treatment with the respective hormone, and both reached their maximum levels at 6 h. The GA_3

induced expression of XET was more obvious at early times than the BL induced expression of gene No. 2994 (Fig. 5).

Discussion

The use of cDNA microarrays for monitoring gene expression provides an efficient high-throughput approach to assessing the possible functions of large numbers of genes. Recently, several groups have reported microarray analyses of BR-regulated gene expression in the dicot plant *Arabidopsis* using Affymetrix GeneChips (Goda et al. 2002; Müssig et al. 2002; Yin et al. 2002), but the results obtained by the different groups were quite different. This may be due to differences in the experimental conditions and materials they used. As far as we are aware, there is only one report on the microarray analysis of GA-regulated gene expression in the germinating *Arabidopsis* seed (Ogawa et al. 2003).

In this present study, a cDNA microarray containing 4000 clones randomly selected from a rice cDNA library was analyzed for expression differences in rice seedlings that had been treated with BL and GA_3. The results indicate that 1.1% and 2.4% of the 4000 randomly selected clones are affected by treatment with exogenous BL and GA_3, respectively. Nine and 29 unique cDNA clones were identified as being up-regulated (Tables 2 and 4), while 32 and 42 unique cDNA clones were identified as down-regulated (Tables 3 and 5) by BL and GA_3, respectively.

Fewer genes respond to BL than to GA_3. One-third of the 41 genes identified as BL responsive have potential functions in signal transduction, transcription, metabolism, cellular organization, and defense or anti-stress responses. A gene encoding a transcription factor of the Myb family was identified as being up-regulated by BL. MYB proteins constitute a diverse class of DNA binding proteins and function in regulating the cell cycle,

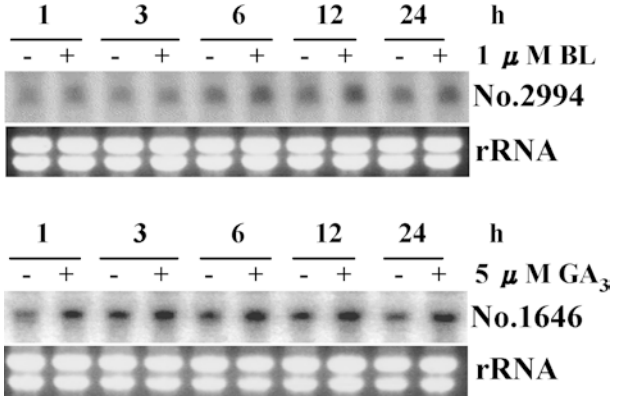


Fig. 5 Kinetics of induction of gene No. 2994 by BL, and *XET* by GA_3. Leaf sheath tissue from 1-week-old seedlings was treated with 1 μM BL (*upper panel*) or with 5 μM GA_3 (*lower panel*) for 1, 3, 6, 12 and 24 h respectively. Total RNAs were extracted and probed with PCR-amplified full-length inserts from clones 1646 and 2994. rRNA stained with ethidium bromide was used as a loading control

and cellular morphogenesis, and in controlling secondary metabolism (Jin and Martin 1999). Previous microarray analyses revealed that several *Myb* genes were up- or down-regulated by BL in *Arabidopsi* s (Goda et al. 2002; Müssig et al. 2002). That BL differentially regulates different *Myb* genes may provide important insights into the action of BRs. Application of exogenous BRs to plant tissues can enhance stress tolerance (Mandava 1988). One of the stress-related genes identified in our analysis as being responsive to BL is *PR1*. In the BR deficient *Arabidopsis* mutant *cpd*, the expression of *PR1* was reduced remarkably compared to that in the wild type, while overexpression of *CDP* cDNA resulted in a significant induction of *PR1* expression (Szekeres et al. 1996). These results are consistent and also shed light on the molecular mechanisms that underlie the physiological effect of BRs on anti-stress responses.

A total of 71 unique genes were identified as GA_3 responsive, of which 19 genes have putative functions based on BLAST homology searches, while the functions of more than two-thirds of the GA_3 responsive genes are unknown. Those genes that have putative functions fall into functional catalogs classified above, indicating that GA_3 is involved in regulating a wide range of growth and development processes. Four clones representing a single XET gene were induced by GA_3, implying a role in regulating cell elongation and cell wall organization. Leaf sheaths from 2-week-old rice seedlings treated with 5 μM GA_3 for 24 h were elongated by almost 100% (Shen et al. 2003). This suggests that the increased expression of *XET* in response to treatment with exogenous GA_3 is one of the molecular bases for this physiological effect of GA_3. *XET* s comprise a large gene family, and they are differentially regulated by GA_3 and/or BL (Uozu et al. 2000; Goda et al. 2002). The *XET* identified in our microarray was specifically affected by GA_3 but not by BL, and Northern analysis also confirmed that its expression could not be increased by BL treatment (data not shown), indicating that multiple BR and GA signaling pathways act to regulate *XET* genes.

Seven stress-related genes, including genes for HSP82, PBZ1, beta 1,3-glucanase, chitinase, SAMT, salT and MT-like proteins were found to be down-regulated by exogenous GA_3. Although relatively little is known about the relationship between GA and stress-related genes, ABA has been demonstrated to be involved in gene activation in response to various abiotic and biotic stresses. Exogenous ABA could induce the transcription of genes for salT, chitinase, HSP, and MT-like proteins (Garcia et al. 1998; Campbell et al. 2001; Dong and Dunstan 1996; Hong and Hwang 2002). Considering the antagonistic effects of GA and ABA, it is reasonable that GA_3 down-regulates some of the stress-related genes as identified in our microarray results.

Expression analyses of four genes up-regulated by BL or GA_3 confirmed our microarray results. These four selected genes showed not only hormonal dose dependent expression, but also significant differences in their

degree of expression in different organs and tissues. The *XET* gene up-regulated by GA_3 was expressed in all examined organs of seedlings and callus, but at an especially high level in the fast-elongating leaf sheath, indicating its role in regulating cell elongation and cell wall organization.

Cross talk between phytohormones plays an important role in regulating plant growth and development (Davies 1995). Thus, it has been demonstrated that many genes are regulated by more than one kind of hormone. The four genes selected for Northern analysis were specifically up-regulated by BL or GA_3, and not by other growth promoting phytohormones. Besides, ABA showed an antagonist effect on the two GA_3-regulated genes. Therefore, these two GA_3 up-regulated genes can also be used as marker genes for studying the action of GAs.

In conclusion, using a cDNA microarray we have constructed, some new BR and GA regulated genes were identified in rice seedlings. These genes display increased or decreased expression in response to treatment with BL or GA_3. However, P450 genes and some other genes involved in BR and GA biosynthesis (Goda et al. 2002; Müssig et al. 2002), which may be subject to feedback regulation, were not identified in our microarray analysis—perhaps because these genes are not included in our arrays. It should be noted that the number of unique genes in our cDNA microarray is less than 4000, given that several of the genes analyzed here are represented by multiple clones. In order to identify more BR- and GA-regulated genes in rice, the use of microarrays containing more genes, and detailed analyses on BR and GA deficient and insensitive mutants, and on the timing and tissue specificity of expression are required. Besides, further detailed analysis of the functions of newly identified genes should provide insight into the actions of BR and GA and advance our understanding of the mechanisms underlying their actions.

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