

Microarray Analysis of Brassinosteroid-Regulated Genes in Arabidopsis

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Brassinosteroids (BRs) are steroidal plant hormones that are essential for growth and development. Although insights into the functions of BRs have been provided by recent studies of biosynthesis and sensitivity mutants, the mode of action of BRs is poorly understood. With the use of DNA microarray analysis, we identified BR-regulated genes in the wild type (WT; Columbia) of Arabidopsis and in the BR-deficient mutant, *det2*. BR-regulated genes generally responded more potently in the *det2* mutant than in the WT, and they showed only limited response in a BR-insensitive mutant, *bri1*. A small group of genes showed stronger responses in the WT than in the *det2*. Exposure of plants to brassinolide and brassinazole, which is a specific inhibitor of BR biosynthesis, elicited opposite effects on gene expression of the identified genes. The list of BR-regulated genes is constituted of transcription factor genes including the phytochrome-interacting factor 3, auxin-related genes, P450 genes, and genes implicated in cell elongation and cell wall organization. The results presented here provide comprehensive view of the physiological functions of BRs using BR-regulated genes as molecular markers. The list of BR-regulated genes will be useful in the characterization of new mutants and new growth-regulating compounds that are associated with BR function.

Exogenous application of brassinosteroids (BRs) at nanomolar to micromolar concentrations to plants induces a wide range of physiological effects, including promotion of cell elongation and division, tracheary element differentiation, gravitropic tissue bending, ethylene biosynthesis, and stress resistance as well as retardation of abscission (Mandava, 1988; Clouse and Sasse, 1998; Sasse, 1999). BR-deficient or -insensitive mutants of Arabidopsis, pea (*Pisum sativum*), or tomato (*Lycopersicon esculentum*) exhibit pleiotropic phenotypes such as dwarfism, dark green leaves, reduced fertility, prolonged life span, and abnormal skotomorphogenesis (Szekeres and Koncz, 1998; Clouse and Feldmann, 1999; Müssig and Altmann, 1999; Bishop and Yokota, 2001). Recent molecular studies thus have indicated that promotion of cell expansion and regulation of photomorphogenic responses are among the most important roles of BRs. The mechanisms by which BRs exert these actions, however, are poorly understood. This study addresses both of these issues.

The expression of several genes has been shown to be regulated by BRs, especially within relatively short periods after BR treatment (Xu et al., 1995, 1996; Szekeres et al., 1996; Goetz et al., 2000; Hu et al., 2000; Müssig et al., 2000; Jiang and Clouse, 2001). To gain insight into the mechanism of BR action at the molecular level, we have now screened for genes in Arabidopsis that respond relatively rapidly to BR

exposure. We exposed a BR-deficient mutant, *det2*, or wild type (WT) to either 10 nM brassinolide (BL) or mock treatment and then used a highly reproducible oligonucleotide-based array, the Arabidopsis Genome Array (Affymetrix, Santa Clara, CA; Lockhart and Winzeler, 2000) to compare the abundance of transcripts corresponding to >8,000 genes between the two treatment groups. Our results represent the most comprehensive study to date of the BR-regulated genes.

RESULTS AND DISCUSSION

Identification of BR-Regulated Genes in the *det2* Mutant and WT

We exposed the BR-deficient mutant, *det2*, to either 10 nM BL or a mock treatment for both 15 min and 3 h and then compared the abundance of >8,000 gene transcripts between the two treatment groups using the Affymetrix Arabidopsis Genome Array. Hybridization was performed with biotin-labeled cRNA samples prepared from different plant samples in three independent experiments. The fold change (FC) values, which represent ratios of hybridization signals (the average differences [AvDf]) between mock- and BL-treated plants, were calculated using Microarray Suite software (Affymetrix). Down-regulated genes (Table I) and up-regulated genes (Table II) are listed with their FC values, which were obtained with or without signal amplification using an anti-streptavidin antibody. No genes responded in a reproducible manner to a 15-min BR treatment.

A single GeneChip experiment, for example for the 3-h exposure experiment, revealed that 1,009 of 8,300

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Table 1. Genes down-regulated by BRs in the *det2* mutant

When genes have names on publications or on database, we adopted their names. Otherwise, we designated genes from BRD1 to BRD12. Amplified and No-Amp., Before and after signal amplification with antibody, respectively.

Gene Name	Accession No.	Function or Comment	Affymetrix No.	<i>det2</i>		WT	
				Amplified	No-Amp.	Amplified	No-Amp.
				FC ± SE	FC ± SE	FC ± SE	FC ± SE
DWF4/CYP90B1	AF044216	BR biosynthesis (P450)	13870_at	-7.5 ± 0.76	-3.4 ± 0.29	-2.8 ± 0.40	-1.8 ± 0.10
CPD/CYP90A1	X87367	BR biosynthesis (P450)	16042_s_at	-2.5 ± 0.66	-3.7 ± 0.37	-4.0 ± 1.30	-4.0 ± 0.40
ROT3/CYP90C1	AB008097	Leaf polar elongation (P450)	16535_s_at	-4.5 ± 0.45	-3.8 ± 0.17	-2.1 ± 0.10	-2.3 ± 0.05
CYP71B3	D78602	Expression in older leaves (P450)	17039_at	-2.6 ± 0.20	-2.3 ± 0.49	-2.3 ± 0.80	-1.3 ± 0.25
PIF3	AF100166	PIF3	14630_s_at	-2.1 ± 0.07	-2.1 ± 0.09	-1.1 ± 0.05	-0.1 ± 1.05
NIA1/NR1	X13434	Nitrate reductase (cytokinin-regulated type)	14240_s_at	-3.0 ± 0.62	-4.0 ± 0.32	-4.2 ± 0.15	-4.6 ± 0.05
XTR7	U43489	Putative xyloglucan endotransglycosylase	15178_s_at	-3.1 ± 0.46	-3.5 ± 0.55	-1.7 ± 0.05	-1.5 ± 0.00
AAP3	X77499	Amino acid transporter (His uptake complement)	12372_at	-4.5 ± 0.59	-4.5 ± 0.99	-2.0 ± 0.10	-2.1 ± 0.05
AAP4	X77500	Amino acid transporter (His uptake complement)	16522_at	-2.3 ± 0.12	-2.4 ± 0.35	-1.7 ± 0.10	-2.0 ± 0.05
AtKUP1	AF029876	High-affinity potassium transporter	16119_s_at	-6.5 ± 0.56	-6.3 ± 0.50	-2.3 ± 0.00	-2.2 ± 0.30
AKT2	U40154	Potassium channel	16163_s_at	-4.4 ± 0.71	-2.6 ± 0.23	-2.5 ± 0.10	-1.7 ± 0.00
AtNAS1	AB021934	Nicotianamine synthase	20547_at	-3.3 ± 0.12	-3.3 ± 0.31	-2.2 ± 0.20	-2.3 ± 0.25
AtmybL2	Z68157	Leaf-specific myb-related protein	20362_at	-2.9 ± 0.42	-2.5 ± 0.33	-2.1 ± 0.70	-1.9 ± 0.55
Athb-5	X67033	Athb family Leu-zipper protein	20389_at	-2.4 ± 0.12	-2.2 ± 0.31	-2.4 ± 0.25	-2.1 ± 0.00
MS2-like protein	X99923	Fatty acyl CoA reductase homolog	19177_at	-2.9 ± 0.43	-3.3 ± 0.35	-8.7 ± 2.60	-4.6 ± 2.10
CYP78A6	AC006418/F13A10.19	P450 monooxygenase	18190_at	-7.3 ± 0.73	-2.9 ± 0.59	-3.7 ± 0.50	-1.5 ± 0.30
CYP81H1	Z99707/C7A10.50	P450 monooxygenase	20271_at	-3.5 ± 0.18	-3.1 ± 0.59	-2.6 ± 0.00	-1.5 ± 0.35
CYP710A	AC004481/F13P17.32	P450 monooxygenase	14856_at	-4.1 ± 0.20	-4.4 ± 0.50	-3.9 ± 0.05	-3.4 ± 0.60
CYP96A1	AC002391/T20D16.19	P450 monooxygenase	19281_i_at	-3.2 ± 0.12	-2.5 ± 0.65	-2.0 ± 0.40	-1.9 ± 0.55
CYP96A9	AL078620/F23D16.110	P450 monooxygenase	19730_at	-2.5 ± 0.23	-2.2 ± 0.38	-1.5 ± 0.00	-1.6 ± 0.00
PAP1/IAA26	AF088281	IAA gene family/putative phytochrome-associated protein	16078_at	-3.4 ± 0.49	-2.7 ± 0.07	-2.6 ± 0.20	-2.7 ± 0.25
iaglu	U81293	Putative indole-3-acetate β-D-glucosyltransferase	16603_s_at	-3.1 ± 0.42	-2.9 ± 0.24	-1.3 ± 0.20	-1.4 ± 0.15
PIN7	AF087820	Putative auxin efflux carrier protein	17576_at	-2.2 ± 0.06	-2.2 ± 0.17	-1.7 ± 0.00	-1.6 ± 0.00
MYB55	AF176000	MYB-like gene	17977_at	-5.0 ± 0.37	-4.5 ± 1.00	-3.7 ± 0.95	-2.4 ± 0.05
BRD1	AL049746/T23J7.130	Aldose 1-epimerase like protein	12998_at	-2.5 ± 0.20	-2.3 ± 0.38	-2.0 ± 0.00	-1.7 ± 0.10
BRD2	AC006836/F19B11.20	Putative sulfotransferase	19127_at	-2.5 ± 0.29	-2.5 ± 0.29	-2.0 ± 0.10	-2.2 ± 0.35
BRD3	AL049171/T25K17.30	Putative caffeoyl-CoA O-methyltransferase	19348_at	-2.3 ± 0.25	-2.3 ± 0.26	-1.2 ± 0.05	0.0 ± 1.15
BRD4	AL035528/F18A5.290	Leu-rich repeat protein	14110_i_at	-4.0 ± 0.35	-1.9 ± 0.38	-2.0 ± 0.10	-1.3 ± 0.10
BRD5/SAUR36 ^a	AC002387/F4L23.28	Auxin-inducible SAUR gene homolog	14448_at	-4.4 ± 0.85	-2.9 ± 0.70	-3.0 ± 0.20	-1.7 ± 0.25
BRD6	AL049659/T29H11.120	Putative DNA-binding protein (BTB domain)	19977_at	-4.1 ± 0.47	-3.8 ± 0.50	-2.1 ± 0.30	-2.3 ± 0.50
BRD7	AC002335/T01O24.18	MYB-like gene	18780_at	-4.2 ± 0.82	-2.7 ± 0.15	-2.2 ± 0.45	-1.2 ± 0.05
BRD8	AC007659/T14P1.14	GATA-type zinc finger protein	13168_i_at	-2.2 ± 0.13	-2.2 ± 0.06	-1.6 ± 0.05	-1.7 ± 0.35
BRD9	Z99708/C7A10.580	Unknown	19221_at	-3.2 ± 0.15	-3.1 ± 0.23	-2.4 ± 0.40	-2.4 ± 0.15
BRD10	AL035605/F19F18.30	Unknown	19398_at	-2.9 ± 0.18	-2.9 ± 0.15	-2.2 ± 0.05	-2.3 ± 0.10
BRD11	AC006224/MFL8.8	Unknown	20174_at	-5.2 ± 0.27	-4.2 ± 0.46	-3.6 ± 0.45	-2.4 ± 0.15
BRD12	AC003033/T21L14.19	Unknown	19889_at	-2.6 ± 0.22	-2.5 ± 0.28	-0.1 ± 1.35	-0.1 ± 1.15

^a Gene name for SAUR36 is from Hagen and Guilfoyle (2002).

Table II. Genes up-regulated by BRs

When genes have names on publications or on database, we adopted their names. Otherwise, we designated genes from BRU2 to BRU43. Ampified and No-Amp., Before and after signal amplification with antibody, respectively.

Gene Name	Accession No.	Function or Comment	Affymetrix No.	<i>det2</i>		WT	
				Amplified	No-Amp.	Amplified	No-Amp.
				FC \pm SE	FC \pm SE	FC \pm SE	FC \pm SE
IAA3/SHY2	U18406	Auxin-inducible nuclear protein/suppressor of phyB	13301_at	2.4 \pm 0.42	2.7 \pm 0.29	1.3 \pm 0.10	1.3 \pm 0.10
IAA5	U18407	Auxin-inducible nuclear protein	13660_i_at	16.8 \pm 4.64	7.8 \pm 1.02	3.3 \pm 0.35	1.8 \pm 0.00
SAUR-AC1/-15	S70188	Small auxin up RNA from Arabidopsis Columbia	12608_i_at	9.9 \pm 0.55	9.6 \pm 1.22	3.3 \pm 0.00	3.7 \pm 0.25
IAA19	U49075	Member of IAA gene family/IAA1 binding protein	13296_at	3.1 \pm 0.25	2.9 \pm 0.15	1.8 \pm 0.00	2.1 \pm 0.20
BAS1/CYP72B1	AC003105/F18A8.8	Activation-tagged suppressor of phyB	12543_at	3.8 \pm 0.27	4.2 \pm 1.16	2.7 \pm 0.05	3.1 \pm 0.00
TCH2	AF026473	Touch-induced gene 2/calmodulin-related protein	18300_at	2.5 \pm 0.19	2.7 \pm 0.24	1.9 \pm 0.15	1.8 \pm 0.05
TCH4	AF051338	Touch-induced gene 4/xyloglucan endotransglycosylase	16620_s_at	8.8 \pm 0.81	16.8 \pm 4.62	6.5 \pm 0.35	8.2 \pm 0.75
XTR6	U43488	Xyloglucan endotransglycosylase-related protein 6	17533_s_at	9.0 \pm 1.10	5.7 \pm 0.96	5.5 \pm 0.45	1.4 \pm 2.50
MSS3	AC004450/F14B2.23	Suppressor of snf4/calmodulin-like protein (EF-hand)	20689_at	3.2 \pm 0.35	3.8 \pm 0.58	1.6 \pm 0.15	1.8 \pm 0.25
ZAT7	X98676	Zinc finger protein	15778_at	4.2 \pm 0.47	2.0 \pm 0.15	1.4 \pm 0.15	0.1 \pm 1.20
ATPA2	X99952	Root-specific peroxidase	12356_at	4.0 \pm 2.09	3.0 \pm 0.43	1.4 \pm 0.20	1.7 \pm 0.40
KCS1	AF053345	Fatty acid elongase 3-ketoacyl-CoA synthase 1	17961_at	2.7 \pm 0.28	3.0 \pm 0.29	1.3 \pm 0.05	1.3 \pm 0.10
OPR1	U92460	12-Oxophytodienoate reductase (jasmonate biosynthesis)	18253_s_at	2.0 \pm 0.03	2.1 \pm 0.03	1.4 \pm 0.10	1.4 \pm 0.10
AtPMEpcrB	AF033206	Pectin methylesterase homolog	19267_s_at	4.1 \pm 0.32	4.4 \pm 0.46	3.3 \pm 0.15	3.5 \pm 0.10
ATP24a	Y11788	Peroxidase	18946_at	2.3 \pm 0.15	2.4 \pm 0.30	0.0 \pm 1.05	1.1 \pm 0.05
CYP94C1	AC005824/F15K20.21	P450 monooxygenase	19288_at	10.1 \pm 3.32	4.0 \pm 0.97	2.0 \pm 0.20	1.7 \pm 0.25
CYP86A2	AF013293/A_IG005110.21	P450 monooxygenase	17966_at	2.0 \pm 0.09	2.2 \pm 0.15	1.3 \pm 0.00	1.3 \pm 0.00
BRU2/SAUR-25	AL035528/F18A5.180	Auxin-inducible SAUR gene family	13395_at	5.3 \pm 0.63	2.6 \pm 0.38	1.6 \pm 0.05	1.6 \pm 0.50
BRU3/SAUR-9	AL022373/T19K4.240	Auxin-inducible SAUR gene family	12947_at	9.0 \pm 0.21	9.2 \pm 0.65	4.9 \pm 0.35	4.9 \pm 0.20
BRU4/SAUR16	AL035656/T9A14.140	Auxin-inducible SAUR gene family	13322_at	5.8 \pm 0.68	6.5 \pm 1.00	2.2 \pm 0.05	2.5 \pm 0.30
BRU5/SAUR-10	AC006201/T27K22.12	Auxin-inducible SAUR gene family	13781_at	3.0 \pm 0.66	3.0 \pm 0.06	1.6 \pm 0.15	1.7 \pm 0.20
BRU6/GH3-2 ^a	AL035601/F6G17.40	Auxin-inducible GH3 gene family	13565_at	2.3 \pm 0.17	2.2 \pm 0.15	1.3 \pm 0.05	1.8 \pm 0.35
BRU7/GH3-10	AC005275/F4C21.36	Auxin-inducible GH3 gene family	13812_at	2.2 \pm 0.21	2.3 \pm 0.15	1.7 \pm 0.20	1.8 \pm 0.40
BRU8	AC004512/T8F5.9	Putative xyloglucan endotransglycosylase (TCH4 homolog)	17960_at	7.7 \pm 0.48	8.0 \pm 0.43	2.1 \pm 0.35	2.0 \pm 0.30
BRU9	AC007067/T10O24.17	Putative endoxyloglucan transferase (EXGT-A3 homolog)	19490_at	22.0 \pm 9.17	10.9 \pm 2.54	5.2 \pm 0.10	3.7 \pm 0.00
AtExp8	AC002336/T2P4.4	Putative expansin (expansin2-homolog)	19660_at	3.3 \pm 0.57	3.7 \pm 0.57	1.3 \pm 0.05	1.3 \pm 0.05
BRU11	AL035539/F22I13.170	Putative expansin (pollen allergen protein homolog)	19976_at	6.2 \pm 0.42	7.2 \pm 0.92	2.0 \pm 0.00	2.1 \pm 0.10
BRU12	AC005169/F6F22.19	Putative extensin with Leu-rich repeat (LRX1 homolog)	18525_at	3.3 \pm 0.09	2.4 \pm 0.45	1.3 \pm 0.20	-0.1 \pm 1.10
BRU13	AC005698/T3P18.1	Putative extensin with Leu-rich repeat (LRX1 homolog)	18998_at	2.2 \pm 0.15	2.3 \pm 0.29	2.0 \pm 0.40	2.0 \pm 0.50
BRU14	AL049608/T9E8.80	Putative extensin with Leu-rich repeat (LRX1 homolog)	20537_at	2.8 \pm 0.15	3.1 \pm 0.15	1.6 \pm 0.00	1.8 \pm 0.00
BRU15	AC003027/F21M11.20	Putative arabinogalactan protein (fasciclin-domain)	16438_at	0.6 \pm 0.80	2.2 \pm 0.10	1.6 \pm 0.10	1.7 \pm 0.10
BRU16	AL035601/F6GT17.100	Putative arabinogalactan protein	14947_at	1.5 \pm 0.28	2.5 \pm 0.18	1.8 \pm 0.00	1.8 \pm 0.00
BRU17	AF060874	Putative arabinogalactan protein (AtAGP4 homolog)	15107_s_at	1.7 \pm 0.37	2.4 \pm 0.24	1.5 \pm 0.00	1.5 \pm 0.00
BRU18	AF069298/T14P8.14	Putative pectinesterase	14612_at	4.0 \pm 0.18	4.2 \pm 0.26	3.3 \pm 0.40	3.6 \pm 0.55

(Table continues on following page.)

Table II. (Continued from previous page.)

Gene Name	Accession No.	Function or Comment	Affymetrix No.	<i>det2</i>		WT	
				Amplified	No-Amp.	Amplified	No-Amp.
				FC ± SE	FC ± SE	FC ± SE	FC ± SE
BRU19	AL022580/T5K18.200	Putative pectinacetyltransferase	19905_at	3.0 ± 0.28	3.6 ± 0.73	2.5 ± 0.45	3.3 ± 0.95
BRU20	AC004077/T31E10.15	Endo-1,4-β-xylanase homolog	15271_at	2.7 ± 0.17	2.9 ± 0.12	1.5 ± 0.05	1.6 ± 0.10
BRU21	AL030978/M4122.170	Ca ²⁺ -binding protein homolog	15431_at	3.1 ± 0.25	3.6 ± 0.49	2.3 ± 0.10	2.2 ± 0.00
BRU22	AL022198/F6118.90	Putative calmodulin-binding protein	19857_at	2.7 ± 0.20	2.7 ± 0.17	1.7 ± 0.10	2.0 ± 0.20
BRU23	AC005970/T6P5.14	Putative protein kinase	14030_at	2.2 ± 0.10	2.4 ± 0.33	1.5 ± 0.15	1.7 ± 0.15
BRU24	AC002339/T11A7.8	Putative receptor kinase (CLV1 homolog)	14112_at	3.4 ± 0.90	2.4 ± 0.32	1.7 ± 0.25	1.8 ± 0.10
BRU25	AC004238/F19I3.16	Leu-rich repeat domain	12251_at	4.5 ± 0.67	4.7 ± 0.72	3.5 ± 0.85	2.6 ± 0.25
BRU26	AC002354/F6P23.7	NAM/NAC gene homolog	13806_at	2.9 ± 0.44	3.0 ± 0.70	1.8 ± 0.30	1.6 ± 0.05
BRU27	AC006533/F20M17.23	Ethylene-Regulated protein ER33 homolog	15403_s_at	3.7 ± 0.25	3.8 ± 0.29	1.9 ± 0.15	2.1 ± 0.20
BRU28	AF128396/T3H13.3	Phosphate-induced gene phl-1 homolog	14077_at	3.2 ± 0.35	5.8 ± 0.29	3.6 ± 0.30	3.5 ± 0.30
BRU29	AL021711/F13C5.140	Pro-rich protein (APG) homolog (GDSL-motif)	16434_at	2.8 ± 0.15	3.0 ± 0.03	2.4 ± 0.40	2.4 ± 0.35
BRU30	AL035353/F16A16.110	Pro-rich protein (APG) homolog (GDSL-motif)	17196_at	3.9 ± 0.38	4.7 ± 0.48	1.9 ± 0.20	2.3 ± 0.25
BRU31	AC005917/F3P11.22	Pollen-specific protein (SF21) homolog	15892_at	2.4 ± 0.18	2.7 ± 0.32	1.8 ± 0.15	1.8 ± 0.25
BRU32	AC005679/F9K20.9	Carrot (<i>Daucus carota</i>) EP1 homolog	17440_i_at	2.7 ± 0.32	3.2 ± 0.55	1.6 ± 0.20	1.6 ± 0.25
BRU33	AC007060/T5I8.14	Phosphatidylinositol/phosphatidylcholine transfer protein homolog	12046_at	3.6 ± 0.34	3.8 ± 0.25	1.9 ± 0.10	1.9 ± 0.15
BRU34	AP007269/A_IG002N01.14	Unknown	12953_at	3.1 ± 0.35	3.3 ± 0.49	1.6 ± 0.05	1.8 ± 0.05
BRU35	AC005169/F6F22.17	Unknown	13916_at	2.2 ± 0.12	3.6 ± 0.20	3.6 ± 0.20	3.8 ± 0.45
BRU36	AC005106/T25N20.6	Unknown	14913_at	3.7 ± 0.34	3.4 ± 0.47	0.1 ± 1.05	0.0 ± 1.10
BRU37	AL049481/T5L19.20	Unknown	14951_at	2.6 ± 0.26	2.8 ± 0.32	2.0 ± 0.10	2.2 ± 0.20
BRU38	AC002535/T30B22.26	Unknown	19880_at	2.6 ± 0.15	2.9 ± 0.25	1.6 ± 0.20	1.6 ± 0.10
BRU39	AC007138/T7B11.21	Unknown	19992_at	3.0 ± 0.21	3.4 ± 0.32	1.6 ± 0.10	1.6 ± 0.05
BRU40	AL022224/F1C12.90	Unknown	12027_at	2.8 ± 0.32	2.5 ± 0.54	1.2 ± 0.05	0.0 ± 1.05
BRU41	AC000348/T7N9.22	Unknown	14443_at	2.1 ± 0.17	2.1 ± 0.12	1.8 ± 0.20	2.0 ± 0.25
BRU42	AC006921/F2H17.17	Unknown	18885_at	2.0 ± 0.12	2.1 ± 0.12	2.0 ± 0.00	2.2 ± 0.10
BRU43	AC004521/F411.31	Unknown	12584_at	2.1 ± 0.15	2.8 ± 0.57	1.4 ± 0.10	1.5 ± 0.45

^a Gene names for SAUR and GH3 genes are from Hagen and Guilfoyle (2002).

genes (12%) were up- or down-regulated by BL, as defined by a more than 2-fold difference in the FC values (before or after signal amplification). We used absolute call and difference call values calculated by the Microarray Suite software to exclude false-positive signals that resulted from cross-hybridization or noise. Taking into account these two parameters, 655 of 1,009 genes (65%) were excluded from the list for this experiment. Genes that were reproducibly regulated by BL in three independent experiments were compiled into a final list of 59 up-regulated genes and 36 genes down-regulated genes (Tables I and II). On the other hand, we also processed raw signal data (AvDf) from triplicate GeneChip analysis using Welch's approximate *t* test. This analysis identified 155 BL-regulated genes (as defined by more than 2-fold difference in the mean AvDf values) at a significance level of $P < 0.05$. Given that 84 of these 155 genes were included in the list of 95 BL-regulated genes that were identified in our initial analysis, we consider that our threshold is reliable and more stringent than the conventional approach based on Welch's approximate *t* test.

We also exposed a WT plant to either 10 nM BL or a mock treatment for 3 h and then compared the abundance of transcripts between the two treatment groups using the Affymetrix GeneChips. Fifty-one genes were identified as the BR-regulated genes in the WT. Fifteen of these identified as novel BR-regulated loci, because they did not appear in the BR-regulated genes in Table I or II. Interestingly, all of these genes were down-regulated by BL, and these genes generally showed milder responses to BL in the *det2* mutant background than in the WT background (Table III). The signal log ratio values, which represent ratios of hybridization signals (AvDf values) between mock- and BL-treated plants on a log scale (base 2), were calculated using Microarray Suite software. A signal log ratio of 1, for example, indicates a 2-fold increase in the transcript level and -1 indicates a 2-fold decrease. The signal log ratio values from the WT experiments were plotted against those from the *det2* mutant experiments for all of the genes listed in Tables I to III (Fig. 1A). In general, most of the genes responded to BL similarly in the

Table III. Genes down-regulated by BRs in WT

When genes have names on publications or on database, we adopted their names. Otherwise, we designed genes from BRD13 to BRD21. Amplified and No-Amp., before and after signal amplification with antibody, respectively.

Gene Name	Accession No.	Function or Comment	Affymetrix No.	<i>det2</i>		WT	
				Amplified	No-Amp.	Amplified	No-Amp.
				FC \pm SE	FC \pm SE	FC \pm SE	FC \pm SE
CYP79B2	AF069495	Cytochrome P450	20479_i_at	-0.4 \pm 0.70	-0.4 \pm 0.68	-1.6 \pm 0.35	-2.2 \pm 0.05
CYP81D8	AL035601/F6G17.20	Cytochrome P450	14032_at	-2.0 \pm 0.50	-0.7 \pm 1.07	-3.7 \pm 0.20	-1.9 \pm 0.15
4HPPD	AF047834	4-Hydroxyphenylpyruvate dioxygenase	15669_s_at	-2.4 \pm 0.30	-2.3 \pm 0.30	-6.9 \pm 1.60	-3.0 \pm 0.30
CHI	M86358	Chalcone isomerase	20413_at	-1.4 \pm 0.15	-1.3 \pm 0.12	-2.2 \pm 0.05	-2.3 \pm 0.45
BRI1	AF017056	BRI1	20209_at	-1.8 \pm 0.06	-1.9 \pm 0.13	-2.0 \pm 0.05	-2.0 \pm 0.20
Eli3-1	X67816	Cinnamyl-alcohol dehydrogenase	13242_at	-1.9 \pm 0.25	-1.9 \pm 0.15	-1.7 \pm 0.00	-2.1 \pm 0.05
BRD13	AL021713/T9A21.60	Putative protein	13514_s_at	-1.9 \pm 0.74	-1.8 \pm 0.03	-4.0 \pm 0.50	-2.0 \pm 0.35
BRD14	AC006085/F11M15.3	Putative Ser/Thr protein kinase	16878_at	-3.3 \pm 0.56	-1.8 \pm 0.74	-4.0 \pm 0.10	-1.8 \pm 0.00
BRD15	AC012562/F17O14.23	Osmotic stress-inducible kinase like-protein	13246_at	-1.8 \pm 0.39	-0.6 \pm 0.83	-3.8 \pm 1.10	-1.9 \pm 0.70
BRD16	AL049480/F14M19.60	Putative pathogenesis-related protein	18755_at	-2.1 \pm 0.38	-2.0 \pm 0.68	-2.6 \pm 0.50	-2.0 \pm 0.25
BRD17	AL033545/F7K2.50	Extensin-like protein	12115_at	-2.2 \pm 0.19	-1.8 \pm 0.10	-2.4 \pm 0.25	-2.3 \pm 0.05
BRD18	AC005662/F13H10.7	Putative embryo-abundant protein	14083_at	-2.0 \pm 0.15	-1.8 \pm 0.27	-2.4 \pm 0.15	-2.2 \pm 0.15
BRD19	Y12776	Late embryogenesis-abundant protein like-protein	18594_at	-1.5 \pm 0.09	-1.6 \pm 0.09	-2.1 \pm 0.05	-2.0 \pm 0.00
BRD20	AL021811/F10M6.70	Putative protein	14574_at	-2.8 \pm 0.38	-2.4 \pm 0.52	-1.8 \pm 0.15	-2.2 \pm 0.15
BRD21	AC007661/T8P21.9	Putative protein	15487_at	-1.6 \pm 0.12	-1.6 \pm 0.29	-1.8 \pm 0.55	-2.1 \pm 0.05

WT and *det2* mutant, although some genes responded poorly in the WT. The correlation coefficient for the WT and *det2* experiments was 0.908, which indicates a strong positive correlation between the WT and *det2* experiments. The inclination of the regression line in Figure 1A is 0.59. We therefore conclude that BR-regulated genes generally respond to BL in a similar fashion in the WT and *det2* but that the *det2* response to BL is more sensitive than that of the WT. We speculate that the *det2* mutant has a stronger response to exogenous BL than WT, because the *det2* mutant accumulates lower levels of endogenous BRs (Fujioka et al., 1997).

Analysis of BR Response in the *bri1* Mutant

It has been shown that BRI1 is a critical component of the BR receptor (Wang et al., 2001). We have examined the effect of BR on a *bri1-5* mutant, a weak mutant allele of *BR-insensitive 1* (Noguchi et al., 1999). The *bri1-5* seedlings were exposed to either 10 nM BL or a mock treatment for 3 h, and transcript abundance was analyzed using the Affymetrix GeneChip. Only three genes were identified to respond to BR in *bri1-5* mutant, namely *DWF4/CYP90B1*, *AtKUP1*, and *MS2-like* genes. These genes are among the genes with the strongest response in the *det2* and WT plants (Table I). All three genes showed weaker responses in the *bri1* than in *det2* background (data not shown). The signal log ratio values of the *bri1* experiments were plotted against those of the *det2* experiments for all the genes listed in Tables I to III

(Fig. 1B). In the *bri1* mutant, most of the genes responded to BL in a similar fashion to those in the *det2* mutant. The correlation coefficient for the *bri1* and *det2* experiments is 0.697, indicating a positive correlation between the *bri1* and *det2* experiments. The inclination of the regression line in Figure 1B is 0.19. These results indicate that BR-regulated genes generally respond to BL similarly in the *bri1* and *det2*, but the responses in *bri1* are weaker than those in *det2* for all BR-regulated genes. This observation provides good evidence that the BL responses of the genes listed in Tables I to III are dependent on the *BRI1* gene. However, this does not necessarily mean that the listed genes are all regulated via a *BRI1*-dependent-signaling pathway. It may be the case that the *bri1* mutant is less sensitive to exogenously applied BRs, because the *bri1* mutant accumulates enormous amounts of endogenous BRs, i.e. BL, castasterone, and typhasterol (Noguchi et al., 1999). Hu et al. (2000) suggested the existence of a *BRI1*-independent-signaling pathway. Moreover, there are several *bri1* homologs in the Arabidopsis genome. However, the existence of a *BRI1*-independent-signaling pathway has yet to be verified.

Evaluation of Effects of Brassinazole (Brz), a BR-Specific Biosynthesis Inhibitor

Brz, a synthetic chemical developed in our laboratory, is a triazole-type BR biosynthesis inhibitor that induces dwarfism (Asami and Yoshida, 1999). We analyzed BR-regulated genes to evaluate the effects

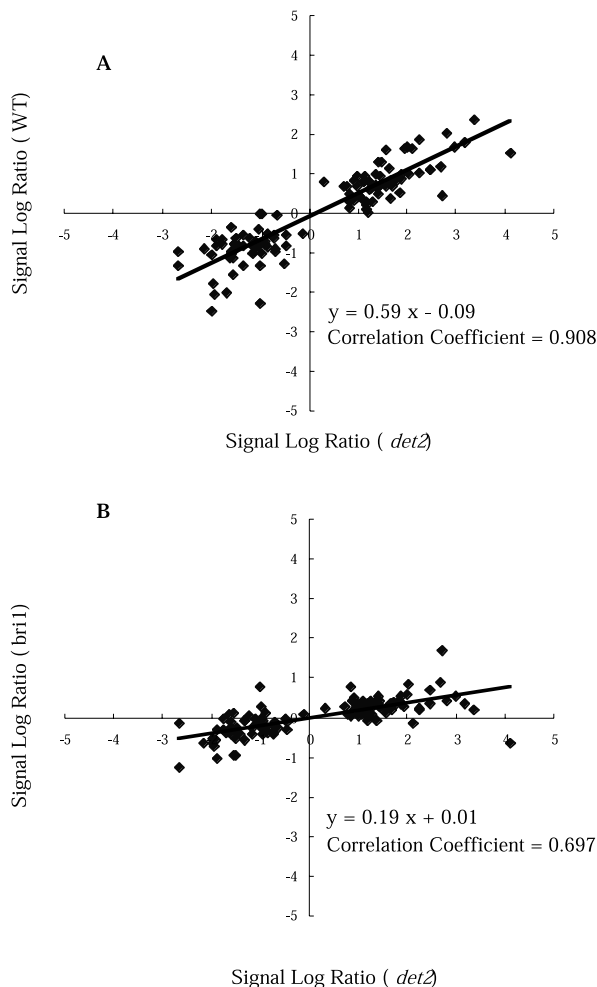


Figure 1. Comparison of BL treatment on the WT, *bri1*, and *det2* seedlings with the use of GeneChip. A, The distribution of the signal log ratio values for WT (y axis) and *det2* (x axis) are shown for the BR-regulated genes, listed in Tables I to III. B, The distribution of signal log ratio values for *bri1* (y axis) and *det2* (x axis) are shown for the BR-regulated genes, listed in Tables I to III. The signal log ratio represents the ratio of hybridization signals between BL- and mock-treated samples using a log (base 2) scale. A signal log ratio of 1 represents a gene that shows a 2-fold increase in expression by BL treatment; a signal log ratio of -1 represents a gene that shows a 2-fold reduction by BL treatment.

of newly developed Brz, Brz220 (Sekimata et al., 2002), which has the strongest and the most specific effects on Arabidopsis among the Brz inhibitors (our unpublished data). WT seedlings were exposed to either 3×10^{-6} M Brz or a mock treatment for 3 h, and the abundance of transcripts was compared using GeneChip. The signal log ratio values from the Brz experiments were plotted against the BL experiments for all genes (Fig. 2A) or for BR-regulated genes listed in Tables I and II (Fig. 2D). The correlation coefficient for the Brz and BL experiments was -0.004 for all genes ($n > 8,000$), indicating that there was no significant correlation between the BL and Brz treatments. On the other hand, a correlation coefficient of

-0.793 was calculated for the BR-regulated genes, suggesting a strong inverse correlation between the BL and Brz treatments. These observations indicate that our list of BR-regulated genes presented here is useful to evaluate new mutants as well as new growth-regulating compounds that are related to BR function. Our observations contrarily suggest that a short (3 h) and moderate concentration (3×10^{-6} M) of Brz treatment decreased the levels of endogenous active BRs within 3 h. This observation, together with our previous observation that Brz selectively interacts with a putative BR 22-hydroxylase (DWF4; Asami et al., 2001), also suggests rapid depletion of the BR-precursor pool, i.e. the BR precursors are metabolized to BL and/or degraded to the inactive form during the 3-h treatment of Brz.

Modulation of the PIF3 (Phytochrome-Interacting Factor 3) Gene

Given that they are fixed in space, plants have evolved highly flexible programs to adapt to the changing environment. Light is one of the most important environmental signals regulating the programs of growth and development. Although interactions between light and hormonal signals have been studied extensively (von Arnim and Deng, 1996; Fankhauser and Chory, 1997; Kraepiel and Miginiac, 1997), the molecular mechanisms that underlie connectivity between the respective signaling pathways remain unclear. In addition, plant hormones have been widely regarded as signaling molecules that act downstream of light signal. BR-deficient or -insensitive mutants and plants treated with Brz, which is an inhibitor of BR biosynthesis, exhibit abnormal skotomorphogenesis or dark green leaves when grown in light (Fankhauser and Chory, 1997; Szekeres and Koncz, 1998; Asami and Yoshida, 1999; Clouse and Feldmann, 1999; Müssig and Altmann, 1999; Nagata et al., 2000). These observations have led to the proposal that light might alter either the concentration of BRs or the responsiveness of cells to these steroids (Fankhauser and Chory, 1997; Kraepiel and Miginiac, 1997).

Our microarray analysis has now demonstrated that BL down-regulates the gene for PIF3 (Table I), a well-characterized transcription factor that functions at the upstream end of the light-signaling pathway (Ni et al., 1998). PIF3 is a basic helix-loop-helix protein that is localized to the nucleus and both interacts with the biologically active-type photoreceptors, Pfrs (Ni et al., 1999; Fairchild et al., 2000; Zhu et al., 2000), and binds to light-regulated promoters through the G-box sequence motif (CACGTG; Friedrichsen et al., 2000; Martinez-Garcia et al., 2000). Quantitative reverse transcription (RT)-PCR analysis revealed that repression of *PIF3* expression was apparent within 15 min after BL treatment, and the expression was reduced by two-thirds within 2 to 3 h (Fig. 3A). Dose-response analysis revealed that inhibition of *PIF3*

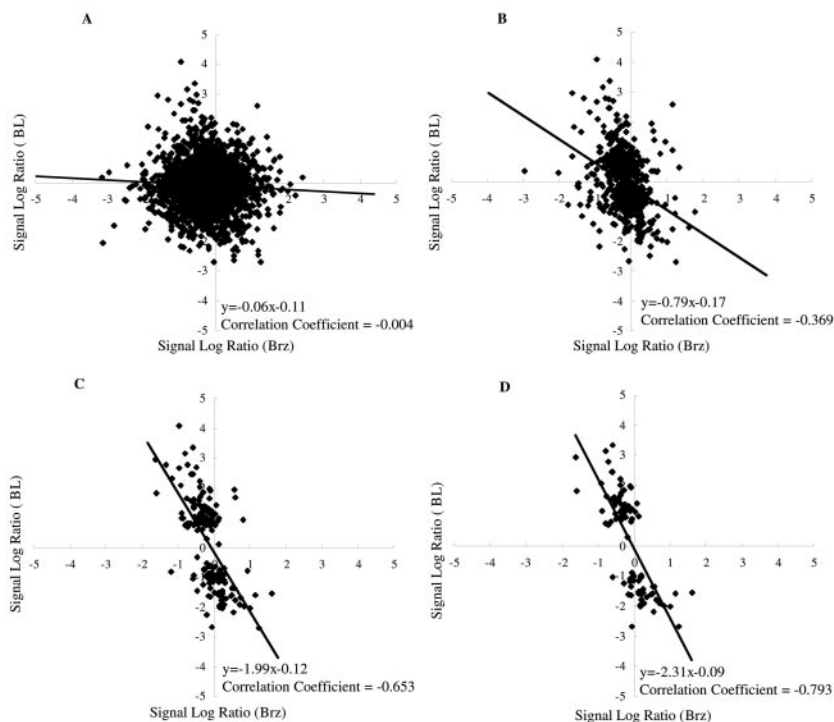


Figure 2. Comparison of BL and Brz treatment with the use of GeneChip experiment. The distribution of signal log ratio values for treatments with BL (y axis) and Brz (x axis) are shown. A, All of the genes (>8,000) on the GeneChip are plotted. B, Genes that are induced or reduced more than 2-fold in a single GeneChip experiment are plotted. C, Genes that are induced or reduced more than 2-fold in two of the three GeneChip experiments are plotted. D, The BR-regulated genes (induced or reduced more than 2-fold in three GeneChip experiments, listed in Tables I and II) are plotted. The signal log ratio represents the ratios of hybridization signals using a log (base 2) scale. A signal log ratio of 1 represents a gene whose expression is increased 2-fold by treatment with either BL or Brz, and a signal log ratio of -1 represents a gene whose expression is reduced 2-fold by treatment with either BL or Brz.

expression by BL was apparent at 0.1 nM BL and was maximal at 100 nM BL (data not shown), indicating that BL regulates the expression of this gene at physiological concentrations.

On the basis of these observations, we examined the effects of BL on the abundance of transcripts derived from *Lhcb1.3* (Jansson, 1999) and *rbcS-1A* (Krebbers et al., 1998), which encode light-harvesting chlorophyll *a/b*-binding protein (Lhc) and the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*), respectively. The promoter regions of both *Lhcb1.3* and *rbcS-1A* contain G-box sequence motifs, which bind to PIF3 protein. The expression of each of these genes was repressed by BL treatment after a lag period of approximately 1 h; transcript levels were reduced by about two-thirds after 24 h (Fig. 3, B and C), at which time the chlorophyll content of BL-treated plants was about one-half that of mock-treated plants, without a significant change in the chlorophyll *a/b* ratio (Table IV). The relative temporal patterns of the expression profiles of *PIF3* and *Lhcb1.3* coincided with those of regulatory genes and their putative output genes in former microarray studies (e.g. Seki et al., 2001; Tepperman et al., 2001). Because light-induced expression of *Lhcb1.3* (*CAB3*) was reduced in plants expressing *PIF3* antisense RNA (Ni et al., 1998), our results suggest that the BL-induced repression of *Lhcb1.3* expression is mediated, at least in part, by the effect of this BR on *PIF3* expression. Our data also suggest that BRs modulate the light-regulated plant development by affecting *PIF3* expression. In other words, BRs may act as regulators of the light-signaling pathway, in addition

to or rather than functioning as downstream mediators of light signal transduction. On the other hand, it has been controversial whether BR-induced photomorphogenic responses, such as de-etiolation and dark green leaves, are secondary effects of the retarded cell elongation in BR-deficient plants (Bishop and Yokota, 2001). It is noteworthy that repression of *PIF3* gene is apparent in 15 min of BR treatment. The speed of this response is similar to that of BR-biosynthetic genes and quicker than that of genes implicated in cell elongation or cell wall organization (discussed below).

To determine whether the down-regulation of *PIF3* by BL is dependent on de novo protein synthesis, we examined the effect of CHX, an inhibitor of cytosolic protein synthesis. The amount of *PIF3* mRNA was unexpectedly moderately increased by treatment of plants with 100 μ M CHX for 1 h (Fig. 3D). Furthermore, in plants exposed to CHX, *PIF3* expression did not respond to subsequent treatment with BL. These results suggest that CHX might prevent the de novo synthesis of a short-lived repressor of *PIF3* expression that acts at the transcriptional or posttranscriptional level. The results further suggest that BL might function upstream of this putative repressor. Recent studies have indicated the importance of short-lived repressors, whose degradation is mediated by the ubiquitin system (Estelle, 2001; Schwechheimer and Deng, 2001). Early auxin-inducible genes have been suggested to be regulated by the ubiquitin-mediated system (Gray and Estelle, 2000; Leyser, 2001; Ramos et al., 2001). The possible involvement of such regu-

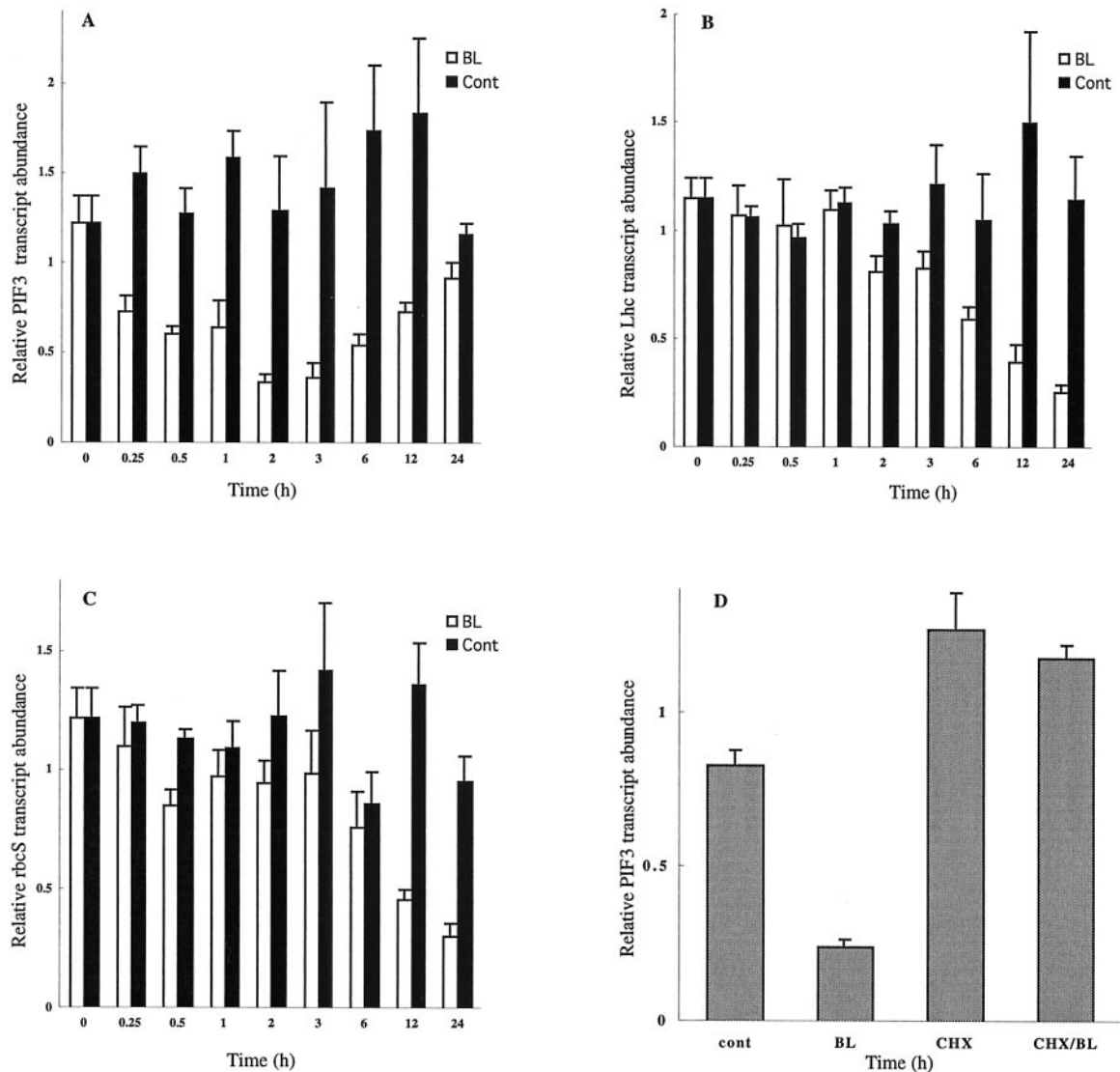


Figure 3. Regulation of *PIF3*, *Lhcb1.3*, and *rbcS-1A* gene expression by BL. Time courses of the inhibition of *PIF3* (A), *Lhcb1.3* (B), and *rbcS-1A* (C) gene expression by BL. Light-grown *det2* seedlings were treated with 10 nM BL or were mock treated (cont) for the indicated times, and transcript abundance was analyzed by quantitative Taq-Man RT-PCR. D, Effects of cycloheximide (CHX) on *PIF3* expression. Seedlings were treated with 100 μ M CHX or mock treatment for 1 h, and then for an additional 3 h with 10 nM BL or mock treatment in the continued presence of CHX. The amount of *PIF3* mRNA was determined by quantitative Taq-Man RT-PCR analysis. Transcript abundance levels are presented as relative values that are normalized with respect to the levels of 18S ribosomal RNA. Data are means \pm SE from three different plant samples.

latory system in BR-signaling pathway is presented in the following section.

Modulation of Early Auxin-Inducible Genes

Some effects of BRs have been thought to be related to auxin action (Mandava, 1988; Sasse, 1999). For example, the application of BRs together with auxins in the rice lamina inclination test or hypocotyl elongation test results in synergistic responses (Mandava, 1988; Sasse, 1999). However, although numerous physiological studies have addressed the interactions between BRs and auxins, little is known about the

underlying molecular mechanisms. Clouse and colleagues performed comparisons of the physiological effects of BRs and auxins, as well as molecular analyses of auxin-inducible genes and auxin-insensitive

Table IV. Chlorophyll contents after BL treatment

Data are means \pm SE. Chl, Chlorophyll.			
	Chl a	Chl b	Chl a/b
<i>mg g⁻¹ fresh wt.</i>			
BL	0.26 \pm 0.04	0.08 \pm 0.01	3.22 \pm 0.04
Cont	0.50 \pm 0.03	0.16 \pm 0.01	3.19 \pm 0.06

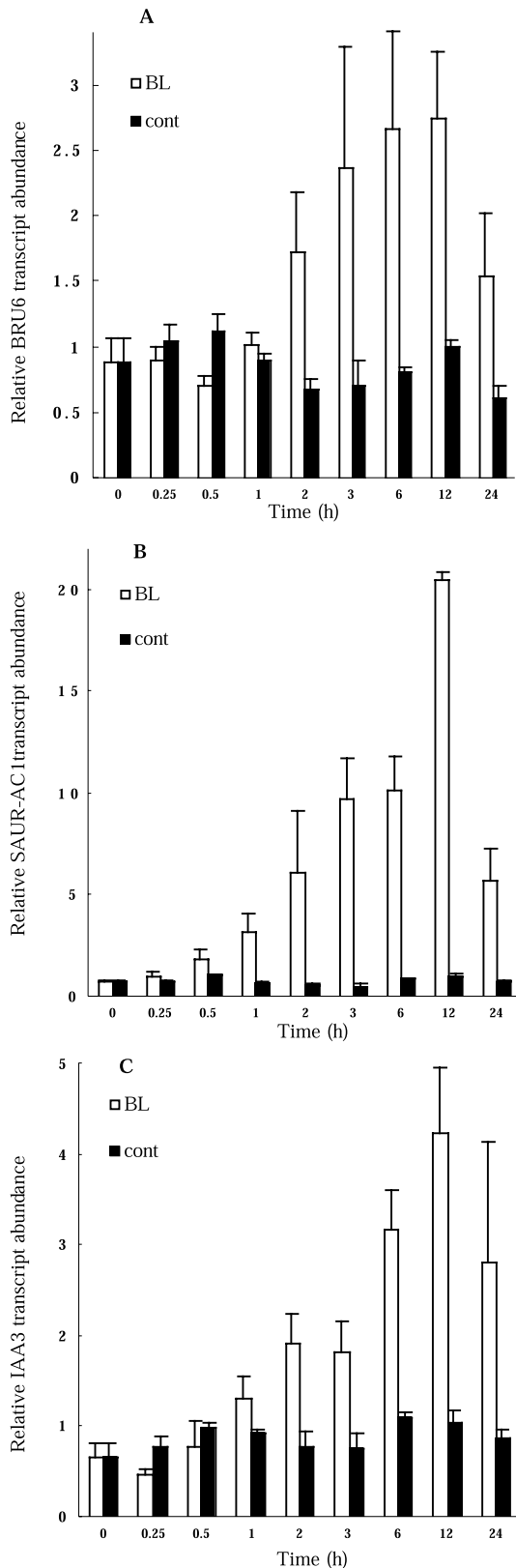


Figure 4. Induction of early auxin-inducible genes by BL. Kinetics of the induction of a *GH3* homolog, *BRU6* (A), *SAUR-AC1* (B), and *IAA3* (C) by BL are shown. Light-grown *det2* seedlings were treated with 10 nM BL or were mock treated (cont) for the indicated times,

mutants in soybean (*Glycine max*) and tomato. They concluded that the promotion of cell elongation by BRs is not likely mediated by the auxin-signaling pathway (Clouse et al., 1992, 1993; Zurek et al., 1994). On the other hand, our comprehensive microarray analysis, which encompasses most of the known auxin-inducible genes and various homologs of these genes, revealed a marked overlap in the BR- and auxin-signaling pathways in Arabidopsis.

BL induced the expression of the early auxin-inducible genes *indole-3-acetic acid (IAA)3*, *IAA5* (Abel et al., 1995), and *SAUR-AC1* (Gil et al., 1994) and that of homologs *IAA*, *GH3*, and *SAUR* genes (Table II). Although the functions of the proteins encoded by early auxin-inducible genes remain controversial, these proteins are thought to serve as important factors in auxin signaling (Abel and Theologis, 1996; Gray and Estelle, 2000; Reed, 2001). Because the BL-induced genes include members of the three most well-characterized families of early auxin-inducible genes (Abel and Theologis, 1996), we chose one member from each family, namely, *IAA3*, *SAUR-AC1*, and a *GH3* homolog (*BRU6* in Table II), for further analysis. Before the analysis of the BL-induced expression, we confirmed that these genes exhibited a more than 2-fold increase in gene expression within 15 min of exposure to auxin (Abel et al., 1995; our unpublished data).

RT-PCR analysis revealed similar kinetics for the induction by BL of all three genes; the maximal level of expression was apparent between 6 and 12 h (Fig. 4, A–C). The *IAA3* and *GH3* homolog (*BRU6*) genes showed limited induction within 1 h of BL treatment, whereas *SAUR-AC1* showed significant induction within 30 min. Because none of the genes was BR-regulated within 15 min, we conclude that *SAUR-AC1* is one of the early BR-inducible genes. Clouse and colleagues (1992) reported that members of the *SAUR* and *GH3* gene family were not rapidly induced during BR-promoted cell expansion but were induced by BR at later time points with different kinetics than when treated with auxin (Zurek et al., 1994). They concluded that these genes were not required for initial elongation in BR-treated tissue. On the other hand, we demonstrate here that some members of these gene families are induced more quickly by BL than previously described. The BL-induction kinetics of these genes are similar to those of genes involved in cell elongation and cell wall organization (see following section). It is not currently clear whether BRs activate auxin biosynthesis or activate a part of the auxin-signaling pathway or whether these genes represent shared signaling components of the auxin- and BR-signaling pathways.

after which transcript abundance was analyzed by Taq-Man RT-PCR. Transcript abundance levels are presented as relative values that are normalized with respect to the levels of 18S ribosomal RNA. Data are means \pm SE from three different plant samples.

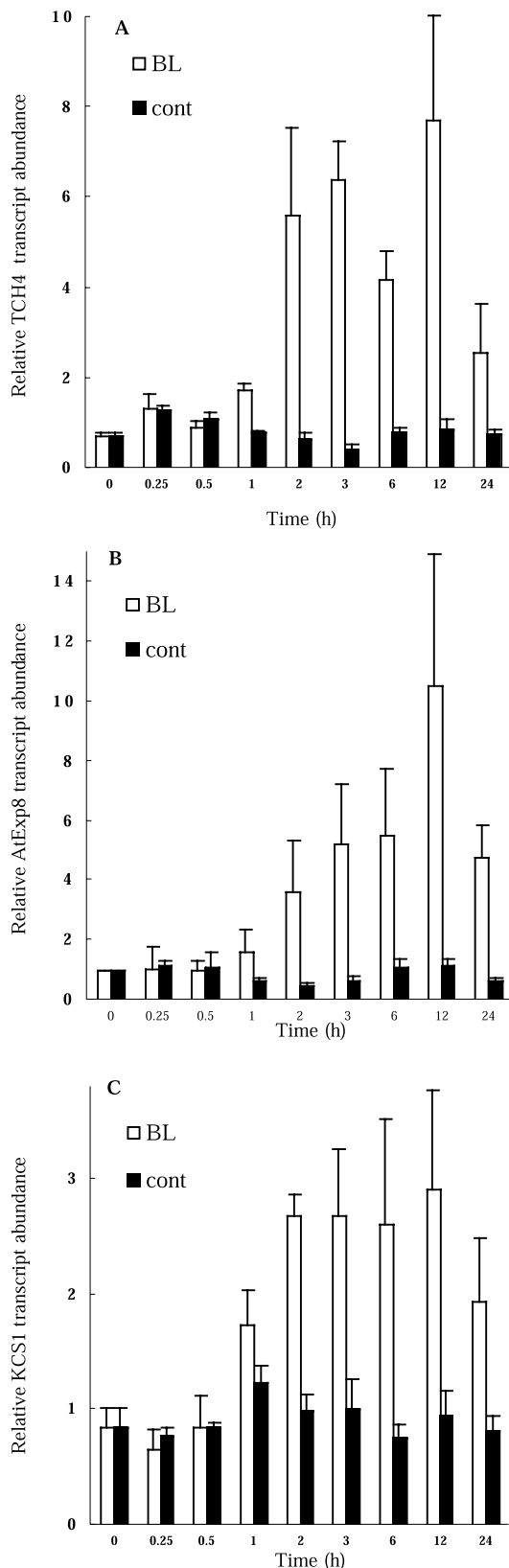


Figure 5. BL induction of genes that are implicated in cell elongation or cell wall organization. Kinetics of induction by BL of *TCH4* (A), a putative expansin gene *AtExp8* (B), and *KCS1* (C) are shown. Light-grown *det2* seedlings were treated with 10 nM BL or were mock

In contrast to the BL induction of some early auxin-inducible genes, none of the late auxin-inducible genes is significantly induced by BL within the 3-h treatment period, even though a number of probes for such genes are present on the Arabidopsis Genome Array. It remains to be determined whether longer exposure to BRs results in the activation of late auxin-inducible genes. On the other hand, neither *IAA1* nor *IAA2*, both of which are early auxin-inducible genes (Abel et al., 1995), was induced by exposure of Arabidopsis to BL for 3 h. The expression of *PAP1/IAA26* and a *SAUR* homolog (*BRD5*), both of which are homologs of auxin-inducible genes, was repressed by BL treatment (Table I); the responses of these genes to auxin have not been known. These observations indicate that members of the *IAA*, *GH3*, and *SAUR* gene families can be classified into at least four groups: (a) those that are induced specifically by auxin (such as *IAA1* and *IAA2*); (b) those that are induced both by auxin and by BRs (*IAA3*, *IAA5*); (c) those that are induced by BRs, but not by auxins (*GH3* homolog [*BRU7*; auxin-insensitive data not shown]); and (d) those that are repressed by BRs (*PAP1*, *SAUR* homolog [*BRD5*]). Further analysis is needed to enable classification of members of the auxin-inducible gene families, depending on their dose- or time-dependent responses to auxin and BRs. Such classification should provide insight into functions of these genes, and it will further extend understanding involvement of these genes in the regulation of growth and development, including photomorphogenesis (Reed, 2001). Functional characterization of these genes should also provide insights into the functional divergence between auxins and BRs.

Control of Cell Elongation and Cell Wall Organization

Microarray analysis revealed that BL induces the expression of various genes implicated in cell elongation or cell wall organization (Table II). As far as we are aware, this list contains the largest number of genes in this class shown to be regulated by a single signaling molecule. Most of these genes have not previously been shown to be under the control of any hormone. We chose three of the genes for further analysis of their induction kinetics: *TCH4*, which encodes xyloglucan endotransglycosylase (XET; Xu et al., 1995); a putative expansin gene (*AtExp8*) that is highly homologous to *ZmExp2* (Im et al., 2000); and *KCS1*, which encodes a fatty acid elongase 3-ketoacyl-CoA synthase that functions in wax biosynthesis (Todd et al., 1999). RT-PCR analysis revealed that the induction of these three genes was apparent

treated (cont) for the indicated times, after which transcript abundance was analyzed by Taq-Man RT-PCR. Transcript abundance levels are presented as relative values that are normalized with respect to the levels of 18S ribosomal RNA. Data are shown as means \pm SE from three different plant samples.

after a lag period of 30 min to 1 h, with maximal expression observed between 6 and 12 h (Fig. 5, A–C). The kinetics of induction of these genes were thus similar to those of BL-induced expression of the early auxin-inducible genes (Fig. 4) and to those of BR-induced cell elongation (Sasse, 1999), i.e. the lag period for the response to BRs is generally longer than that for responses to auxins. The lag period of BL-induced *TCH4* expression was similar to that demonstrated previously (Xu et al., 1995).

On the other hand, the expression of *XTR7*, a member of the *XET/XTH* gene family, was down-regulated by BL (Table I), whereas the expression of this gene was previously shown to be independent of BR and auxin regulation (Xu et al., 1996). We demonstrated that BL reduced the abundance of *XTR7* transcripts in a dose-dependent manner between 10^{-10} and 10^{-5} M by RT-PCR, but 10^{-6} M auxin increased the levels of *XTR7* transcripts (data not shown). Our observations thus suggest the existence of multiple BR-signaling pathways that regulate *XET/XTH* gene family: One regulates the *TCH4* gene in the manner observed for auxin induction, and the other regulates the *XTR7* gene in a manner that is opposite to that observed with auxin. These observations pave the way for the exploration of the complex interactions between auxin- and BR-signaling pathways.

P450s and BR Metabolic Enzymes

In contrast to animals and yeast, plants possess a large family of P450 genes that contribute to the synthesis of secondary metabolites. The Arabidopsis genome thus contains nearly 300 P450 genes (Arabidopsis Genome Initiative, 2000). Recent studies into the specific functions of the proteins encoded by these genes have revealed that they are involved in the biosynthesis of a number of signaling molecules in plants. CYP85A (Bishop et al., 1999; Shimada et al., 2001), CYP90A (Szekeres et al., 1996), and CYP90B (Choe et al., 1998) participate in BR biosynthesis, CYP701A3 (Helliwell et al., 1998) and CYP88A3 (Helliwell et al., 2001) in gibberellin (GA) biosynthesis, and CYP79B2 (Hull et al., 2000) and CYP83B1 (Barlier et al., 2000; Bak et al., 2001) in auxin metabolism. It is important to know how BRs regulate P450 genes because it will help elucidate signaling network cross-talk between BRs and other signaling molecules. Furthermore, the oxygenation steps in the BR biosynthetic pathway, which are probably catalyzed by P450 enzymes, have yet to be characterized. Using the DNA array analysis, we examined the effects of BL on the expression of P450 genes, including those that contribute to BR metabolic pathways. Many P450 genes were regulated by BL (Fig. 6A). Such information will be useful in the future characterization of their specific functions. BL represses the expression of CYP79B2 and CYP81D8 (Table III). The CYP79B2 has shown to convert Trp to indole-3-

acetaldoxime, the first step of Trp-independent IAA biosynthesis. We have recently identified that the CYP81D8 is the only auxin-inducible P450 gene by the comprehensive screening of early auxin-inducible genes using DNA microarray (S. Sawa and our unpublished data). These P450 genes may account for the physiological interactions between auxin and BRs.

Among P450 genes, a cluster consisting of the CYP85 and CYP90 families attracted our interest. Three members of these families have been shown to contribute to BR biosynthesis, and we found that the expression of the clustered genes was all repressed by BL treatment (Fig. 6, B–F). The gene cluster included *ROT3/CYP90C*, which functions in polar cell elongation in leaf cells (Kim et al., 1998), and *CYP90D*, a novel P450 gene (the full-length sequence of which has determined and has been submitted to GenBank; accession no. AB066286). These data suggest that all of the genes in the cluster encode BR-related enzymes. The observation that BL represses the expression of several BR biosynthetic genes indicates that the BR biosynthetic pathway is subject to feedback regulation at multiple points to ensure homeostasis of endogenous BRs. The rate of repression of *CPD* gene in Figure 4B was slower than that described in a previous report (Mathur et al., 1998), probably because of the low concentration of BL used in our experiments. On the other hand, our microarray experiments revealed that the expression of genes for enzymes that function upstream in the pathway of BR biosynthesis, including *DWF7/STE1* (Choe et al., 1999), *DIM/DWF1* (Klahre et al., 1998), and *DET2* (Li et al., 1996), was not significantly affected by BL treatment (data not shown). The results indicate that the genes encoding upper step enzymes are less likely to be regulated by BL in a feedback regulatory manner. Furthermore, we observed that *BAS1/CYP72B1*, which encodes a repressor of a *phyB* mutant, was induced by BL treatment (Fig. 6G). Although CYP72B1 has been shown to catalyze C26 hydroxylation of BL (Neff et al., 1999), its physiological function is not fully understood. Our results imply that the expression of this gene was induced to promote the catabolism of the exogenously applied BL.

CONCLUSIONS

Very recently, three independent groups reported microarray analysis of BR-regulated genes (Hu et al., 2001; Müssig et al., 2002; Yin et al., 2002). The presented list of BR-regulated genes have significant differences with each other. Such differences may be attributable to different experimental conditions, but further studies may be required to account for the discrepancy. We present here the largest list of the BR-regulated genes, which may provide comprehensive view of the physiological functions of BRs using BR-regulated genes as molecular markers. P450 genes and transcription factor genes predominated

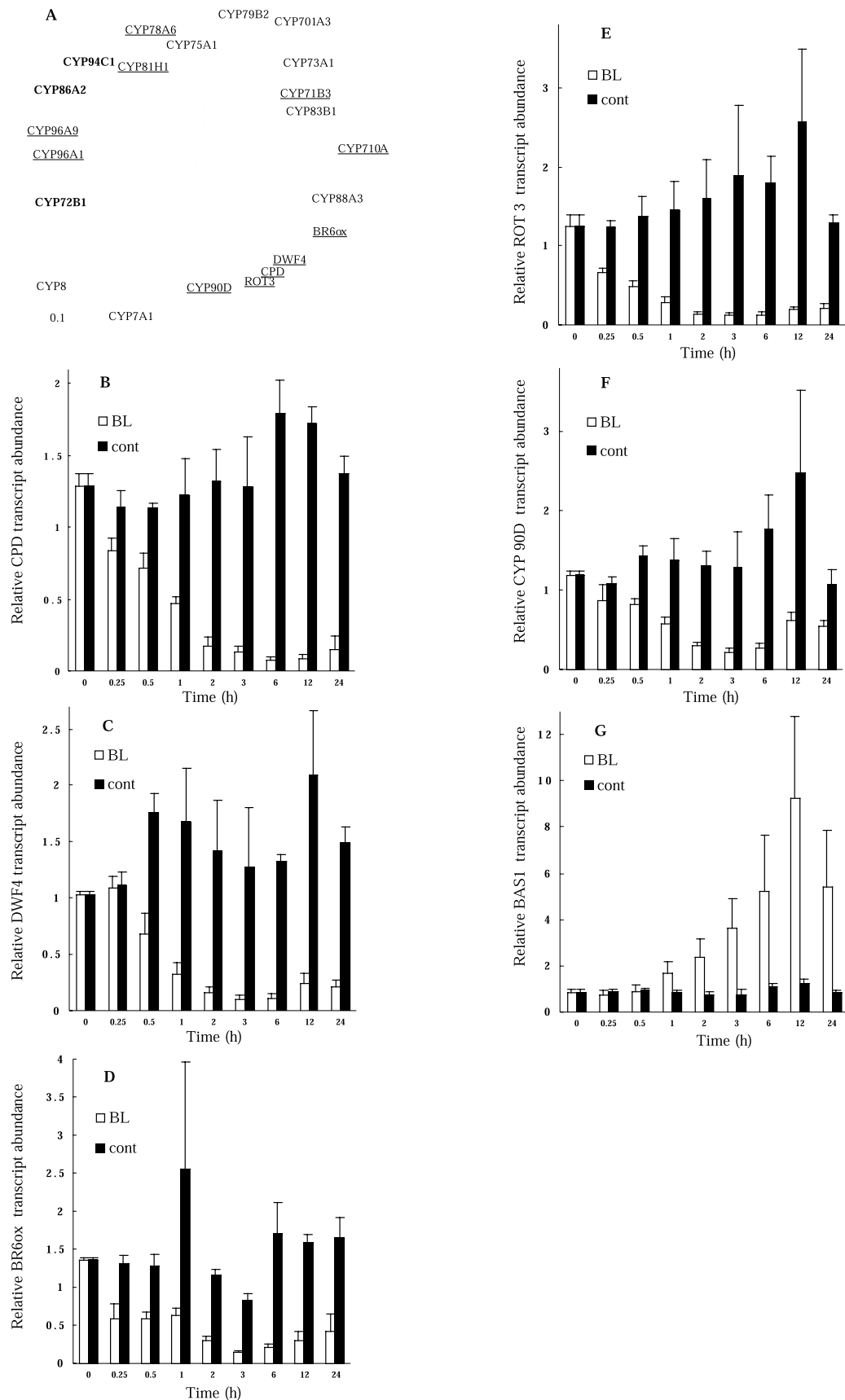


Figure 6. (Legend appears on facing page.)

among down-regulated genes (Fig. 7A). Auxin-related genes and genes that are implicated in cell elongation or cell wall organization predominated among up-regulated genes (Fig. 7B). We focused some of these genes for further characterization (Figs. 3–6). We did not discuss all of the genes shown to be regulated by BL in our microarray analysis (Tables I and II). These additional genes also provide insight into the actions of BRs and their underlying mechanisms. For example, the microarray results suggest possible mechanisms through which BRs interact with auxin. The expression of *PIN7*, a homolog of the *PIN1* and *PIN2* genes for putative auxin-efflux carrier proteins (Galweiler et al., 1998; Muller et al., 1998), was repressed by BL treatment, suggesting that BL might control auxin efflux. The expression of *iaglu*, which encodes a putative indole-3-acetate β -D-glucosyltransferase, was also repressed by BL, suggesting that BL might regulate auxin metabolism. BR signaling also appears to exhibit cross-talk with that of other plant hormones, including jasmonic acid (*OPR1* in Table II; Biesgen and Weiler, 1999) and an unidentified ligand of a putative receptor kinase (*BRU23* in Table II). Some of the remaining genes encoded proteins with Leu-rich repeats that lacked kinase domains, either with or without an extensin-like domain (Tables I and II). Genes that potentially related to signaling molecules, such as calmodulin-related proteins and their putative binding proteins, were regulated by BL (Tables I and II). Finally, BL controlled the expression of genes potentially regulating cell elongation, such as a K^+ channel, a K^+ transporter, and other transporters (Table I). BRs exert a wide variety of effects on both growth and development in plants. It is likely that many BR-regulated genes, presented here, contribute to these pleiotropic effects. Further analysis of the functions of these genes will provide insights into BR activities and will facilitate the understanding functions of the steroidal hormone in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis ecotype Columbia was used as WT in this study. The Arabidopsis mutant *det2-1* (Chory et al., 1991) was used as BR-deficient mutant. Seedlings were grown for 7 d at 22°C under continuous light in one-half-strength Murashige and Skoog (1962) liquid medium (Invitrogen, Carlsbad, CA) supplemented with 1.5% (w/v) Suc. The seedlings were then treated with 10 nM BL or mock-treated with dimethyl sulfoxide (final concentration, 0.1%, v/v), after which they were immediately frozen in liquid nitrogen and stored at –80°C until RNA isolation.

DNA Microarray Analysis

Total RNA was isolated from seedlings by the acid-guanidinium-phenol-chloroform method (Sambrook et al., 1989). Polyadenylated RNA was purified with the Oligotex-dT30 kit (Roche Diagnostics, Indianapolis) and converted into double-stranded cDNA with the use of a Super Script Choice cDNA Synthesis kit (Invitrogen) and with an oligo(dT)₂₄ primer containing a T7 polymerase promoter site at its 3' end (Amersham Biosciences AB, Uppsala). Biotin-labeled cRNA was generated from the double-stranded cDNA using the BioArray High Yield RNA transcript labeling kit (Enzo Diagnostics, New York) and was then purified with the use of the RNeasy RNA purification kit (Qiagen USA, Valencia, CA). Each cRNA sample (20 μ g) was fragmented by incubation for 35 min at 94°C in fragmentation buffer (40 mM Tris-acetate [pH 8.1], 100 mM potassium acetate, and 30 mM magnesium acetate). The hybridization mixture comprised 15 μ g of fragmented cRNA in 300 μ L of a solution containing 100 mM MES, 1 M [Na⁺], 20 mM EDTA, 0.01% (v/v) Tween 20, 0.1 mg mL⁻¹ herring sperm DNA, 0.5 mg mL⁻¹ acetylated bovine serum albumin, and control cRNA (Eukaryotic Hybridization Control Kit, Affymetrix). Portions (200 μ L) of each mixture were subjected to hybridization with the Arabidopsis Genome Array (Affymetrix) for 16 h at 45°C with rotation at 60 rpm. Each array was then washed consecutively with non-stringent wash buffer (6 \times SSPE [Sambrook et al., 1989], 0.01% [v/v] Tween 20, and 0.005% [v/v] Antifoam) and stringent wash buffer (100 mM MES, 0.1 M [Na⁺], and 0.01% [w/v] Tween 20). Hybridization complexes were then detected by consecutive exposure to phycoerythrin-streptavidin (Molecular Probes, Eugene, OR), biotinylated antibodies to streptavidin (Vector Laboratories, Burlingame, CA), and phycoerythrin-streptavidin, after which each array was washed again with non-stringent wash buffer. All washing and staining procedures were performed with a Fluidics Station 400 (Affymetrix). The array was scanned by a confocal microscope scanner (HP Genome Array Scanner, Affymetrix) at a wavelength of 570 nm.

DNA microarray technology is developing continually, but technical problems, such as a narrow signal dynamic range and aberrant detection of cross-hybridization or noise, remain to be resolved. We used the following methods to solve these problems. We chose GeneChip system (Affymetrix) as a DNA microarray system. The protocols for data analysis of the GeneChip system and the issues of sensitivity and quantitation have been described previously (Lockhart et al., 1996). In brief, each gene is represented

Figure 6. Regulation of P450 genes by BL. A, Phylogenetic relationships among BR-regulated and other P450 genes. Proteins whose genes were shown to be up- or down-regulated by BL are indicated in bold and underlined, respectively (Tables I and II). BR6ox (Arabidopsis, CYP85A, AB035868), CPD (Arabidopsis, CYP90A, X87367), ROT3 (Arabidopsis, CYP90C, AB008097), DWF4 (Arabidopsis, CYP90B, AF044216), and CYP90D (Arabidopsis, AB066286) belong to the CYP85 or CYP90 families. CYP72B1 (Arabidopsis, BAS1, AC003105) is a suppressor of the *phyB* mutant. CYP88A3 (Arabidopsis, AtKAO1, AF318500) and CYP701A3 (Arabidopsis, GA3, AF047720) participate in GA biosynthesis. CYP83B1 (Arabidopsis, D78598) and CYP79B2 (Arabidopsis, AF069495) participate in auxin metabolism. The genes for CYP73A1 (*Helianthus* sp., cinnamate 4-hydroxylase, Z17369) and CYP75A1 (*Petunia* sp., flavonoid-3', 5'-hydroxylase, D14588) were the first P450 genes to be identified functionally in higher plants and belong to the higher plant-specific group A of P450 genes. The accession numbers of the other P450 family members are D30718 (CYP8) and M93133 (CYP7A1). Kinetics of regulation of the CPD (B), DWF4 (C), BR6ox (D), ROT3 (E), CYP90D (F), CYP72, CYP85, CYP90, and BAS1 (G) genes by BL are shown. Light-grown *det2* seedlings were treated with 10 nM BL or were mock treated (cont) for the indicated times, after which transcript abundance was analyzed by Taq-Man RT-PCR. Transcript abundance levels are presented as relative values that are normalized with respect to the levels of 18S ribosomal RNA. Data are shown as means \pm SE from three different plant samples.

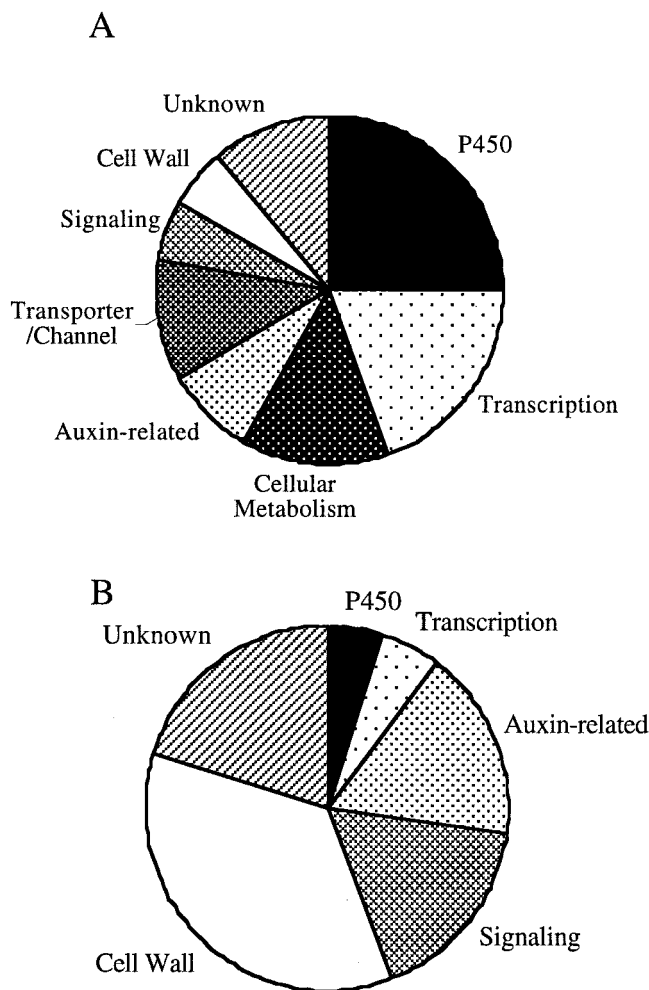


Figure 7. Frequencies of up-regulated and down-regulated genes. A, Distribution of BR-down-regulated genes that are listed in Table I. B, Distribution of BR-up-regulated genes that are listed in Table II. The BR-regulated genes are classified into the functional categories based on their established or putative functions.

on the array as a set of 16 oligonucleotide probes that match the sequence of the gene exactly. The specificity of hybridization is verified by inclusion on the array of the same set of probes each with a single-nucleotide mismatch in the center of its sequence. The difference between the hybridization signal obtained with the matching set of probes and that obtained with the mismatched probes is proportional to the abundance of the corresponding transcript and is calculated as the AvDf value. Analysis of absolute and differential gene expression was performed with the GeneChip software, Microarray Suite (v4.0, Affymetrix). AvDf values of highly abundant transcripts that are obtained with the standard GeneChip protocol are frequently affected by signal saturation when antibody amplification is used. To achieve a higher signal dynamic range, we scanned each chip before and after signal amplification using an anti-streptavidin antibody. Each chip was normalized relative to the sum of the AvDf values, and then each gene was compared between the control and BL-treated samples. Genes that were up- or down-regulated as reflected by a more than 2-fold difference in their AvDf values and were assigned to an “increase” or “decrease” in the difference call of the comparison analysis by Microarray Suite were identified. Furthermore, genes with an “absent” value in the absolute call of baseline data and with a decrease value in the difference call were excluded from the list. Genes with an absent value in the absolute call of experimental data and with an increase value in the difference call were conversely also excluded from the list. To ensure the reproducibility of results, we performed three independent experiments with different plant samples, and genes that showed the same responses in all three experiments were classified as genes regulated by BR (Tables I–III). The *BR6ox* and *CYP90D* genes were identified with the use of Arabidopsis GEM1 microarrays (Incyte Systems, Palo Alto, CA) containing 7,942 cDNA clones.

Taq-Man RT-PCR Analysis

Total RNAs were isolated as described above and then treated with DNase I. They were then converted to cDNAs using a Super Script first-strand synthesis system (Invitrogen). Quantitative RT-PCR was performed with the use of real-time Taq-Man technology (Holland et al., 1991) and a sequence detector (model 7700, Applied Biosystems, Foster City, CA). Gene-specific primers and Taq-Man probes (Table V) were used to analyze transcript abundance. The 18S ribosomal RNA was analyzed as an internal control and was used to normalize the values for transcript abundance. We performed three independent experiments with different plant samples. Each experiment was normalized relative to the median of the experiment, and then means and ses of three experiments were calculated.

Sequence Analysis

DNA sequences were determined with an automated DNA sequencer (model 373A DNA Sequencing System, Applied Biosystems). Nucleotide

Table V. Primers and TaqMan probes used for RT-PCR

Gene Name	Forward Primer Sequence	Reverse Primer Sequence	TaqMan Probe Sequence
CPD	CCCAAACCACTCAAAGATGCT	GGCCTGTGCTTACCGAGTT	TCTGCCATCTCCAAGGGTTGAAAGTGC
DWF4	GTGATCTCAGCCGTACATTTGGA	CACGTCGAAAACTACCACTTCCT	CAGCAAAAACAACGGAGCGTCATCG
ROT3	ATTGGCGCGTTCTCAGAT	CAAGACGCCAAAGTGAGAACAA	CTCACCTCAAAGACCGGATCACTCGAGA
BR6ox	TGGCCAATCTTTGGCGAA	TCCCGTATCGGAGTCTTTGGT	ACCGAGITTTCTCAAACAAGGCCCAAC
CYP90D	CTCATTACCCTTGCCGTCAAA	CAGCTTCATGTTTTCTCCGTTAG	CCTCTCTGATTCTCTGCTGCCCTCAAT
BAS1	TTGGCTTCATACCGTTTGGC	TTACAGCGAGTGTCAATTTGGC	CGGAGTTCGTACATGATTGGTCAGAATC
Lhcb1.3	GGAGCTCAAGAACGGAAGATTG	GGTTCTCTATCGGTCCCTTACCA	TGGATTTCTCGTTCAAGCCATCGTCA
rbcS1A	GCTCTCTCCGCTACTATGGTTG	AAGGCAGCGGAGGACTTAAAGT	TCAGGCCACTATGGTCGCTCCTTTCA
TCH4	CAAGAACATGGAGTCTCTAGGCACT	GTGAAAGGAGCTTTAGACCAATCG	ACTCGAGTCTTTGGAACGCTGATGATTGG
PIF3	TCAGGCTCACAAAGCTAAGC	CACACCAGCTCCACAACCTCA	AATCTGCTCAAGACAGGAACCTTCTCCAC
GH3 homolog (BRU6)	CGTATATCCAACGGCGATTGT	CCAGCAGATGTTCTGAGCTT	TCTTCTACCCCATCACCGAGTTTCTCA
SAUR-AC1	GAGGATTCATGGCGGTCTATG	GTTAAGCCGCCATTGGAT	TGGTGCCGGTTTCACTTAACCAACGCT
IAA3	AGCTAAACCTTTGGCTTCTGA	GGTGATTGATGCTCATTGGT	CTTGCACGTACATGAAACATCTCCCATGG
XTR7	TCGACGAGTTTGACCTCACTTG	GTCCAGGCACAAGACAGCATA	ACCACAGAGGCAAAATCTTCAACGGAGG
AtExp8	CAACCATCACCGTCACAGCTA	TGAAGAGGAGGATTGCACCAA	AAACTTTTGCCCACTAACCCCTGGCCT
KCS1	GCTCAAATACGTGAAGCTTGGAA	CAGCACGGTTCGGTAAA	CAACTCTGCAACGTGACCACCACTCTCT
18S ribosomal RNA	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC

sequences were compiled and analyzed with GENETYX-Mac software (Software Development Co. Ltd., Tokyo). The BLAST program (Altschul et al., 1990) was used to search for entries of homologous sequences in the DNA data bank of Japan. The ClustalW program on the server at DNA data bank of Japan was used to align amino acid sequences and to derive phylogenetic relations based on the neighbor-joining method (Saitou and Nei, 1987).

Determination of Chlorophyll Content

Acetone was added to the seedlings to bring the final concentration to 80%. After homogenization, the solution was centrifuged at 10,000g for 5 min, and chlorophyll was determined according to the method of Arnon (1949). Chlorophyll contents were normalized with respect to the fresh weight of the seedlings.

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