Microarray and Proteomic Analysis of Brassinosteroid- and Gibberellin-Regulated Gene and Protein Expression in Rice

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Brassinosteroid (BR) and gibberellin (GA) are two groups of plant growth regulators essential for normal plant growth and development. To gain insight into the molecular mechanism by which BR and GA regulate the growth and development of plants, especially the monocot plant rice, it is necessary to identify and analyze more genes and proteins that are regulated by them. With the availability of draft sequences of two major types, *japonica* and *indica* rice, it has become possible to analyze expression changes of genes and proteins at genome scale. In this review, we summarize rice functional genomic research by using microarray and proteomic approaches and our recent research results focusing on the comparison of cDNA microarray and proteomic analyses of BR- and GA-regulated gene and protein expression in rice. We believe our findings have important implications for understanding the mechanism by which BR and GA regulate the growth and development of rice.

Key words: brassinosteroid, gibberellin, microarray, proteome, rice

Introduction

Rice (*Oryza sativa* L.) is an important crop in eastern Asia; it is also widely accepted as a good model for the studies of monocot plants because of its small genome (430 Mb), well-established protocols for transformation and high degrees of synteny among other crop plants of *Poaceae* species (*1*). With the publication of draft sequences of *Oryza sativa* L. ssp. *indica* and *japonica* genomes (*2*, *3*) and completion of sequencing of chromosomes 1 and 4 by IRGSP (*4*, *5*), we have entered into an era of functional genomics in the research on rice. Functional genomics can be considered as any technique or approach that identifies gene function and/or the role of a gene in plant biology (*6*, *7*). Knowing the exact sequence and location of all genes of a given organism is only the first step towards understanding how all parts of a biological system work together. Although 25,426 genes have been identified in *Arabidopsis*, less than 10% have been documented experimentally (*8*). Rice genome is predicted to have approximately 50,000 to 55,000 protein-coding genes (*2*, *3*). About 76% of the 28 Kb full-length rice cDNA clones could be assigned tentative function by gene ontology term (*9*). However, gene function predication by sequence comparison will not always lead to conclusive results. Knowing the class of protein that a gene belongs to does not immediately tell us the exact function of a gene. For example, in the *Arabidopsis* genome, at least 610 genes have been identified as encoding leucine-rich-repeat class of receptor-like kinases (LRR-RLKs), but only a few of their biological functions have been determined (*10*). Therefore, the gene function must be inferred by analyzing the phenotype of mutant and by studying the expression of the gene in question over the course of developmental process or in response to certain biotic and abiotic stimuli.

To assign function to unknown genes, different functional genomic methodologies, which are termed phenomics, transcriptomics, proteomics and metabolomics, are currently being developed and used (*6*). We have been systematically analyzing changes induced by the phytohormones brassinosteroid (BR) and gibberellin (GA) both at transcriptional and translational levels in rice seedlings by using cDNA microarray and proteomic approaches. In this review, we summarize rice functional genomic research by using microarray and proteomic approaches and our recent research results focusing on the comparison of cDNA microarray and proteomic analyses of BR- and

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GA-regulated gene and protein expression in rice.

Rice Functional Genomic Research by Microarray and Proteomic Approaches

Transcriptional profiling using microarray has developed into the most prominent tool for functional genomics. Changes in mRNA abundance, which are related to changes in protein levels, are indicative of changes in environmental and developmental program or reflect response to all kinds of stimuli. In the past few years, although research on expression profiling in rice was quite limited in contrast to *Arabidopsis*, microarray analyses in rice have approached various biological questions such as grain filling (*11*), pollination and fertilization (*12*), responses to biotic (*13*) and abiotic stresses (*14*, *15*), and phytohormones (*16*, *17*) by interrogating an increasing number of genes. Proteomics seeks to measure the expression of all proteins within an organism and monitor changes in response to developmental and environmental cues. In principal, variations between protein patterns are either due to differences in gene expression levels modulating protein concentrations, or may result from post-translational modifications, which change the structural properties of proteins. In rice, several studies dealt with the construction of proteome at cell, tissue and whole plant levels (*18*, *19*), and analyses of defense-associated response, blast fungus infection of rice grown under different levels of nitrogen fertilization have been published (*19*). As a part of Rice Genome Research Project of NIAS, Japan, the rice cDNA microarray and proteome databases are now available to the public (http://microarray.rice.dna.affrc.go.jp and http://gene64.dna.affrc.go.jp/RPD). Currently, rice cDNA microarray has 9,000 unique ESTs and 28 Kb full-length cDNA versions. Recently, a high-density rice array is available to the public (http://www.ricearray.org). Around 11,941 identified protein spots corresponding to 4,180 separate protein entries have been deposited in the database (*19*). The information on amino acid sequences is updated weekly.

Molecular Mechanism of Brassinosteroid and Gibberellin Actions

BR and GA are two groups of plant growth regulators essential for normal plant growth and development (*20*, *21*). While rapid progress has been made in studies of the biosynthetic pathways, metabolism and signaling of BR (*22*) and GA (*23*) using biochemical techniques as well as by the characterization of their biosynthetic mutants in *Arabidopsis*, not much is known about how they regulate the wide variety of physiological processes at the molecular levels. It is now believed that the binding of BR and its receptor BRI1 at cell membrane will lead to inactivation of a negative regulator BIN2, and the inactivated BIN2 allows the unphosphorylated BES1 and BZR1 to accumulate and move to the nucleus where they activate target genes transcription (*24*). Several important positive and negative regulastors of GA signaling have also been identified. The DELLA proteins function as negative regulator of GA signaling, and their degradation through the ubiquitin/proteasome pathway is considered as a key event in the regulation of GA-stimulated processes (*25*). GA is believed to bind to an unidentified receptor and activates G proteins that enhance the GA signaling. As a consequence, PHOR1 is translocated into the uncleus, where it acts as a positive regulator. GA signal also activates protein kinase and GID2/SLY1-mediated degradation of DELLA protein SLR1/RGA, which otherwise inhibits the expression of GA-induced genes (*25*).

Major effects of BR and GA on plant growth and development are mediated through gene expression modulation because RNA and protein synthesis inhibitors interfere with these processes. To further understand the molecular mechanism of BR and GA actions, especially in monocot rice, it is necessary to identify and analyze more genes that are controlled by them, and characterization of individual gene will help us to understand how BR and GA regulate the growth and development of plants.

Brassinosteroid-Regulated Gene and Protein Expression in Rice

Recent studies have proven that BR plays an essential role in rice growth and development (*26–28*), but there is still no report on BR-regulated gene and protein expression in rice. The bending of the second leaf and its leaf sheath (lamina joint) in rice is very sensitive to the concentration of BR. This unique characteristic of rice leaves has been used as a quantitative bioassay for BR (*29*, *30*). Initially, we adopted this model system for analyzing BR effect on the changes of gene and protein expression.

Microarray analysis of brassinosteroid-regulated gene expression

First, a cDNA microarray containing 1,265 independent rice genes arrayed in duplicates that were randomly selected from 9,000 ESTs was used to analyze differential gene expression in lamina joint tissue caused by brassinolide (BL), the most active form of BR (*31*). Twelve clones were found to be up-regulated after 1 μM BL treatment for 24 h (Table 1). Among them, 5 clones had homologies based on the search in GenBank database using the BLAST program. A vacuolar H^+-transporting ATPase homologue showed higher expression in the BL-treated lamina joint, suggesting a role in BL-mediated cell expansion. One clone showed homology to a sorghum mRNA for ACC oxidase-related protein. Clone 97 was a putative kinetochore protein homologue, a protein involved in mitosis machinery. Clones 165 and 250 were homologous to the *Arabidopsis* p23 co-chaperon and ubiquitin-conjugating enzyme respectively, which are involved in protein metabolism. The other 7 clones had no significant homologies in the database (*31*). Second, to enrich the BR-induced genes, a cDNA library was constructed with mRNAs isolated from seedlings treated with BL. A microarray containing 4,000 clones randomly selected from this library was analyzed with an aim to identify new genes that exhibit transcription regulation by BR. This time, we were able to identify another nine new BL-up-regulated genes and 32 BL-down-regulated genes (*16*).

Two novel BL-up-regulated genes *OsBLE1* and *OsBLE2* identified from our first time microarray analysis were selected for detailed characterization. OsBLE1 is a small protein with 81 amino acid residues while *OsBLE2* encodes a predicted polypeptide of 761 amino acid residues and nine possible transmembrane regions. *OsBLE2* expression was most responsive to BL in the lamina joint and leaf sheath in rice seedlings. BL did not enhance its expression in transgenic rice expressing antisense *BRI1*, a BR receptor, indicating that BR signaling to the enhanced expression of *OsBLE2* is through BRI1. BL effect in the *d1* mutant rice was much weaker than that in its wild type control, suggesting that heterotrimeric Gα protein may be a component of BR signaling. Transgenic rice expressing antisense *OsBLE1* and *OsBLE2* exhibits various degrees of repressed growth. Our results demonstrated that OsBLE1 and OsBLE2 play important roles in BL-regulated growth processes in rice (*31*, *32*).

Table 1 BL-up-Regulated Genes in Rice Lamina Joint

Clone No.	Putative gene identification	Accession No.
67	Unknown	AU077480
97	mRNA for putative kinetochore protein	AU068983
145	Vacuolar H^+-transporting ATPase	AU085745
165	mRNA for p23 co-chaperon	AU069113
203	Unknown	AU063127
214	Unknown	C97224
250	Ubiquitin conjugating enzyme	C97278
550	Unknown	AU082386
654	Unknown	AU085926
973	Unknown	AU088678
1029	ACC oxidase related protein	AU032762
1190	Unknown	AU175763

Proteomic analysis of proteins regulated by brassinosteroid

Proteins extracted from lamina joints that had been treated with 1 μM BL and water control for 48 h were separated and compared by 2D-PAGE followed by amino acid analysis (*33*). Eight protein spots representing seven kinds of proteins were increased in quantity in lamina joints treated with BL when compared to water control. These proteins are tubulin, glyceroaldehyde 3-phosphate dehydrogenase (Spot 417), homeodomain leucine zipper protein (Spot 528), dihydroflavonol 4-reductase (Spot 566), Pyruvate decarboxylase 1 (Spot 591), glutathione S-transferase (Spot 595), and RuBisCO LSU (Spots 738 and 742), which fall into categories of transcription factor, cell structure, metabolism, photosynthesis, and stress response (Table 2). This result suggests that BL has wide range of influence on many cellular processes through regulating expression of different proteins.

Gibberellin-Regulated Gene and Protein Expression in Rice

Regulation of rice plant height is important for lodging resistance and thus improving grain yield. GA is one of the major factors determining plant height including monocot rice. However, the mechanism of how GA regulates this process remained unclear. Rice seedlings leaf sheath is very sensitive to GA, and two-week-old rice treated with 5 μM GA_3 for 48 h elongated twice in length (*34*). We used this system to

analyze GA-regulated changes in gene and protein expression.

Microarray analysis of gibberellin-regulated gene expression

A cDNA microarray containing 9,000 independent rice ESTs was used to analyze differential gene expression in leaf sheath treated by GA_3. Seventeen genes were up-regulated after 5 μM GA treatment for 12 h (Table 3). Among them, the functions of four clones were unknown. The other 13 genes encode various proteins involved in many cellular processes. Glyceroaldehyde 3-phosphate dehydrogenase (Clone 1404) and malate dehydrogenase (Clone 1421) are two important enzymes in carbon metabolism. Components of cytoskeleton including actin (Clone 5820) and tubulin (Clone 216) were also up-regulated by GA_3. The Golgi COPI coatomer components (Clones 5887 and 6364) are proteins involved in intracellular protein trafficking process while NADH dehydrogenase (Clone 1459) and cytochrome C (Clone 1710) are the members of mitochondrial electron transport chain. Other GA_3-up-regulated genes that were identified in this microarray analysis encode protein phosphatase 2A regulatory subunit A (Clone 1298), mitochondrial 2-oxoglutarate/malate translocator (Clone 1390), topoisomerase IV subunit A (Clone 6175), adenylate kinase A (Clone 5951) and farnesyl diphosphate synthase (Clone 1644) (*35*).

We also used a cDNA microarray containing 4,000 clones randomly selected from a rice cDNA library

Table 2 BL-up-Regulated Proteins in Rice Lamina Joint

Spot No.	Homologous protein	Accession No.
384	Tubulin	AF030548
417	Glyceroaldehyde 3-phosphate dehydrogenase	Q59800
528	Homeodomain leucine zipper protein	AF145727
566	Dihydroflavonol 4-reductase	AB003496
591	Pyruvate decarboxylase I	U07339
595	Glutathione S-transferase	P31671
738	RuBisCO LSU	P30828
742	RuBisCO LSU	P30828

Table 3 GA_3-up-Regulated Genes in Rice Leaf Sheath

Clone No.	Putative gene identification	Accession No.
216	Tubulin	AU102148
1173	Unknown	C96918
1298	Protein phosphatase 2A regulatory subunit A	AU166798
1386	Unknown	AU108788
1390	Mitochondrial 2-oxoglutarate/malate translocator	AU176430
1404	Glyceroaldehyde 3-phosphate dehydrogenase	AU108826
1421	Malate dehydrogenase	AU092479
1459	NADH dehydrogenase	AU100811
1644	Farnesyl diphosphate synthase	C27703
1710	Cytochrome C	AU100900
5820	actin	AU164488
5887	COP alpha	AU031660
5951	Adenylate kinase A	D23938
6236	Unknown	AU031768
6175	Topoisomerase IV subunit A	AU173178
6237	Unknown	AU173243
6364	Coatomer complex subunit	AU031850

prepared from seedlings treated with GA_3 to analyze gene expression differences caused by GA_3 in rice seedlings. Twenty-nine and forty-two unique cDNA clones were found up- and down-regulated by GA_3 this time, respectively (*16*). Four clones encoding the same isotype of xyloglucan endotransglucosylases/hydrolases (XTHs) were GA_3-up-regulated. XTHs are encoded by a gene family of 29 members in rice (*36*) and mediate the cleavage and rejoining of the β-(1-4)-xyloglucans of the primary cell wall. It is considered that XTHs play an important role in the construction and restructuring of xyloglucan cross-links and thus are essential for regulating cell elongation. The GA_3-up-regulated XTH identified here was designated *OsXTH8,* cloned and characterized. *OsXTH8* was preferentially expressed in rice leaf sheath in response to GA_3. *In situ* hybridization and *OsXTH8* promoter GUS fusion analysis revealed that *OsXTH8* was highly expressed in vascular bundles of leaf sheath and young nodal roots, where the cells are actively undergoing elongation and differentiation. *OsXTH8* gene expression was specifically up-regulated by GA_3. In two genetic mutants of rice with abnormal height, the expression of *OsXTH8* positively correlated with the height of the mutants. Transgenic rice expressing an RNAi construct of *OsXTH8* exhibited repressed growth. These results indicate that *OsXTH8* is differentially expressed in rice in relation to GA and potentially involved in cell elongation processes (*37*).

Proteomic analysis of proteins regulated by GA_3

Proteins extracted from leaf sheath that had been treated with 5 μM GA_3 and water control for 48 h were separated and compared by 2D-PAGE followed by amino acid analysis (*34*). A total of 21 protein spots representing 18 kinds of proteins were increased in quantity in leaf sheath treated with GA_3 when compared to water control. Among them, seven proteins did not have homologies or only hit to hypothetical proteins in the database (Table 4). These identified proteins include phosphatidylinositol 4 kinase (Spot 004), Calcium binding protein (Spot 006), lumental binding protein (Spots 030 and 034), calreticulin (Spot 079), β-tubulin (Spot 089), RuBisCO LSU (Spots 093 and 094), RuBsCO activase (Spots 129 and 162), tetraphosphate phosphorylase II (Spot 239), ascorbate peroxidase (Spot 278), GSH-dependent dehydroascorbate reductase (Spot 309), and histone HI (Spot 313).

Table 4 GA_3-up-Regulated Proteins in Rice Leaf Sheath

Spot No.	Homologous protein	Accession No.
004	Phosphatidylinositol 4-kinase	AAK18831
005	Unknown	
006	Hypothetical protein	BAA95862
008	Calcium-binding protein 1	P42529
030	Lumental binding protein	AAB63469
034	Lumental binding protein	AAB63469
056	Unknown	
079	Calreticulin	BBA889900
089	Tubulin-1α chain	P28752
093	RuBisCO LSU	P12089
095	RuBisCO LSU	P12089
096	Unknown	
108	Unknown	
129	RuBisCO activase	P93431
162	RuBisCO activase	P93431
212	Hypothetical protein	P31545
226	Unknown	
239	Tetraphosphate phosphorylase II	P49348
278	Ascorbate peroxidase	BAB17666
309	GSH-dependent dehydroascorbate	BAA90672
313	Histone H1	S59589

RuBisCO activase, a key enzyme in carbon assimilation during photosymthesis, was identified here as a GA_3-up-regulated protein. RuBisCO activase has been shown to function as GA-binding protein in rice (*38*), inducing two independent cytosolic Ca^{2+}-dependent protein kinase signaling components downstream to the RuBisCO activase, suggesting their roles in the GA signaling (*39*).

Tubulin-1α chain was also identified as GA_3-up-regulated protein. Therefore, We have found that some isotypes of both β-tubulin and α-tubulin are up-regulated by GA_3 using microarray as well as proteomic approaches, suggesting that tubulins play important roles in GA-regulated growth processes in rice. Microtubules are involved in many cellular processes, such as cell division and cell elongation in plants. Tubulins are the major protein in the microtubules, which are composed of repeating heterodimers of β-tubulin and α-tubulin that exist in many isotypic forms encoded by different genes. In *Arabidopsis,* during seed germination, β-2,4-tubulin was found to be increased by GA (*40*). We first selected β-tubulins as our target for detailed characterization. Homology search within the rice ESTs

and genome sequence database identified at least eight β-tubulin isotypes and were designed *OsTUB1-8*, including three novel genes. Northern analysis using specific probes to 3′-UTR of β-tubulin isotypes showed differential and tissue-specific expression. Seven out of eight *OsTUB* genes were dominantly expressed in leaf sheath, while *OsTUB8* was preferentially expressed in anther, including mature pollens. The existence of anther-specific β-tubulin suggests its unique role in the formation of microtubules during the anther and pollen development or pollen tube growth. Furthermore, transcripts of *OsTUB5, OsTUB6* and *OsTUB7* genes were significantly enhanced by GA_3 but all eight *OsTUB* genes were repressed by abscisic acid. Our results imply that *OsTUB* genes are differentially regulated by developmental and hormonal signals and different *OsTUB* isotypes might play special role in the growth and development of specific organ in rice (*41*).

Conclusion

We have identified some BR- and GA-regulated genes and proteins by using cDNA microarray and proteomic approaches in rice. While some of them are previously reported BR- and GA-regulated genes or proteins, most identified genes/proteins, including some functionally unknown genes, are found to be regulated by BR and GA for the first time. Therefore, we believe our findings have important implications for understanding the mechanism by which BR and GA regulate the growth and development of rice, and the information obtained will be helpful in assigning functions to some unknown genes.

One problem we encountered is that we did not find any gene/protein overlapped in our cDNA microarray and proteomic analyses. There are many reasons that lead to this consequence. First, the number of genes in our array and tissues analyzed are limited; changed protein identified by proteomic approach may not be included in our array. Second, transcript products of some genes are too low in quantities to be detected by 2D-PAGE, therefore gene expression changed at mRNA level could not be reflected at protein levels. In order to comprehensively analyze BR- and GA-regulated genes in rice, using microarray of more genes contained in the whole genome of rice is necessary. Besides, detailed analyses are required, such as using BR and GA deficient and insensitive mutants, specific tissues, and timing of expression analysis. Furthermore, detailed analysis of the functions of newly identified genes should provide insight into the actions of BR and GA and facilitate our understanding of the underlying mechanisms of their actions. But information on post-translational modifications such as phosphorylation, glycosylation and other modifications that affect gene product activity or destiny can only be obtained by proteomic approach. Therefore, to study the mechanism of BR and GA actions, both microarray and proteomics, and all means of other functional genomic methodologies are important. Such studies will provide us with increasing knowledge about the regulation of agronomically important traits and accelerate breeding crops with high productivity, good quality and broad stress resistance.

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