J. Microbiol. Biotechnol. (2006), **16**(12), 1990–1994

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Proteome Analysis of Waito-c Rice Seedlings Treated with Culture Fluid of Gibberellin-producing Fungus, *Fusarium proliferatum* KGL0401

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Received: October 3, 2005
Accepted: January 14, 2006

Abstract *Fusarium proliferatum* KGL0401 was previously isolated from *Physalis alkekengi* var. *francheti* plant roots and exhibited a high GA productivity. A gas chromatography-mass spectrometry (GC-MS) analysis of extracts of the culture fluid of *F. proliferatum* KGL0401 also revealed the presence of GA_1, GA_3, GA_4, GA_7, GA_{20}, and GA_{24}. Therefore, the present study conducted a proteome analysis of waito-c rice treated with the culture fluid of the isolated *F. proliferatum* KGL0401 to identify the protein expression triggered by the GA-containing culture fluid. The results revealed the overexpression of 180 protein spots in the sample treated with the culture fluid. Among them, 75 induced proteins were selected and analyzed by MALDI-TOF (matrix-assisted laser desorption-ionization time-of-flight) mass spectrometry, followed by database searching, and 51 proteins were identified.

Key words: Gibberellin, *Fusarium proliferatum* KGL0401, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry, two-dimensional polyacrylamide gel electrophoresis

Gibberellins (GAs) are a large family of isoprenoid plant hormones, and the term GA was first used in 1935 to describe the substance produced by the fungus *Gibberella fujikuroi* that caused overgrowth symptoms in rice, referred to as bakanae disease [10]. GAs control many aspects of plant development, including seed germination, shoot elongation, flower formation and development, fruit-setting, seed development, sex determination, and the chlorophyll content [11].

The use of a sensitive physicochemical method of analysis, which principally combines gas chromatography and mass spectrometry (GC-MS), has revealed that GAs are an amazingly large group of natural products, currently composed of 130 different compounds, found in higher plants, fungi, and bacteria. The bioactive GAs include GA_1, GA_3, GA_4, and GA_7 [4].

Seven genes related to GA biosynthesis are clustered in the fungus *G. fujikuroi*, and the functions of five of these genes have already been determined [20]. As such, genes encoding geranylgeranyl diphosphate synthase (*ggs2*), ent-kaurene synthase (*cpk/ks*), desaturase gene (*des*), and cytochrome p450 monooxygenase genes (*p450-1* to *p450-4*) have been shown to be closely linked in a gene cluster [20], and the cloning of these seven GA biosynthetic genes allows further study of the factors involved in controlling their expression.

Generally, after a genome has been fully sequenced, the key task is to study the modification, functions, and regulation of all the encoded proteins. Thus, proteomics is now an important factor in the study of complex cellular proteins [19].

The analysis of protein mixtures that contain proteins involved in stem growth requires the utilization of multidimensional separation methods, where the samples are separated according to their isoelectric point in the first dimension and their molecular weight in the second dimension.

F. proliferatum KGL0401 was previously isolated from *Physalis alkekengi* var. *francheti* plant roots, and a GC-MS analysis revealed that *F. proliferatum* KGL0401 had a higher GA productivity than the wild-type *G. fujikuroi*, plus *F. proliferatum* KGL0401 was found to be 2 times stronger than the wild-type *G. fujikuroi* as regards overgrowth symptoms in waito-c [13]. Accordingly, the present study analyzed the proteins in waito-c rice after treatment with the culture fluid of *F. proliferatum* KGL0401, so as to identify the proteins induced by the addition of a culture fluid containing GAs.

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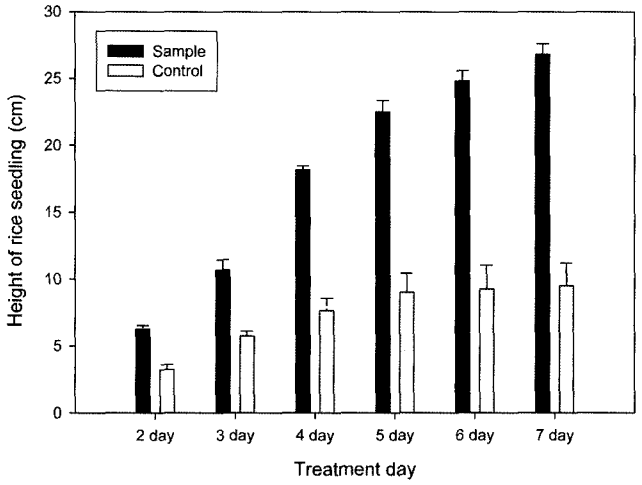

Fig. 1. Effect of *F. proliferatum* KGL0401 culture fluid and media on waito-c rice.
The height of the waito-c rice treated with the culture fluid of *F. proliferatum* KGL0401 (sample) and medium (control) gradually increased for 7 days, and reached 26 cm in the case of treatment with the culture fluid after 7 days at 30°C, 120 rpm.

The surface of the rice(waito-c) seeds was sterilized in a spotac (Aventis cropsciences) solution with Uniconazol (20 ppm) for 1 day, after being washed with distilled water. The surface-sterilized watio-c rice seeds were then placed on a water agar (0.8%).

The isolated *F. proliferatum* KGL0401 was cultured in 40 ml of Czapek's liquid medium (1% glucose, 1% peptone, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and pH 7.2±0.2) at 30°C, 120 rpm for seven days. The culture fluid was then concentrated 30-fold and applied to 2-leaf waito-c rice seedlings that had been grown for 7 days at 30°C (Fig. 1).

To determine the proteins related to the effect of the *F. proliferatum* KGL0401 culture fluid on the waito-c rice, the proteins were analyzed by electrophoresis. The rice seedlings were grown for 7 days, then directly homogenated using a motor-driven homogenizer (PowerGen125, Fisher Scientific) in a sample lysis solution composed of 7 M urea, 2 M Thiourea, containing 4% (w/v) 3-[(3-cholamidopropy) dimethyammonio]-1-pro-panesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT), and 2% (v/v) pharmalyte, and 1 mM of benzamidine. The proteins were extracted for 1 h at room temperature with vortexing. After centrifugation at 15,000 ×g for one hour at 15°C, the insoluble material was discarded and the soluble fraction used for two-dimensional gel electrophoresis. The protein loading was normalized by a Bradford assay [1]. A large number of protein spots were separated on the 2-D PAGE gel, and most of the proteins identified from the control treated with the medium and those from the sample treated with the culture fluid of *F. proliferatum* KGL0401 were separated with a similar electrophoretic mobility in the 2-D PAGE (Fig. 2A, 2B). However, 180 protein spots were overexpressed in the sample treated with the culture fluid.

A quantitative analysis of the digitized images was carried out using PDQuest software (version 7.0, BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by the total valid spot intensity. Protein spots with a significant expression variation of over two-fold compared with the control or normal samples were also selected.

The results revealed increased levels of 51 proteins after treatment with the culture fluid of *F. proliferatum* KGL0401 (Fig. 2C).

The protein spots were digested enzymatically in a gel in a manner similar to that previously described by Shevchenko *et al.* [16]. Thereafter, the gel pieces were washed with 50% acetonitrile to remove the SDS, salt, and stain, dried to remove the solvent, then rehydrated with trypsin (8–10 ng/μl) and incubated for 8–10 h at 37°C. The proteolytic reaction was terminated by the addition of 5 μl of 0.5% trifluoroacetic acid. The tryptic peptides were recovered by combining the aqueous phase from several extracted gel pieces with 50% aqueous acetonitrile. After desalting the concentration of the peptide mixture using C_{18}ZipTips (Millipore), the peptides were eluted in 1–5 μl of acetonitrile. An aliquot of this solution was then mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile, and 1 μl of mixture spotted on a target plate.

An MALDI-TOF analysis and database search were carried out (Table 1), and the protein analysis performed using an Ettan MALDI-TOF (Amersham Biosciences). The peptides were evaporated with a N_2 laser at 337 nm, where a delayed extraction approach was used. Plus, the peptides were accelerated with a 20 Kv injection pulse for a time-of-flight analysis. Each spectrum was the cumulative average of 300 laser shots. The search program ProFound, developed by Rockefeller University (http://129.85.19.192/profound_bin/WebProFound.exe), was used for the protein identification by peptide mass fingerprinting. The spectra were calibrated with a trypsin auto-digestion ion peak *m/z* (842.510, 2211.1046) as the internal standard.

The pathways involved in GA biosynthesis and signal transduction have been elucidated over the past several years [12]. Plus, at the gene expression level, several GA-regulated genes have already been identified in the shoots, leaves, flowers, and streams of various plants [17, 18, 21, 22]. However, little is still know about the proteins regulated by GAs in rice, and only a few proteomic analyses of the effect of GAs on plant growth have been reported. The role of GAs in the germination of Arabidosis seeds has showed that α-tubulin, a cytoskeleton component, is a prime target of GAs in this system [3].

In this study, 2D-PAGE was used to identify the GA-regulated proteins after treatment with *F. proliferatum*

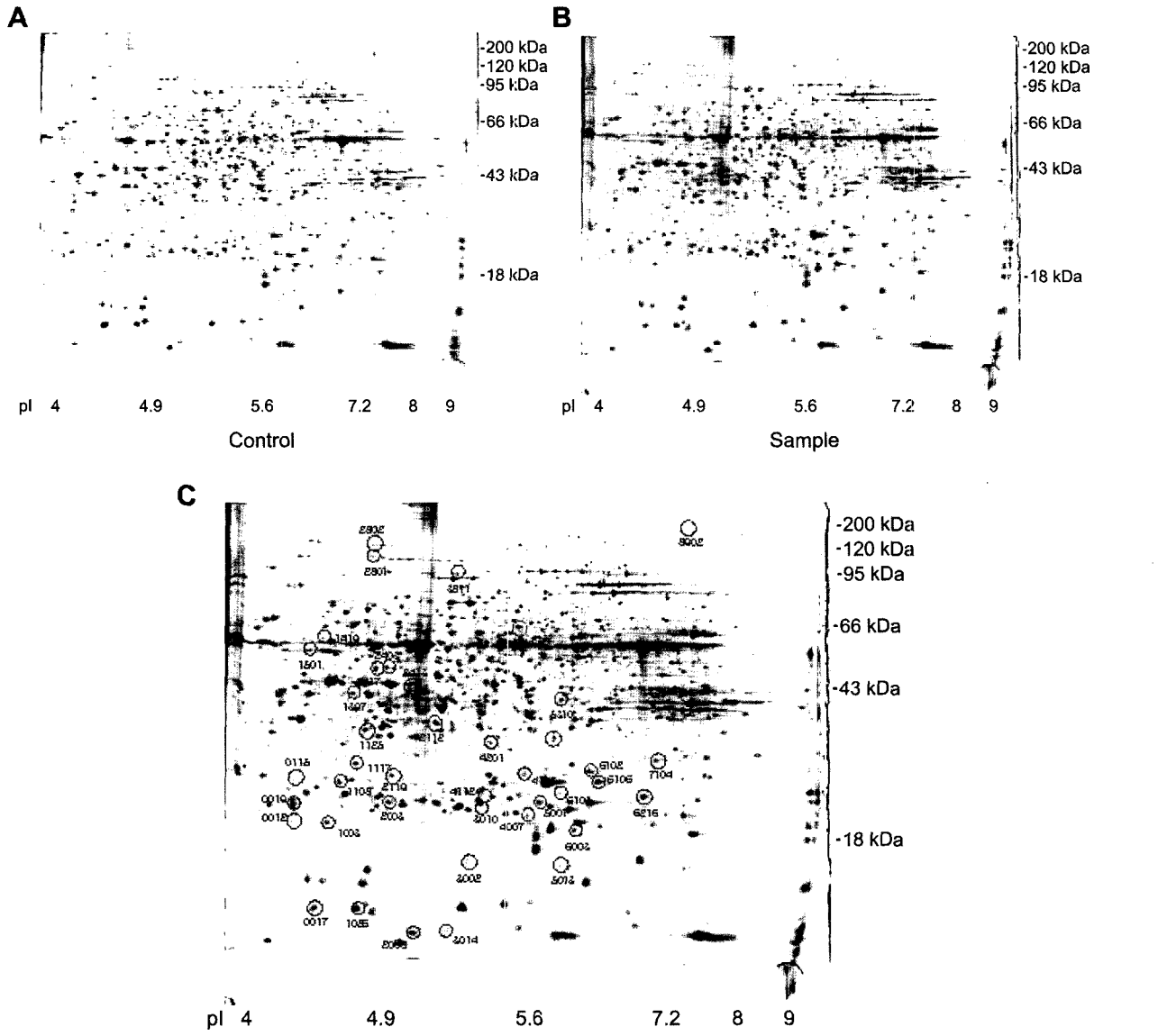

Fig. 2. 2-D PAGE patterns of waito-c rice proteins.
(**A**) and (**B**): proteins extracted from waito-c rice incubated for 7 days in treated medium and *F. proliferatum* KGL0401 culture fluid, respectively, and separated by 2-D PAGE, (**C**): proteins selected for MALDI-TOF, where the up and down-regulated proteins are marked.

KGL0401 (Fig. 2), and 51 proteins exhibited upregulated protein levels in the culture fluid of *F. proliferatum* KGL0401. These regulated proteins were involved in several functions and included glutathione S-transferase, a hypothetical protein, actin, heat-shock protein, tubulin alpha-1 chain, Ribulose-1, 5 bisphosphate carboxylase/oxygenase (RuBisCO), RuBisCO activase, and unknown proteins. In particular, RuBisCO activase, which is the key enzyme in carbon assimilation during photosynthesis, has already been identified as a GA-binding protein in rice. RuBisCO activase is a nuclear-encoded chloroplast regulatory protein that controls the activity of RuBisCO. Plus, RuBisCO activase has been shown to be phosphorylated with Ca^{2+}, Mg^{2+}, and ATP from rice leaves grown in the presence of GAs, thereby suggesting a role in GA signaling [14]. The tubulin alpha-1 chain and actin have also been identified as GA_3-upregulated proteins. Microtubules are involved in many cellular processes, such as cell division, cell transport, and cell elongation in plants, and tubulins are the major protein in microtubules [23]. Rice has at least eight actin genes, which play an important role in cytoplasmic streaming, cell shape determination, cell division, organelle movement, and extension growth. Furthermore, proteins of unknown function were also included in the list of GA-regulated proteins. Therefore, the information revealed by the treatment of waito-c rice with the *F. proliferatum* KGL0401 culture

Table 1. Identification of waito-c rice proteins by MALDI-TOF mass spectrometry.

Spot No	pI	kDa	Homologus protein[a]	Homology (%)	Accession
0012	4.4	20.6	drought-induced S-like ribonuclease	31	AY061961
0017	4.5	10.8	ribulose 1,5-bisphosphate carboxylase/oxygenase	16	NP039391
0019	4.3	18.5	RNase S-like protein	27	AAL17717
0115	4.3	23.8	putative caffeoyl-CoA O-methyltransferase 1	18	XP483167
0618	4.5	55.7	putative terpene synthase	15	AAR87368
1003	4.6	18.5	Proteasome subunit beta type 3	15	Q9LST7
1023	4.9	20.5	20S proteasome beta 4 subunit	32	AAQ19367
1108	4.7	23.9	putative caffeoyl-CoA O-methyltransferase 1	24	BAA81774
1117	4.8	26.7	hypersensitive-induced response protein	28	AAK54610
1125	4.9	31.5	glyoxalase I	29	XP480480
1126	4.9	21.7	putative glutathione S-transferase	31	AAM12323
1307	4.8	41.2	Actin	18	AAK84456
1417	4.9	47.7	OSJNBa0072K14.5	18	CAD40552
1419	4.8	44.9	putative actin	15	BAB63635
1501	4.6	52.9	Tubulin alpha-1 chain	25	P28752
2003	5.0	21.1	Ferritin	12	AAN74942
2008	5.1	9.3	ribulose 1,5-bisphosphate carboxylase	17	AAA84592
2102	4.9	22.7	OSJNBa0070M12.12	34	XP474434
2112	5.0	29.6	putative ATP synthase gamma chain 1	23	XP478377
2113	5.1	31.5	glyoxalase I	31	BAA36759
2119	5.1	30.6	sucrose-UDP glucosyltransferase 2	12	NP909830
2206	4.9	34.9	OSJNBb0018J12.16	22	CAE04830
2403	4.9	48.0	monodehydroascorbate reductase	16	BAA77282
2413	5.0	43.7	actin 1-rice	24	ATRZ1
2801	4.9	92.6	putative heat-shock protein	18	BAB32902
2802	4.9	99.6	putative carbamoyl phosphate synthetase	20	XP463327
3002	5.1	15.1	putative alcohol dehydrogenase	17	XP919944
3010	5.4	20.9	putative glutathione S-transferase GST 24	32	BAB86196
3014	5.2	9.4	Ribulose bisphosphate carboxylase	18	P05347
3203	5.2	33.1	probable enoyl-[acyl-carrier-protein] reductase	17	T03735
3620	5.3	63.5	putative transcription factor IIIA	9	XP475509
3811	5.3	84.7	pyruvate, phosphate dikinase	7	T02979
4003	5.5	19.6	putative glutathione S-transferase OsGSTU6	18	AAG32469
4007	5.6	19.3	unknown	15	AAO72697
4103	5.4	30.8	myosin-like protein	15	AAO72674
4112	5.5	21.6	Proteasome subunit alpha type 3	22	Q9LSU0
4116	5.6	25.2	putative glyoxalase II	14	BAB90501
4201	5.0	34.0	similar to putative adenylate kinase	27	XP493821
4724	6.0	65.0	putative phosphoglycerate dehydrogenase	14	XP482675
5013	6.0	14.0	hypothetical protein	44	NP920319
5310	6.0	40.0	DNA-directed RNA polymerase	11	RNZMA
5215	6.0	33.0	putative serine/threonine kinase -related protein	10	XP478599
6001	6.0	21.0	unnamed protein product	15	BAA89582
6003	6.0	18.0	putative quinone oxidoreductase	21	CAD31838
6103	6.0	26.0	glucanase	20	AAK16694
6106	6.0	24.0	putative transcription factor	16	AAL79687
6216	6.0	33.0	Ribulose bisphosphate carboxylase/oxygenase activase	14	P93431
7010	7.0	19.0	putative 1,4-benzoquinone reductase	34	NP916411
7104	6.6	32.3	putative NADPH-thioredoxin reductase	12	XP467446
7405	6.6	50.4	26S proteasome regulatory particle triple-A ATPase subunit1	20	XP468146
8902	7.3	113.1	putative S-locus protein 4	6	XP480436

[a]Pro Found Ver4.10.5 was used for the protein identification.

fluid will be helpful in predicting the functions of many other proteins in response to environmental challenges, and for molecular cloning in future experiments.

Acknowledgement

This work was supported by a grant from the BioGreen 21 program of the Rural Development Administration, Republic of Korea.

REFERENCES

1. Bradford, M. M. 1976. A rapid and sensitive method for the quantities of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
2. Choi, W. Y., S. O. Rim, J. H. Lee, J. M. Lee, I. J. Lee, K. J. Cho, I. K. Rhee, J. B. Kwon, and J. G. Kim. 2005. Isolation of gibberellins-producing fungi from the root of several sesamum indicum plants. *J. Microbiol. Biotechnol.* **15:** 22–28.
3. Gallardo, K., C. Job, S. P. C. Goot, M. Puype, H. Demol, J. Vandekerckhove, and D. Job. 2002. Proteomic of arabidopsis seed germination. a comparative study of wild-type and gibberellin-deficient seeds. *Plant Physiol.* **129:** 823–837.
4. Hedden, P. and A. L. Phillips. 2000. Gibberellin metabolism: New insights revealed by the genes. *Trands in Plant Sci.* **5:** 523–530.
5. Kim, M. J., H. J. Chung, S. M. Park, S. G. Park, D. K. Chung, M. S. Yang, and D. H. Kim. 2004. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-based cloning of enolase, ENO1, from *Cryphonectria parasitica*. *J. Microbiol. Biotechnol.* **14:** 620–627.
6. Koller, A., M. P. Washburn, B. M. Lange, N. L. Andon, C. Deciu, P. A. Haynes, L. Hays, D. Schieltz, R. Ulaszek, J. Wei, D. Wolters, and J. R. Yates. 2002. Proteomic survey of metabolic pathways in rice. *Plant Biol.* **18:** 11969–11974.
7. Komatsu, S., A. Muhammad, and R. Rakwal. 1999. Separation and characterization of proteins from green and etiolated shoots of rice (*Oryza sativa* L.): Towards a rice proteome. *Electrophoresis.* **20:** 630–636.
8. Komatsu, S., H. Konishi, S. Shen, and G. Yang. 2003. Rice proteomics: A step toward functional analysis of the rice genome. *Mol. Cell. protemics.* **2:** 2–10.
9. Komatsu, S., T. Matsuda, and H. Hirano. 1996. Rice gibberellin-binding phosphoprotein structurally related to ribulose-1, 5-bisphosphate carboxylase/oxygenase activase. *FEBS Lett.* **384:** 167–171.
10. Lange, T. 1998. Molecular biology of gibberellin synthesis. *Planta* **204:** 409–419.
11. Metzger, J. D. 1995. Hormones and reproductive development, pp. 617–648. *In* P. J. Davies (ed.), Plant Hormones, Dordrecht, The Netherlands: Kluwer Academic Publishers.
12. Olszewski, N., T.-p. Sun, and F. Gubler. 2002. Gibberellin signaling: Biosynthesis, catabolism, and response pathways. *Plant Cell* **14:** 61–80.
13. Rim, S. O., J.-H. Lee, W.-Y. Choi, S.-K. Hwang, S.-J. Suh, I.-J. Lee, I.-K. Rhee, and J.-G. Kim. 2005. *Fusarium prolifertum* KGL0401 as a new gibberellin-producing fungus. *J. Microbiol. Biotechnol.* **15:** 809-814.
14. Sharma, A. and S. Komatsu. 2002. Involvement of Ca2+ dependent protein kinase component downstream to the gibberellin-binding phosphoprotein, RuBisCO Activase, in Rice. *Biochemical and Biophysical Research Communications* **290:** 690–695.
15. Shen, S., A. Sharma, and S. Komatsu. 2003. Characterization of proteins responsive to gibberellin in the leaf-sheath of rice (*Oryza sativa* L.) seedlings using proteome analysis. *Biol. Pharm. Bull.* **26:** 129–136.
16. Shevchenko, A., M. Wilm, O. Vorm, and M. Mann. 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem.* **68:** 850–858.
17. Shi, L., R. T. Gast, M. Gopalraj, and N. E. Olszewski. 1992. Characterization of a shoot-specific, GA_3 and ABA-regulated gene from tomato. *Plant J.* **2:** 153–159.
18. Speulman, E. and F. Salamini. 1995. GA_3-regulated cDNA from *Hordeum vulgare* leaves. *Plant Mol. Biol.* **28:** 915–926.
19. Tanaka, N., H. Konishi, M. M. K. Khan, and S. Komatsu. 2004. Proteome analysis of rice tissues by two-dimensional electrophoresis: An approach to the investigation of gibberellin regulated proteins. *Mol. Gen. Genomics* **270:** 485–496.
20. Tudzynski, B., M. Mihlan, M. C. Rojas, P. Linnemannstons, P. Gaskin II, and P. Hedden II. 2003. Characterization of the final two genes of the gibberellin biosynthesis gene cluster of *Gibberella fujikuroi*: Des and P450-3 encode GA4desaturase and the 13-hydroxylase, respectively, *J. Biol. Chem.* **278:** 28635–28643.
21. Van den Heuvel, K. J., R. H. Van Lipzig, G. W. Barendse, and G. J. Wullems. 2002. Regulation of expression of two novel flower-specific genes from tomato (Solanum lycopersicum) by gibberellin. *J. Exp. Bot.* **53:** 51–59.
22. Van der Knaa, E., J. H. Kim, and H. Kende. 2000. A novel gibberellin induced gene from rice and its potential regulatory role in stem growth. *Plant Physiol.* **122:** 695–704.
23. Yang, G. and S. Komatsu. 2004. Microarray and proteomic analysis of Brassinosteroid- and Gibberellin-regulated gene and protein expression in rice. *Geno. Prot. Bioinfo.* **2:** 77–83.