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RESEARCH ARTICLE

A proteomic approach to investigating the promotive effects of brassinolide on root growth of rice seedlings

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Abstract **Brassinolide influenced rice (*Oryza sativa* L.) root growth in a concentration-dependent manner. Roots grown in 0.1 nmol/L BL were 28% longer than those in the control. On the other hand, the roots grown in 1000 nmol/L BL were 65% shorter in comparison with those in the control. To investigate how BL influences rice root growth, proteome analysis techniques were applied. After BL treatments, total proteins from rice root were extracted separately. Extracted proteins were separated by two-dimensional polyacrylamide gel electrophoresis and analyzed using an automated protein sequencer and mass spectrometer. There were 33 proteins that showed differences in the accumulation levels as a result of treatments with BL. Proteins related to stress tolerance, enzymes, and cell structure were mainly found in the root. There were many proteins regulated by other hormone or light also. Results suggest that the physiologic functions of these proteins detected using powerful proteome analysis are implicated in root elongation triggered by BL. High-level brassinosteriod (BR) indicated that IAA amidohydrolase, which can release active IAA from IAA pool was increased, and the IAA level was so high that the root length was inhibited.**

Keywords rice, proteomics, brassinolide, root growth

Introduction

Root growth regulation is highly important for plants and is controlled by plant hormones. Brassinosteriod (BR) is one of the important hormones of plants. It participates in the regulation of a large variety of developmental processes, from seed germination to cell elongation, fruit ripening, organ senescence, abscission, and stress protection (Mandava, 1988). More and more scientists have recognized the importance of BRs during plant development and identified genes involved in BR biosynthesis and signal transduction. Many groups are beginning to study other related genes involved in BR signal transduction using genetic screening in *Arabidopsis*. Up to now, the basic pathway of BR signal is clear already in *Arabidopsis*. In contrast with the rapid progress in understanding how the plant steroid signals reach the nucleus to regulate gene activities, little is known about how BRs induce nongenomic responses at the cell membrane or in the cytosol.

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Proteomics is used to analyze gene products in various tissues and physiologic states of cells (Pandey and Mann, 2000), and it has become very important in the functional genomic field now that several genomes have been sequenced completely and analytical methods for protein characterization have been developed. Progress in plant proteomics has largely been made possible by two-dimensional (2D) gel electrophoresis-based proteomic approaches (van Wijk, 2001).

In this study, after BR treatment, the total protein from rice root, rice suspension cells, and two BR-related rice mutants was isolated and analyzed using two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF MS). In an attempt to identify the proteins involved in BR response, we evaluated BR-induced changes in the isolated proteins. 2D gel analysis revealed that 33 proteins were either upregulated or downregulated by BR treatment. Of them, the actin depolymerizing factor, glycine-rich RNA binding proteins, and IAA amidohydrolase may play important roles in the BR-regulated effect. As a result, we concluded that the proteomic approach may be an effective means of identifying novel proteins implicated in hormone response.

Materials and methods

Plant growth assay

When the root length grew to 1 cm, germinated rice seeds (*Oryza sativa* L. japonica) were sown in basins filled with liquid culture medium of 0.1, 1.0, 10, 100, and 1000 nmol/L BL separately. The culture medium containing the same volume of 80% ethanol was used as the control. These basins were put in the greenhouse with the medium exchanged everyday to keep the culture medium invariable. Seven days later, the seedlings were removed from each basin, and the root and shoot lengths were measured.

Plant material and growth condition

Rice seedlings were cultured with liquid culture medium and maintained in the culture box. Seven-day-old seedlings were transplanted into the liquid culture medium with 0.1 nmol/L and 1000 nmol/L BL separately. The culture medium of 80% ethanol was used as the control. Root was taken after being treated for 6 h to extract proteins and RNA.

Protein extraction

About 0.1 g root powder (ground under liquid nitrogen) was weighted in a tube, added with 1 mL 10% trichloroacetic acid/acetone, stored at −20°C overnight, and centrifuged at 14000 r/min for 20 min at 4°C. After removing the supernatant and washing the pellet twice in 1 mL ice-cold acetone, the acetone was removed away as possible. Thereafter, the pellet was suspended in 100 μL lysis buffer containing 9 mol/L urea, 4% (w/v) CHAPS, 2% ampholine at pH 3–10 (Amersham Biosciences), and 1% DTT, incubated for 2 h at room temperature and pipetted up and down to dissolve the proteins. Then, it was incubated at 30°C for 1 h and spun in Eppendorf microcentrifuge at 4°C for 20 min at 14000 r/min to obtain the supernatant, which was used to determine protein concentration and identify proteins by 2D-PAGE.

RNA extraction

RNA extraction was conducted by grinding 0.1 g tissue in liquid nitrogen, transferring the powder into 1.5 mL tubes containing 1 mL Trizole, and adding 200 μL chloroform to each tube, which was centrifuged at 12000 r/min for 5 min at 4°C. When aqueous phases were carefully pipetted into clean screw-cap centrifuge tubes (interphase and lower phase used to extract protein), equal volume isopropanol was added to each of the aqueous phase, covered, and mixed by gentle inversion. After centrifugation at 12000 r/min at 4°C for 5 min, the supernatant was discarded, and the pellet was washed twice with 700 μL of 75% ethanol by votexing briefly and then recentrifuged at 12000 r/min at 4°C for 2 min. Moreover, after discarding the supernatant and briefly drying the pellet (5–10 min; not longer), 20 μL DEPC-H_2O was added.

Gel electrophoresis

Prepared samples were separated in the first dimension by isoelectric focusing (IEF) and in the second dimension by sodium dodecyl sulfate (SDS)-PAGE. The protein with its concentration determined was diluted with rehydration buffer of 9 mol/L urea, 4% CHAPS, 0.5% Ampholines (pH 3.0–10.0), 1% DTT, and 0.002% Bromophenol Blue. IPG electrophoresis was carried out at 0 V for 12 h, followed by 500 V for 1 h and 1000 V for 1 h, and 8000 V for 9.5 h. After IPG, SDS-PAGE in the second dimension was performed using 15% polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue (CBB), and the image analysis was performed. Images of 2D-PAGE were synthesized, and the positions of individual proteins on the gels were evaluated automatically using ImageMaster 2D Elite software (Amersham Biosciences). Protein spots whose intensity in the BR-treated medium was altered relative to the untreated control medium were quantitatively analyzed. In addition, those spots whose abundance was varied reproducibly greater than twofold were considered for identification by MALDI-TOF MS.

MALDI-TOF MS and database searching

For protein identification by MALDI-TOF MS, one aliquot of the enzyme digest solution was spotted onto a sample plate with matrix (α-cyano-4-hydroxcinnamic acid, 8 mg/mL in 50% v/v TFA) and allowed to air dry. MALDI-TOF MS acquisition was an Autoflex MALDI mass spectrometer (Bruker Daltonics, Germany), equipped with a flight tube (reflex mode, 2.6 m long), laser (337 nm), and scout 384 targets system. Accelerating voltage was 20 kV and micro-channel plate (MCP) detector worked at 1.6 kV. Mass spectra were acquired in positive mode. Known trypsin autocleavable peptide masses (906.51 Da and 2273.16 Da) were used for a two-point internal calibration for each spectrum. Peptide mass fingerprinting (PMF) was searched against NCBI protein databases using the search engine Matrix Science at http://www.matrixscience.com. *Oryza sativa* was chosen for the taxonomic category. All peptide masses were assumed as monoisotopic $[M + H]^+$ (protonated molecular ions). Searches were conducted using a mass accuracy of ± 100 ppm, and one missed cleavage site was allowed for each search.

RT-PCR

The concentration of RNA was accurately quantified by spectrophotometric measurement, and 1 μg total RNA was separated on 1% formaldehyde agarose gel to check their concentration and to monitor their integrity. Five hundred ng

of total RNA was used in an RT-PCR system together with gene-specific primers. Control RT-PCR was performed with the same amount of total RNA using the primer pair specific to the tublin gene. Twenty microliter of each RT-PCR product was loaded on 0.8% (w/v) agarose gel to visualize the amplified cDNAs.

Results

Effects of different concentration of brassinolide on rice seedlings

Series concentrations of 0.1, 1, 10, 100, and 1000 nmol/L BL solution were separately added to the culture medium of the rice seedlings grown in a greenhouse for five days when their root length reached 1cm. It was found that the root growth was influenced by different concentrations of BL, which promoted or inhibited the rice root elongation, and the influence was concentration dependent. There was approximately 28% increase in root elongation at 0.1 nmol/L BL, and 65% root growth was inhibited at 1000 nmol/L BL as compared with the control, and these differences were significant (Table 1). The rice root was stained using PI and observed with confocal fluorescence microscope, which indicated that the cells of mature zone of the root had shorter length and wider width in 1000 nmol/L BL treatment than in the control. Fewer meristem layers in 1000 nmol/L BL treatment but more meristem layers in 0.1 nmol/L BL treatment were found as compared those in the control. A longer mature zone in that 0, 0.1 nmol/L BL treatment was observed as compared to that in the 1000 nmol/L BL treatment. There were more cells outside the top of the root of BL treatments than that in the control (Fig. 1).

2D-PAGE separation of the total proteins of rice root after BL treatments

The results showed that there was an opposite effect on the rice root growth between 0.1 nmol/L and 1000 nmol/L BL treatment. To find the mechanism of the regulation of BL on rice development, we analyzed the difference of the total proteins of the root treated with 0.1 and 1000 nmol/L BL using proteomic method.

The total protein was extracted from the root tissue and separated in the first dimension by IPG and in the second

Table 1 The root length of rice seedlings after a series of BL treatment for five days

BL(mol/L)	0	1×10^{-10}	1×10^{-9}	1×10^{-8}	1×10^{-7}	1×10^{-6}
Root length/cm	12.54	16.02*	14.82	9.41*	4.76*	4.33*

Note: * indicates a statistically significant difference at $P = 0.05$.

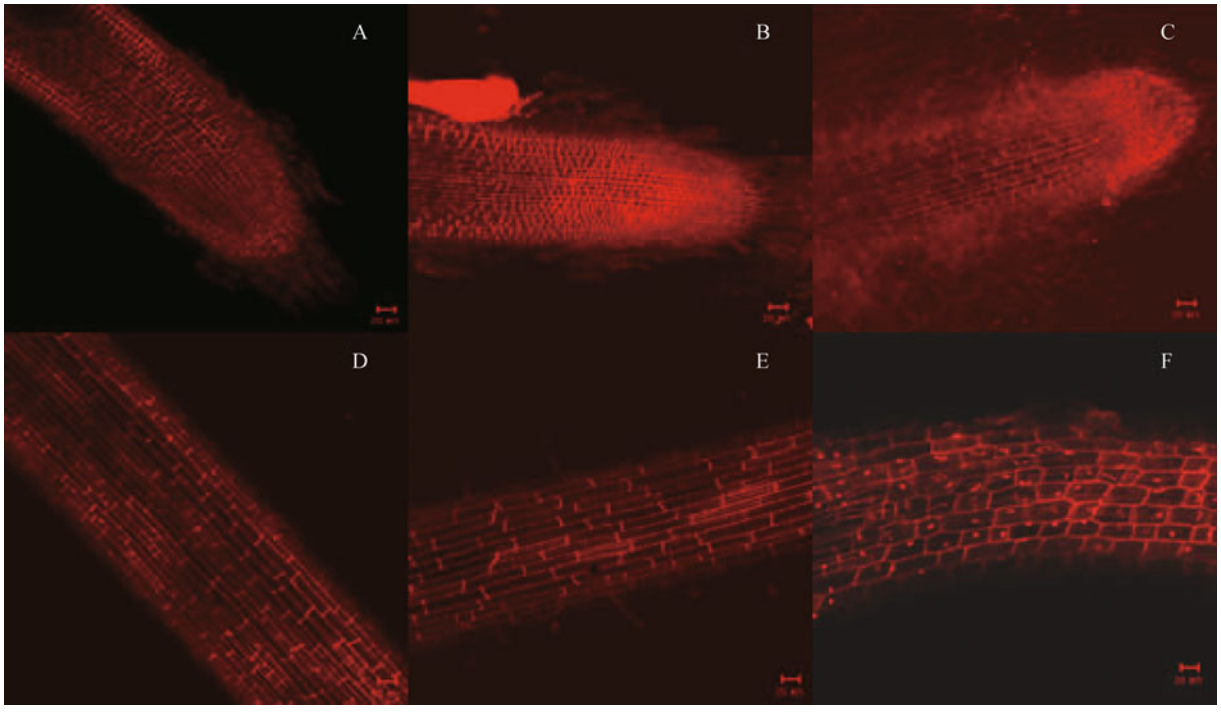

Figure 1 The cell shape in rice root stained using PI and observed with confocal fluorescence microscope. A–C show the root top, representing control, 0.1 nmol/L BL treatment, and 1000 nmol/L BL treatment, respectively. D–F show the mature zone, representing control, 0.1 nmol/L BL treatment, and 1000 nmol/L BL treatment, respectively.

Table 2 Proteins regulated by BR in root total protein after 6 h BL treatment

Spot no.	Code	Protein matched to	Conserved domain	Fold changes		
				0 nmol/L	0.1 nmol/L	1000 nmol/L
1	gi|19920119	Putative retroelement	Reverse transcriptase	−1000000	1000000	−1000000
2	gi|37535140	Mitochondrial chaperonin-60	TCP-1/cpn60 chaperonin family	1000000	−1000000	1000000
3	gi|51535280	Putative PrMC3	Esterase_lipase	−1000000	1000000	−1000000
4	gi|53749382	Putative ferredoxin sulfite reductase	Nitrite and sulphite reductase 4Fe-4S domain	6.86713	−6.86713	−2.67854
5	gi|34894766	putative peroxidase	secretory peroxidases	4.55727	−4.55727	−1.99768
6	gi|50910077	Translational elongation factor Tu	Elongation factor Tu GTP binding domain	12.0737	−12.0737	−1.78652
7	gi|62733235	dnaK-type molecular chaperone hsp70 - rice	Hsp70 protein	−4.97645	−10.7623	10.7623
8	gi|1296955	osr40c1	Not putatively conserved domains	1000000	−1000000	−2.76531
9	gi|50919967	Putative IAA amidohydrolase	Glutamate carboxypeptidases	−3,87622	−1000000	1000000
10	gi|50918345	actin	actin	−1000000	1000000	−1000000
11	gi|57900055	Putative ubiquitin-specific protease 6	Intracellular peptidases	−6.87645	−2.67543	6.87645
12	gi|50931297	Unknown protein	Annexin repeats	−15.78462	15.78462	−1.76231
13	gi|50923903	947OSJNBa0072K14.5	2-oxoacid dehydrogenases acyltransfer	−2.56435	−1.64310	2.56435
14	gi|50924436	OSJNBa0006B20.1	Regulator of chromosome condensation	1000000	−1000000	−1000000
15	gi|50933679	Putative peroxidase 1 precursor	Peroxidase	1000000	−1000000	−3.87465
16	gi|50946591	Putative activator of 90 kDa heat shock protein ATPase homolog 1	Heat shock protein	−20.23759	20.23759	−20.23759
17	gi|53749369	Putative 1,4-benzoquinone reductase	Flavodoxin_1	1000000	−1000000	−1000000
18	gi|34908134	Osmotin-like protein	Thaumatin family	−1.87492	−30.64921	30.64921
19	gi|56784135	Putative Y1 protein	WD40 domain (signal transduction)	−2.98641	1000000	−1000000
20	gi|295885	Actin	Actin	−1.58299	3.652491	−3.652491
21	gi|303844	Eukaryotic initiation factor 4A	DEAD-box helicases, helicase superfamily c-terminal domain	−1000000	1000000	−1000000
22	gi|50918875	Putative UDPglucose dehydrogenase	UDPglucose/GDP-mannose dehydrogenase family	20.38299	−1.99869	−20.38299
23	gi|2662343	EF-1 alpha	Elongation factor Tu GTP binding domain	1000000	−1000000	1000000
24	gi|50940457	Putative fructokinase	Fructokinases	−1000000	−1000000	1000000
25	gi|50898962	Putative D-protein	Kelch motif	2.05292	−2.05292	−0.67239
26	gi|50935067	Putative stationary phase survival protein SurE	Survival protein SurE	1000000	−1000000	1000000
27	gi|49532749	Radc1	Not putatively conserved domains	−0.63927	2.21973	−2.21973
28	gi|46798901	Kelch repeat containing protein	Kelch motif	−1000000	1000000	−1000000
29	gi|50918681	Cysteine synthase	Pyridoxal-phosphate dependent enzyme	2.7625	3.9254	−2.7625
30	gi|50355737	Putative DnaJ like protein	DnaJ domains	−3.01862	−1.09217	3.01862
31	gi|50918709	Putative exoglucanase precursor	Glycosyl hydrolase family 3 domain..	−2.15790	2.15790	−2.08721
32	gi|34897872	Putative phosphogluconate dehydrogenase	6-phosphogluconate dehydrogenase	−1000000	−1000000	1000000
33	gi|34895730	Unknown protein	Mitochondrial glycoprotein	−3.98092	−4.53826	4.53826

dimension by SDS-PAGE. Approximately, 800 protein spots were detected on Comas Blue-stained 2D gel. Analysis of those protein spots whose abundance was varied reproducibly greater than twofold was conducted by MALDI-TOF MS, which led to the identification of 33 different proteins (Table 2). Of the proteins identified, most had their known functions or sequences similar to those of the known proteins, whereas there are some novel peptides that have not been assigned by any functions. All proteins were categorized into classes based on their functions. The BR-regulated proteins interacted in signaling pathways, such as auxin, stress cell development, etc. (Table 2).

BR possibly affected the IAA release from the conjugate form in rice

Our proteomics results show that 1000 nmol/L BL treatment upregulated IAA amidohydrolase, but 0.1nmol/L BL inhibited IAA amidohydrolase (Fig. 2). The RT-PCR results of IAA amidohydrolase also showed that the RNA level of IAA amidohydrolase was decreased when treated with 0.1 nmol/L BL and increased when treated with 1000 nmol/L BL (Fig. 3).

To confirm the IAA content after BL treatments, we identified the free IAA level using HLPC (Table 3). The results showed that the free IAA level of 0.1 nmol/L BL-treated root was decreased by 45.7% lower than that of control, while the IAA level of 1000 nmol/L BL treatment was increased to 17.3% higher than that of control.

Discussion

Indole-3-acetic acid (IAA) is a signaling molecule that modulates division and elongation of plant cells (Davies, 1995). Plants contain little free IAA, and most IAA is found conjugated to amino acids, peptides, sugars, or high molecular weight glycans. These conjugates have been implicated in such processes as storage, transport, and protection from oxidative degradation (Cohen and Bandurski, 1982). Plants produce active IAA by de novo synthesis and by hydrolyzing IAA conjugates (Bartel, 1997; Normanly, 1997). IAA conjugation activity is widely distributed in the plant kingdom from mosses to angiosperms (Sztein et al., 1995). IAA-conjugate hydrolases release free IAA from the conjugate form and thus are likely to play an important role in regulating free IAA levels. These hydrolases have been detected in bacteria and in a variety of plants (Hall and Bandurski, 1986; Kowalczyk and Bandurski, 1990; Chou et al., 1996; Bartel, 1997). Interestingly, the infection with *Plasmodiophora brassicae* (which causes a clubroot disease) correlates with a dramatic increase in the rate of IAA–Asp hydrolysis (Ludwig-Müller et al., 1996). This induction of one specific hydrolytic activity in response to a particular challenge suggests that various conjugate hydrolases might supply free IAA in response to a variety of needs. Several exogenous IAA conjugates mimic IAA (Feung et al., 1977; Hangarter et al., 1980; Šoškić et al., 1995), suggesting that these conjugates are either auxins or hydrolyzed to release

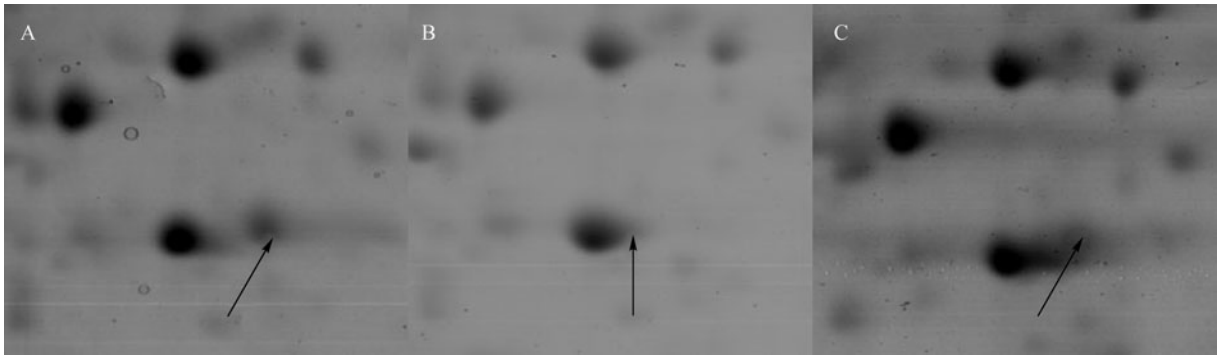

Figure 2 IAA amidohydrolase (part of 2D-PAGE of root protein). A–C represent control, 0.1 nmol/L BL treatment, and 1000 nmol/L BL treatment, respectively.

Figure 3 RT-PCR of IAA amidohydrolase. The left is control, the middle is 0.1 nmol/L BL, and the right is 1000 nmol/L BL.

Table 3 The IAA content of rice root after different concentration BL treatment for six hours

BL treatment	Control	0.1 nmol/L	1000 nmol/L
IAA content(mg/100 g)	0.81	0.44	0.98

IAA. Bartel et al. cloned the *ILR1* gene and found that it was an amidohydrolase, *ILR1* catalyzed IAA-amino acid hydrolysis, thus released active indole-3-acetic acid from conjugates. The *ILR1* (IAA-Leu-resistant) mutant elongates roots at concentrations of IAA-Leu that inhibit wild-type root growth.

The activity of indole-3-acetamide (IAM) hydrolase found in various organs of rice plants is prominent in wild and cultivated rice. In order for the rice amidohydrolase to serve in auxin biosynthesis, high concentrations of substrate must be present endogenously. However, no endogenous IAM was detected from various organs, such as shoots, roots, calli, and young fruits of rice. Therefore, it is unlikely that the rice enzyme is involved in auxin biosynthesis via the IAM pathway. As the enzyme has dual functions as amidase and esterase, therefore, the rice amide hydrolase may serve to control IAA accumulation via hydrolysis of the endogenous esterified IAA.

Our results found that the IAA amidohydrolase decreased when treated with 0.01 nmol/L BL, but it increased when treated with 1000 nmol/L BL. The low concentration of IAA promoted the elongation of root; however, very high concentration of IAA increased the elongation of root. In the 1000 nmol/L BL treatment, the IAA amidohydrolase increased, and the pool IAA was almost activated. In addition, BL stimulated polar auxin transport and modified the distribution of endogenous (Li, 2005), so the concentration of the activated IAA was very high with the phenotype of the root shortened and increased lateral roots when the rice seedling was treated with 1000 nmol/L BL. However, when the rice was treated with 0.01 nmol/L BL, the IAA amidohydrolase was low; therefore, there was a low level of activated IAA, and root elongation was promoted.

Acknowledgements

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