Proteomic analysis of *Arabidopsis* root gravitropism

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**Abstract** In gravitropism of *Arabidopsis* root, the *Arabidopsis* root proteins induced and/or changed by gravitational stimulation were analyzed using a proteomic method. In this study, it was found that the levels of expression of some proteins related to the cytoskeleton and the calcium signal transmission system had changed during gravitational stimulation. In addition, the β subunit of the E1 component of pyruvate dehydrogenase, fructose bisphosphate aldolase, and 20S proteasome β subunit E1 protein appeared in two different molecular weight types during gravitational stimulation. The transitory changes in molecular weight in these three identified proteins were important findings although their functions in the gravity response in the roots of *Arabidopsis* seedlings remain uncertain. As far as we know, this is the first study involving the molecular weight shift-change in the same protein during the gravitropism response in plants using proteomic analysis.

**Key words:** gravity, gravitropism, *Arabidopsis*, root, proteomics

**Introduction** Plants have the ability to adjust to various environments. Of the various environmental factors, such as gravity, moisture, temperature, and touch, gravity is a constant force that guides plant growth direction and development (Darwin 1880, Okada and Shimura 1994). When vertically growing plants are placed in a horizontal orientation, the so-called gravitational stimulation acts on the plants, resulting in the upward and downward growth of the apical shoot and root tip, respectively. This phenomenon in plants is called gravitropism (Massa and Gilroy 2003, Morita and Tasaka 2004). The cells responsible for the perception of gravity in plants are the endodermal cells in the shoot, which are essential for shoot gravitropism, and the columella cells in the root tip, which are essential for root gravitropism (Blancaflor et al. 1998, Fukaki et al. 1998). These gravisensing cells contain plastids filled with starch, namely amyloplasts, which exhibit sedimentary movement in the direction of the gravity vector (Morita and Tasaka 2004). It is thought that the sedimentation of amyloplasts is a likely trigger of subsequent intracellular signaling, and recently, there have begun to understand the molecular mechanisms related to gravity signal transduction and transmission, using gravitropism-defective mutants of *Arabidopsis* (Morita and Tasaka 2004). After gravity signal transduction and transmission, lateral transport of plant hormone, auxin, is induced, and auxin re-distribution is occurred at elongation zone (Cholodny 1927, Went 1933). As a result, the direction of the extension and/or the extension speed of the cell are controlled and the organ of the plant bends.

The analyses related to gravitropism and gravity signal transduction/transmission mechanisms were performed using DNA chip, DNA microarray, and suppression subtractive hybridization (SSH) (Centis-Aubay et al. 2003, Kimbrough et al. 2004, Moseyk et al. 2002). As a result, the contributions of many important genes involved in the gravity response mechanisms in plants have been understood. However, these methods involved the use of mRNA transcripts as analytical samples; hence, the changes that occur at the protein level are not understood.

A related concern is that substantial regulation of cellular events can occur at the protein level with no apparent changes in mRNA abundance. The posttranslational modification of proteins can result in a dramatic increase in protein complexity without a concomitant increase in gene expression. Proteomics may be the most promising technique for identification of proteins that are induced, repressed, and posttranslationally modified during gravity response in plants. Currently, proteomics is widely used in animal and plant studies for understanding physiological and biological mechanisms (Gallardo et al. 2001, Rabilloud 2002, Schiltz et al. 2004, Tsugita et al. 1996). In addition, proteomic analysis of the plants treated with stresses such as salinity and drought, helps in understanding the underlying molecular mechanisms (Dani et al. 2004, Hajheidari et al. 2005). However, proteomics research related to gravity response mechanisms in plants have...
not been reported. In our previous study, the protein expression patterns of Arabidopsis whole seedlings growing stationary condition and clino-rotated condition were compared by two-dimensional electrophoresis (Kamada et al. 2004). Develop more, and in this research, we analyzed and identified the gravity-regulated proteins that function in the gravity response mechanisms using the gravitationally stimulated Arabidopsis root apices.

**Materials and methods**

**Plant materials**

Wild type Arabidopsis thaliana (L.) Heynh. (ecotype Columbia) seeds were sterilized with 2% sodium hypochlorite, rinsed 3 times with sterilized water, and germinated on a 0.5× Murashige and Skoog medium plate containing several vitamins, 5 mM MES (pH 5.8), 1% (w/v) sucrose, and 2% (w/v) agar. The germination was performed according to the method of Soga et al. (2004) with slight modifications. The plates were kept at 4˚C in the dark for 2 days for vernalization and water absorption by the seeds; subsequently, the seeds were allowed to germinate at 23˚C in the light in a vertical stationary position. After 1 day, they were placed at 23˚C in the light in the vertical stationary position. After 1 week, for gravitational stimulation of the Arabidopsis seedling roots measuring ca. 25 mm in length, the orientation of the plates was changed from the vertical to a horizontal position (90 degrees) (Fig. 1). Following gravitational stimulation for several hours, approximately 10 mm of each root of Arabidopsis seedlings was cut from the root tip toward the stem and then frozen using liquid nitrogen. These samples included tissues from the root tip to the elongation zone.

**Extraction and purification of proteins**

For protein extraction, the root tissues of Arabidopsis seedlings were homogenized in 40 mM Tris buffer (pH 8.0) containing 7 M Urea, 2 M Thiourea, and 1% (w/v) ASB-14 detergent, which increases protein solubilization and enables the identification of previously undetected membrane proteins. The extracted sample was centrifuged at 15,000 × G for 10 min; the supernatant was collected and purified using ReadyPrep™ 2-D Cleanup Kit (Bio-Rad, Hercules, CA, USA). The purified samples were dissolved in a lysis buffer [8 M Urea, 2% (w/v) CHAPS, 50 mM DTT, 0.2% (v/v) Bio-Lytes3-10 (Bio-Rad), and 0.001% (w/v) BPB] containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany).

**Gel electrophoresis**

A 100μg aliquot of the total protein that was solubilized in the lysis buffer was separated in the first dimension by isoelectric focusing and in the second dimension by SDS-PAGE. Using an IPG strip gel pH 5.0-8.0 (Bio-Rad), the first dimension electrophoresis was carried out at 500 V for 30 min, followed by 4000 V for 5 h. After the first dimension electrophoresis, the IPG strip gel was treated with 0.375 M Tris buffer (pH 8.8) containing 6 M Urea, 2% (w/v) SDS, 130 mM DTT, and 20% (v/v) glycerol for 15 min; this was followed by treatment with 0.375 M Tris buffer (pH 8.8) containing 6 M Urea, 2% (w/v) SDS, 135 mM Iodoacetamide, and 20% (v/v) glycerol for 15 min. Subsequently, the second dimension SDS-PAGE was performed using a 10%-20% gradient polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan). The gels were stained with colloidal Coomassie blue, and image analysis was performed. Images of the two-dimensional electrophoresis were obtained, and the positions of individual proteins on the gels were evaluated automatically using PDQuest software (Bio-Rad). Three proteins on the gel were used as the internal standard for the protein expression profile. The expression of these three proteins did not change as compared with those in control and assay samples (see Fig. 3). The isoelectric point (pI) and molecular weight (Mr) of each protein were determined using respective markers (Bio-Rad).

**Fig. 1.** Schematic representation of the experimental design. The roots were subjected to gravitational stimulation by changing the orientation of the plates from a vertical to a horizontal position (90 degrees). Approximately 10 mm of each Arabidopsis seedling root was cut from the root tip toward the stem and used in this research.
**MALDI-TOF MS**

The stained protein spots were excised from the gels, destained twice with 25 mM NH₄HCO₃ in 30% Acetonitrile (ACN) for 15 min, followed by destaining twice with 25 mM NH₄HCO₃ in 50% ACN for 15 min. The gel pieces were minced and allowed to dry and then rehydrated in 50 mM NH₄HCO₃ with 1 pmol trypsin (Promega, Madison, WI, USA) at 37°C for 15 min. The digested peptides were extracted thrice from the gel slices with 0.1% Trifluoroacetate (TFA) in 50% ACN/water. The peptide solution thus obtained was dried and reconstituted with 30μl 0.1% TFA in 5% ACN/water and then desalted with ZipTip C18™ pipette tips (Millipore, Bedford, MA, USA). The peptide solution thus obtained was mixed with α-cyano-4-hydroxycinnamic acid. MALDI-TOF MS was performed using a Voyager-DE-STR TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA). The mass spectra were subjected to sequence database search for peptide mass fingerprinting (PMF) analysis using the Protein Prospector software (Regents from the University of California, CA, USA) (Clauser et al. 1999).

**Results and discussion**

First, we measured the gravitropic curvatures of the roots of Arabidopsis seedlings subjected to gravitational stimulation by reorientation (Fig. 2). The Arabidopsis seedling root curvatures toward the gravity vector reached 20 degrees within 0.5 h after reorientation (Fig. 2). Subsequently, the gravitational stimulation was continued for 3 h, and the roots bent by almost 60 degrees; thereafter, the gravitropic angles showed little change (Fig. 2). This result was almost similar to previously reported data (Hou et al. 2004). Therefore, in this study, the roots of Arabidopsis seedlings subjected to gravitational stimulation for 0.5 h and then for 3 h were used for proteomic research.

### Table 1 Proteins identified in the gravity-regulated expression of proteins in Arabidopsis root apices by MALDI-TOF and PMF analysis

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<td>59</td>
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<td>43/7.5</td>
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<td>27</td>
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### Decreased by gravitational stimulation

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<td>14</td>
<td>36</td>
<td>50/9.2</td>
<td>18390831</td>
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*The spot numbers correspond to the numbers in Fig. 3C. The experimental molecular weight and isoelectric point, theoretical molecular weight and isoelectric point, the number of MALDI-TOF-matched peptides, and the percentage of sequence coverage of the protein by the matched peptides are indicated as Exp. Mr/pl, Theo. Mr/pl, No. Match Pep., and % Seq. Cov., respectively.*
Arabidopsis root apices (10 mm) that include from the gravisensing columella cells to elongation zone were harvested to analyze the changes in protein expression in response to gravitational stimulation; this analysis was performed by proteomic approaches. In the two-dimensional electrophoresis, approximately 800 protein spots were detected. In particular, a large number of proteins were detected as compared with those detected after non-gravitational stimulation in a comparative analysis experiment on proteins that appeared immediately after Arabidopsis roots were subjected to gravitational stimulation for 0.5 h (Fig. 3A). In the spots that showed a decreased level of expression due to gravitational stimulation, the spot analysis detected 10 proteins. On the other hand, in the spots that showed an increased level of expression, 6 proteins were detected (Fig. 3B, C). These proteins were identified by MALDI-TOF MS analysis (Table 1). The proteins that showed decreased levels of expression due to gravitational stimulation were identified as actin 2/7 (15242516), tubulin α-6 chain (TUA6; 15233627), alcohol dehydrogenase class III (15240054), aspartate aminotransferase, cytoplasmic isozyme 1 (15239772), ATP synthase β chain 1 (18415909),...
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Fig. 4. Changes in the protein spot patterns obtained from *Arabidopsis* root apices subjected to gravitational stimulation. The panels in A, B, and C show the close-up images representing the molecular weights and isoelectric points in the ranges of 35-40 kDa and 5.6-5.8, 37-40 kDa and 7.2-7.4, and 26-30 kDa and 6.9-7.4, respectively. Arrowheads a1, a2, a3, b1, b2, c1, and c2 show the protein spots where a substantial molecular weight change has occurred.

glycine hydroxymethyltransferase (15236375), isocitrate dehydrogenase (15218869), quercetin 3-O-methyltransferase 1 (15239571), vitamin B12-independent methionine synthase (15238686), and heat shock cognate 70 kDa protein 2 (HSP70-2; 15241847).

On the other hand, the proteins that showed increased levels of expression due to gravitational stimulation were identified as glutathione S-transferase PM24 (24 kDa auxin-binding protein; 15235401), Ca2+-dependent membrane-binding protein annexin (15220216), Lipase/Acylhydrolase with GDSL-motif family (4587541), malate dehydrogenase (18404382), fructose bisphosphate aldolase-like protein (15231715), and elongation factor 1-α (18390831). The results suggested the cytoskeleton systems participated in the regulation of the gravity response in plants (Blancaflor 2002). Using microarray analysis, Kimbrough et al. (2004) have shown that the expression of cytoskeleton-related gene was decreased due to gravitational stimulation. Since these results were verified by our proteomic analysis, the change in the protein expression appears to occur through a change in the gene expression. Moreover, Kimbrough et al. (2004) have shown that the expression of other types of the alcohol dehydrogenase gene, aminotransferase gene, ATP synthase gene, and heat shock protein gene was down-regulated by gravitational stimulation, but that of other types of the elongation factor gene was up-regulated.

Therefore, these molecules appear to be regulated by gravitational stimulation at the level of gene and the protein expression. On the other hand, Kimbrough et al. (2004) have also shown that gravitational stimulation produced both types of effects on the expression of the glutathione S-transferase gene, that is, it was decreased as well as increased, and the expression of the annexin gene, Lipase/Acylhydrolase with GDSL-motif family gene, and malate dehydrogenase gene was down-regulated by gravitational stimulation. However, our results show the up-regulation of these proteins. This difference between the gene and protein expression indicates the presence of a peculiar gene or protein expression control mechanism. The increase or decrease in the expression of the glycine hydroxymethyltransferase gene, isocitrate dehydrogenase gene, quercetin 3-O-methyltransferase gene, vitamin B12-independent methionine synthase gene, and fructose bisphosphate aldolase gene has not been reported in previous microarray studies. The relationship between the decrease or increase in the expression levels of these identified proteins and the gravity response in plants is uncertain. However, it has been shown that the expression levels of genes that belong to the same family of identified proteins are changed by gravitational stimulation. Therefore, although it is necessary to conduct a detailed analysis of the functions of the identified proteins in the future, it seems that these proteins have the key roles immediately and indirectly in gravitropism response mechanisms.

In addition, when the spots were analyzed in detail, it was observed that proteins with different molecular weights appeared transitorily (Fig. 4). In the panel in Fig. 4A, a protein spot, a1, was detected before gravitational stimulation; however, spot a1 disappeared, and a spot about the position of molecular weight a2 was detected approximately 0.5 h after gravitational stimulation. Spot a2 having a low molecular weight disappeared at 3 h after gravitational stimulation; however, spot a3 having a molecular weight similar to that of spot a1 appeared. Using MALDI-TOF MS and PMF analysis, the spots a1, a2, and a3 were identified as the β subunit of E1 component of pyruvate dehydrogenase (15241286), which is an enzyme in the metabolic pathway, i.e., the TCA cycle, that synthesizes energy-ATP-in *vivo* (LeCler et al. 2004). As shown in the panels in Fig. 4B, spot b1 was detected within 0.5 h after gravitational stimulation, but the shift in molecular weight up to the b2 position occurred at 3 h after gravitational stimulation. Spots b1 and b2 were putatively identified as fructose bisphosphate aldolase (15226185), which corresponds to an enzyme involved in carbohydrate metabolism, i.e., in glycolysis (Angeles-Castillejo et al. 2004, Azama et al. 2003). In the panel shown in Fig. 4C, spot c1 was detected at 0.5 h after gravitational stimulation, but the shift in molecular weight up to position c2 occurred at 3 h after gravitational stimulation. Spots c1 and c2 were identified as 20S proteasome β subunit E1 (15222152), which is involved in the degradation/resolution system of proteins in the ubiquitin pathway (Moon et al. 2004, Fu et al. 1998). A change was detected in the molecular weights of the proteins but not the isoelectric point.
Therefore, in the case of the β subunit of E1 component of pyruvate dehydrogenase and fructose bisphosphate aldolase, the change in the molecular weights was not due to the association and/or dissociation of a peptide with a molecular mass of several thousand daltons; it may be due to a difference in the modification of a sugar chain, which is not accompanied with a change in the charge. In fact, the β subunit of E1 component of pyruvate dehydrogenase and fructose bisphosphate aldolase has two putative glycosylation sites in their peptides. Moreover, no difference was identified in either the number or the position of the peptide peaks that could be confirmed by the PMF analysis. The change in the molecular weights of the two proteins may be due to the following two possibilities: (1) firstly, it may be attributed to the modifications that might have occurred in the protein molecule during gravitational stimulation and (2) secondly, it may be due to the posttranslational modification of the protein that was newly translated from mRNA. A study reported that the E1 component of pyruvate dehydrogenase and fructose bisphosphate aldolase participate in the DnaK-ClpB related chaperone systems in E. coli. (Mogk et al. 1999). Therefore, it is suggested that these two types of protein enzymes may participate in the chaperone systems involved in the gravity response in Arabidopsis root. On the other hand, the 20S proteasome β subunit E1 protein was shown to be capable of self-cleavage at the amino-terminal residue in eukaryotes including Arabidopsis (Fu et al. 1998, Baumeister et al. 1998). In addition, this self-cleavage reaction is pH dependent and is caused by both acidity and basicity (Ditzel et al. 1998).

A previous report suggested that during gravitational stimulation of plant roots, the pH of the cytoplasm of the gravisensing columella cells transitorily becomes alkaline (Hou et al. 2004); therefore, it is possible that the 20S proteasome β subunit E1 protein responds to this change in pH. It is likely that this β subunit E1 participates in the ubiquitin-related resolution system of the Aux/IAA protein, which is translated due to the asymmetric distribution of auxin induced by gravitational stimulation (Dharmasiri et al. 2004, Liscum and Reed 2002). These results suggest that the dynamic mechanisms involved in the response to gravity may be regulated by the activation and/or inactivation of chaperone and proteasome systems. However, the relationship between the change in molecular weight and the gravity response in Arabidopsis roots remains uncertain. It is important to verify the relationship between the change in the molecular weights of these proteins and the gravity response mechanisms.

In addition, we are also studying gravity perception, transduction, and/or transmission using large plants (i.e., corn and rice). Moreover, experiments using microarray and quantitative real-time PCR are being performed to examine the change in the mRNA expression levels of the identified proteins.

Conclusions

This is the first study in proteome research that analyzed the protein expression during the gravity response in plants. It yielded many new findings on the gravity-induced protein expression in plants. In particular, the new finding that the molecular weights of proteins change transitorily during gravitational stimulation is thought to be an important contribution to the gravity response research in plants. The dynamic mechanisms involved in the gravity response may be regulated by the activation and/or inactivation of chaperone and proteasome systems due to gravitational stimulation. It appears that the mechanism of this gravity-regulated protein expression could be clarified by a detailed analysis in the future.

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References


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