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Quantitative analysis of auxin-regulated proteins from basal part of leaf sheaths in rice by two-dimensional difference gel electrophoresis

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Abstract

To identify the effects of auxin on rice root formation, proteins induced by exogenous addition of auxin to rice seedlings were analyzed by a proteomic approach. Root formation by rice seedlings was promoted by 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and repressed by 60 μM *p*-chlorophenoxyisobutyric acid (PCIB). Proteins extracted from the basal part of leaf sheaths of rice seedlings treated with 2,4-D or PCIB for 48 h were labeled with Cy3 and Cy5, and separated by two-dimensional polyacrylamide gel electrophoresis. Out of nine proteins up-regulated by 2,4-D and down-regulated by PCIB, five proteins showing significant difference in abundance were used for expression analysis at the transcript abundance level. Transcript abundance of the mitochondrial complex I subunit slightly increased with 2,4-D treatment and were repressed by PCIB. The transcript abundance of EF-1β′, myosin heavy chain and mitochondrial [Mn]SOD increased with 2,4-D treatment but did not decrease with PCIB. The transcript abundance of aldehyde dehydrogenase was not effected by 2,4-D or PCIB. These results indicate that mitochondrial complex I subunit is part of the downstream signal cascade of PCIB, whereas myosin heavy chain, mitochondrial [Mn]SOD and EF-1β′ are involved in the 2,4-D signal cascade but are probably upstream of PCIB.

Keywords: Rice; Auxin; 2,4-D; PCIB; *Oryza sativa*; Gramineae; Proteomics; RT-PCR

1. Introduction

Endogenous phytohormone auxin is essential for root development, and exogenous treatment with auxin induces ectopic formation of lateral and adventitious roots (Berleth and Sachs, 2001). At the cellular level, auxin regulates these processes by controlling cell division and cell expansion via changing the expression pattern of specific genes (Abel and Theologis, 1996). Auxin acts either by exerting direct effects on the physiology of cell, or by influencing patterns of gene expression. In the former case, the perception of auxin at the cell surface modifies the activity of membrane transport processes, ultimately leading to an increase in wall extensibility and cell elongation. These events by auxin are initiated after binding of auxin to specific receptors such as auxin-binding protein 1 (ABP1) (Napier, 1997) and transport inhibitor response 1 (TIR1) (Dharmasiri et al., 2005).

Eleven rice genes were isolated encoding homologous genes to the Arabidopsis auxin response factors (ARFs) (Sato et al., 2001). In these cases, the auxin/indole-3-acetic-acid (Aux/IAA) proteins regulate gene expression by interacting with ARF proteins that function as positive regulators of auxin signaling (Reed, 2001). The loss-of-function mutant of Arabidopsis ARF8 showed increased lateral root formation, and over-expression of ARF8 in transgenic Arabidopsis inhibited lateral root formation (Tian et al., 2004). Based on this evidence, the important

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; PCIB, *p*-chlorophenoxyisobutyric acid; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2D-DIGE, two-dimensional difference gel electrophoresis; SOD, superoxide dismutase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; EF-1β′, elongation factor-1β′.

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role that auxin plays in root development is apparent; however, the targets and molecular mechanisms downstream of ARF and the Aux/IAA proteins in root development are still unresolved. Recently, to gain more insight into lateral root initiation induced by auxin in Arabidopsis, Himanen et al. (2004) conducted transcript profiling of early lateral root initiation by microarray analysis. From the transcript profiling results, six clusters of genes were found to be involved in the initiation of pericycle cell division, by which auxin promotes lateral root formation (Smet et al., 2006). The precise roles of these candidate genes in the pathway between auxin perception and pericycle cell division are still poorly understood.

In rice, Inukai et al. (2005) reported that crown rootless1, a gene essential for crown root formation, is a target of an auxin response factor in auxin signaling. However, compared to the progress on understanding auxin induction of lateral root formation in Arabidopsis, similar progress elucidating the mechanism for rice lateral root formation lags behind. One of the reasons for this discrepancy is lack of an appropriate approach. With increasing sophistication, proteomics has become a promising methodology for the large-scale analysis of proteins in many fields of plant biology (Canovas et al., 2004; Koller et al., 2002; Komatsu et al., 2003). Proteomics, in combination with the availability of genomic sequence data, has opened up the enormous possibility of identifying the total set of expressed proteins, as well as monitoring expression changes during the growth and development and in response to biotic and abiotic stresses (Komatsu and Tanaka, 2004). Using a proteomic approach, several investigators have reported that root formation in rice is regulated by both auxin and zinc (Hossain et al., 1997; Saeki et al., 2000) and that NADPH-dependent oxidoreductase and methylmalonate-semialdehyde dehydrogenase are necessary for auxin and/or zinc induced root formation (Oguchi et al., 2004a,b). Also, Yang et al. (2005) reported that a higher level of EF-1β′ expression is necessary for auxin- and zinc-induced root formation in rice. These studies provide new insight into the auxin response in rice and demonstrate the power of the proteomic approach in this kind of study. Nevertheless, the mechanism for regulating root formation in rice is still poorly understood at the molecular level.

Fortunately, auxin inhibitors play an important role in establishing the function of auxin. To date, there are several known inhibitors of auxin; *N*-(1-naphthyl) phthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA) are inhibitors of auxin transport, and 2,4,6-trichlorophenoxyacetic acid (2,4,6-T) is a non-auxin analog. *p*-Chlorophenoxyisobutyric acid (PCIB) is one of the most often used inhibitors of auxin and is assumed to inhibit auxin action by competing with auxin for the binding site of the auxin receptor (Oono et al., 2003). Although PCIB and auxin are structurally similar, the mode of action for PCIB has not been completely defined. In this study, to enhance our understanding of auxin's role in regulating lateral root formation in rice, exogenous auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) and anti-auxin PCIB (Rahman et al, 2006) were used. For this purpose, a proteomic approach was used to identify more proteins in the auxin signaling pathway in rice. Two-week-old rice seedlings were treated with 2,4-D or PCIB, and proteins were separated by differential display two-dimensional gel electrophoresis (2D-DIGE). Furthermore, genes encoding proteins that showed a significant difference in accumulation between the control and 2,4-D or PCIB treatment, were analyzed for their transcript expression level.

2. Results and discussion

2.1. 2,4-D and PCIB regulate root formation in rice seedlings

Auxin plays important roles in regulating lateral and adventitious root development (Berleth and Sachs, 2001), and PCIB is known as an anti-auxin that is used to inhibit auxin action (Kim et al., 2000; Xie et al., 2000). Because of the structural similarity of PCIB with the synthetic auxin 2,4-D (Jonsson, 1961), and its inhibition of auxin-induced physiological responses, PCIB was proposed to inhibit auxin action by competing with auxin at the binding site of the auxin receptor (Oono et al., 2003). At the present time, the mechanism of PCIB function in rice is not known. Since the basal part of leaf sheaths contains the most vigorous growing tissues and it also contains the tissues from which new adventitious roots initiated (Yang et al., 2005), the basal part of leaf sheaths was considered to be the most suitable tissue part for examining the 2,4-D effect on root formation. In this study, the effects of 2,4-D and PCIB on root formation were examined using basal parts of rice seedlings in nutrient soil either treated with 0.45 μM 2,4-D or 60 μM PCIB for 48 h by soil drenching. Application of 2,4-D increased lateral root formation in rice seedlings, while PCIB had an antagonistic effect (Fig. 1a). In the case of 2,4-D treated plants, the number of lateral roots increased by 30.9%, while for PCIB treated plants the number decreased by 33.7% compared to untreated plants (Fig. 1b). Besides, after PCIB treatment, the length of main root was elongated compared to the non-treated plants or 2,4-D treated plants. These results indicate that PCIB is an inhibitory factor to induction of lateral root formation by auxin. There are a few other reports demonstrating that PCIB inhibits auxin regulated gene expression (Okamoto et al, 1995; Klotz and Lagrimini, 1996; Oono et al., 2003). Thus, PCIB appears to be a useful chemical to elucidate the mechanism of auxin perception and signal transduction and development.

2.2. Nine proteins are up-regulated by 2, 4-D and down-regulated by PCIB

To identify auxin responsive proteins that are related to lateral root formation in rice, proteins from the basal part

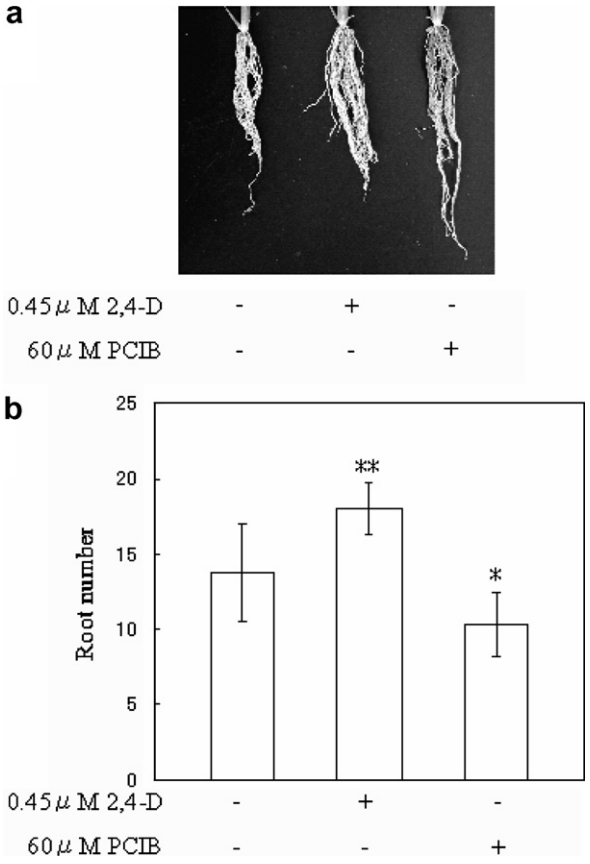

Fig. 1. Effect of 2,4-D and PCIB on root formation in rice seedlings. Two-week-old seedlings of rice were treated with 0.45 μM 2,4-D and 60 μM PCIB for 48 h by soil drenching. (a) Root morphology of plants without 2,4-D and PCIB treatment (left), and with 2,4-D treatment (middle) or PCIB treatment (right). (b) The number of roots counted in seedlings without 2,4-D and PCIB treatment (left), and with 2,4-D treatment (middle) or PCIB treatment (right). Data are shown as the average of three experiments ± SD. Fifteen seedlings were used in each experiment. * $P > 0.05$ and ** $P > 0.01$: significantly different from non-treated plants.

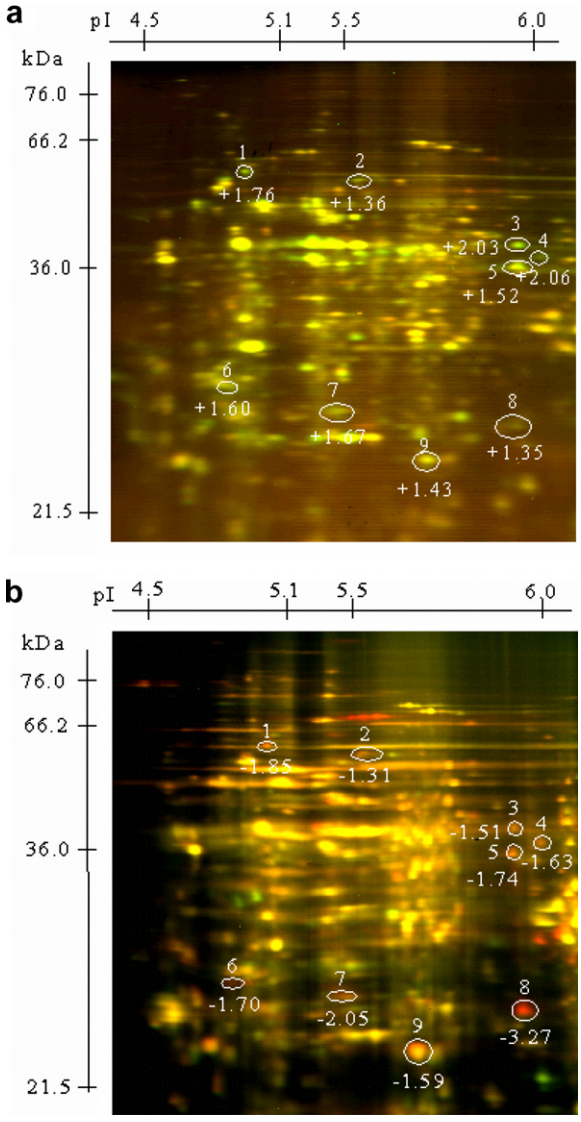

Fig. 2. Changes in protein expressions in the basal parts of leaf sheaths of rice seedlings treated with 2,4-D and PCIB. Proteins were extracted from the basal parts of leaf sheaths of two-week-old seedlings treated with 0.45 μM 2,4-D (a) and 60 μM PCIB (b) for 48 h. Proteins from 2,4-D or PCIB treated plants were labeled with green fluorescent dye Cy3, and those from non-treated plants were labeled with red fluorescent dye Cy5. The protein mixture was separated by 2 D-PAGE. (a) 2D-PAGE pattern showing the expression of proteins responding to 2,4-D treatment. (b) 2D-PAGE pattern showing the expression of proteins responding to PCIB treatment. Proteins on the 2D-PAGE gel were analyzed using DeCyder software. Green spots indicate the proteins increased by 2,4-D or PCIB and red spots indicate the proteins decreased by 2,4-D or PCIB. Circles show proteins up- and down- regulated by 2,4-D (A) and PCIB (B), respectively. The spot numbers are the same as those specified in table 2.

of leaf sheaths of two-week-old seedlings treated with 0.45 μM 2,4-D or 60 μM PCIB for 48 h were separated by 2 D-PAGE. On the 2D gel, 935 proteins were identified that distributed between pI 4 and 6 and molecular masses of 10–100 kDa. Some of the proteins displayed greater than 1.5 fold up- and down-regulation by 2,4-D or PCIB treatment (Fig. 2). The intensities of spots were quantified by DeCyder software. The experiments were repeated three times.

Among these proteins, nine that were up-regulated by 2,4-D (Fig. 2a) and down-regulated by PCIB (Fig. 2b) were quantified. The relative abundance of five proteins, spots 1, 2, 4, 6 and 9, in response to 2,4-D and PCIB was significantly different from the control (Fig. 3). In contrast, the other proteins, spots 3, 5, 7 and 8, showed no significant changes in abundance. These results support the hypothesis that genes for spots 1, 2, 4, 6 and 9 are involved in the auxin responsive pathway.

To identify the nine proteins, the proteins were first analyzed by Edman sequencing that determines the N-terminal amino acid sequences. The N-terminal sequences of spots 5 and 6 were determined (Table 2) probably because the N-terminals of the other seven proteins were blocked by posttranslational modification. For the seven proteins, ESI-Q-TOF MS/MS was used to determine the amino acid sequences of fragmented peptides; however, the identity of only spot number 1 could be determined using this method. Since the mass of the other six proteins

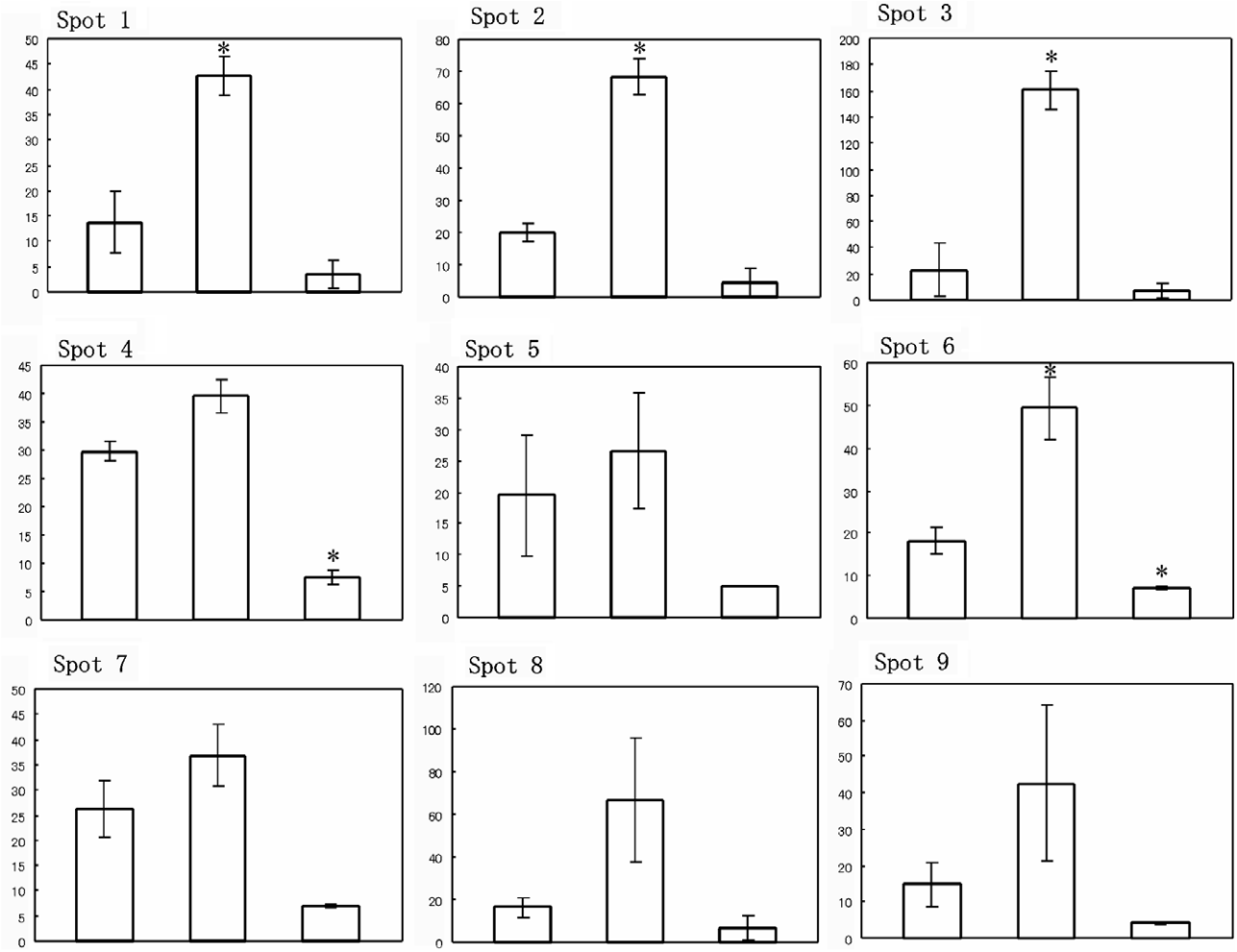

Fig. 3. Protein expression levels changed by 2,4-D and PCIB treatment. The relative abundance ratio of proteins shown in Fig. 2 was determined with ImageMaster 2D Elite software. The amount of a protein spot was expressed as the volume of that spot that was defined as the sum of the intensities of all the pixels that make up the spot. The vertical axis shows the relative volume of the nine proteins between 2,4-D (middle), or PCIB (right) treated plants and non-treated plants (left). Data are shown as the average of three experiments ± SD. *t*-Test was used to test the significance of the difference of protein volume between non-treated plants and 2,4-D or PCIB treated plants. $*P>0.05$: significantly different from non-treated plants.

was extremely low the remaining six spots were subjected to MALDI-TOF MS analysis. These proteins were determined to be myosin heavy chain (spot 1), the 76 kDa mitochondrial complex I subunit (spot 2), ribulose-1,5-bisphosphate carboxylase (RuBisCO) large subunit (spot 3), aldehyde dehydrogenase (spot 4), disulfide-isomerase (spot 5) , elongation factor-1β′(EF-1β′, spot 6), ribosomal protein L27 (spot 7), 20S proteasome β subunit B (spot 8) and mitochondrial manganese-superoxide dismutase ([Mn]SOD) (spot 9).

As mentioned above, among the nine proteins, five proteins showed significant up- and down-regulation in protein expression abundance relative to the control (Fig. 3): myosin heavy chain, the 76 kDa mitochondrial complex I subunit, aldehyde dehydrogenase, EF-1β′ and mitochondrial [Mn]SOD. Myosins are a large superfamily of motor proteins that move along actin filaments, while hydrolyzing ATP. They play important functional roles within plant cells in driving actin-based motility such as intracellular vesicle and membrane transport (Tominaga et al., 2003). The heavy chain of myosin plays a major role in various myosin functions, such as the motor for actin filaments that promotes cytoplasmic streaming by hydrolyzing ATP. Complex I (NADH:Ubiquinone oxidoreductase) catalyses the first step in the mitochondrial electron transport chain, by which electrons from the oxidation of NADH are used to convert oxygen to water, the energy liberated being trapped in ATP formation and ultimately used as energy source for various plant activities. Many plant adaptations to stresses involve mitochondria where complex I is localized (Lee et al., 2002). Aldehyde dehydrogenase is a superfamily of enzymes catalyzing the conversion of various endogenous and exogenous aldehydes to the corresponding carboxylic acids using the coenzyme NAD^+ or $NADP^+$ (Rodrigues et al., 2006). EF-1β′ is a subunit of the EF-1 complex, which is not only a major factor associated with translation, but also an important multifunctional proteins (Ejiri,

2002). Mitochondrial [Mn]SOD is the superoxide dismutase specifically present in mitochondria, with Mn as the prosthetic metal. This enzyme converts superoxide radicals that are generated in mitochondria when plants are exposed to chemicals such as plant hormones, fungal toxin/herbicides, and environmental stresses to molecular oxygen and H_2O_2 (Kwon and An, 2003). Based on the functions of these proteins, the results in this study indicate that the 76 kDa mitochondrial complex I subunit, myosin heavy chain and EF-1β′ are likely involved in cell extension and cell division resulting in lateral root formation by auxin. Aldehyde dehydrogenase and mitochondrial [Mn]SOD are likely involved in the defense system that auxin triggers.

2.3. Myosin heavy chain, EF-1β′ and mitochondrial [Mn]SOD are up-regulated by auxin

As shown in Fig. 1a, lateral root formation by two-week-old seedlings was promoted by 0.45 μM 2,4-D and repressed by 60 μM PCIB treatment for 48 h. Based on 2D-DIGE, five proteins were identified that showed significant differences in accumulation. To understand the expression of genes encoding these five proteins after 24 h and 48 h treatment of 2,4-D or PCIB at the transcriptional level, the abundance of transcripts was investigated by semi-quantitative RT-PCR. Among five proteins that were up-regulated by auxin and down-regulated by PCIB at protein level, the transcripts of myosin heavy chain, EF-1β′ and mitochondrial [Mn]SOD increased after 24 h or 48 h of 2,4-D treatment (Fig. 4, spots 1, 6 and 9). However, the expression of these genes was not repressed by PCIB. This observation indicates that these genes are involved in the auxin signaling cascade but are upstream of PCIB. Considering that these proteins were down-regulated by PCIB, protein(s) encoded by a key gene(s) downstream of PCIB might be involved in the post-transcriptional regulation of these proteins. Post-transcriptional regulation could play an important role for these genes in response to auxin, because it is a common means of regulating genes responding to various abiotic stresses as described by Nakashima and Yamaguchi-Shinozaki (2005). Myosin heavy chain may be involved in reconstruction of the cytoskeleton and EF-1β′ could be involved in translating genes that are needed for cell division. With respect to [Mn]SOD, it likely functions as an antioxidant to scavenge reactive oxygen species (ROS) that increased when plants were treated with 2,4-D.

For aldehyde dehydrogenase, although the protein showed significant differences in accumulation, the gene transcript did not increase by 2,4-D or decrease by PCIB as shown by semi-quantitative RT-PCR. At the present time, we can not explain how aldehyde dehydrogenase might be involved in the auxin signaling pathway. More experimental evidence must be gathered to indicate the importance of this enzyme to auxin signaling.

2.4. The 76 kDa mitochondrial complex I subunit expression is repressed by treatment with PCIB

Expression of the 76 kDa mitochondrial complex I subunit was repressed by treatment with PCIB for 48 h at the transcript abundance level (Fig. 4; spot 2). This result was consistent with protein expression that was repressed after PCIB treatment with PCIB for 48 h, indicating that the 76 kDa mitochondrial complex I subunit could be one of the downstream genes of PCIB. Oguchi et al. (2004) reported that NADPH-dependent oxidoreductase can be induced by auxin in rice and is expressed predominantly in proliferating tissues. In this study, the 76 kDa mitochondrial complex I subunit did not show increased expression when exogenous 2,4-D was applied even though the expression was repressed greatly by PCIB. Binding of auxin to cellular receptors initiates the auxin signaling pathway (Napier, 1997; Dharmasiri et al., 2005), but the number of binding sites per receptor is limited. If receptor binding sites were occupied by endogenous auxin, the addition of exogenous auxin would not show any detectable effect on gene expression by semi-quantitative RT-PCR. The role that the 76 kDa mitochondrial complex I subunit plays in the formation of lateral roots could be to provide energy for cell division.

As described so far, PCIB is a useful inhibitor of 2,4-D activity. There are possibilities for the mechanism underlying this inhibitor: whether PCIB competes directly with auxin at the auxin receptor binding site (Oono et al., 2003), or not. To understand more about the relationship between 2,4-D and PCIB, two-week-old seedlings were treated simultaneously with 2,4-D and PCIB. Fig. 5 shows the transcript abundance of the mitochondrial complex I subunit after the above treatments. Simultaneous treatment of rice seedlings with 2,4-D and PCIB for 24 h increased the abundance of the mitochondrial complex I subunit transcript (Fig. 5); however, expression of this gene was not further enhanced by extension of the PCIB treatment for another 24 h period. As shown in Fig. 4, PCIB represses the action of endogenous auxin. When rice plants are treated with 2,4-D and PCIB simultaneously, PCIB initially represses the action of endogenous auxin. In the absence of endogenous auxin, exogenous 2,4-D can function resulting in increased transcript abundance of the mitochondrial complex I subunit gene. Moreover, extended PCIB treatment did not show any difference in transcript abundance of the mitochondrial complex I subunit, suggesting that PCIB impairment of the auxin-signaling pathway is likely by regulating Aux/IAA protein stability.

3. Concluding remarks

Auxin can affect gene expression within minutes of auxin treatment (Himanen et al., 2004). These rapid responses are direct responses in that they do not require

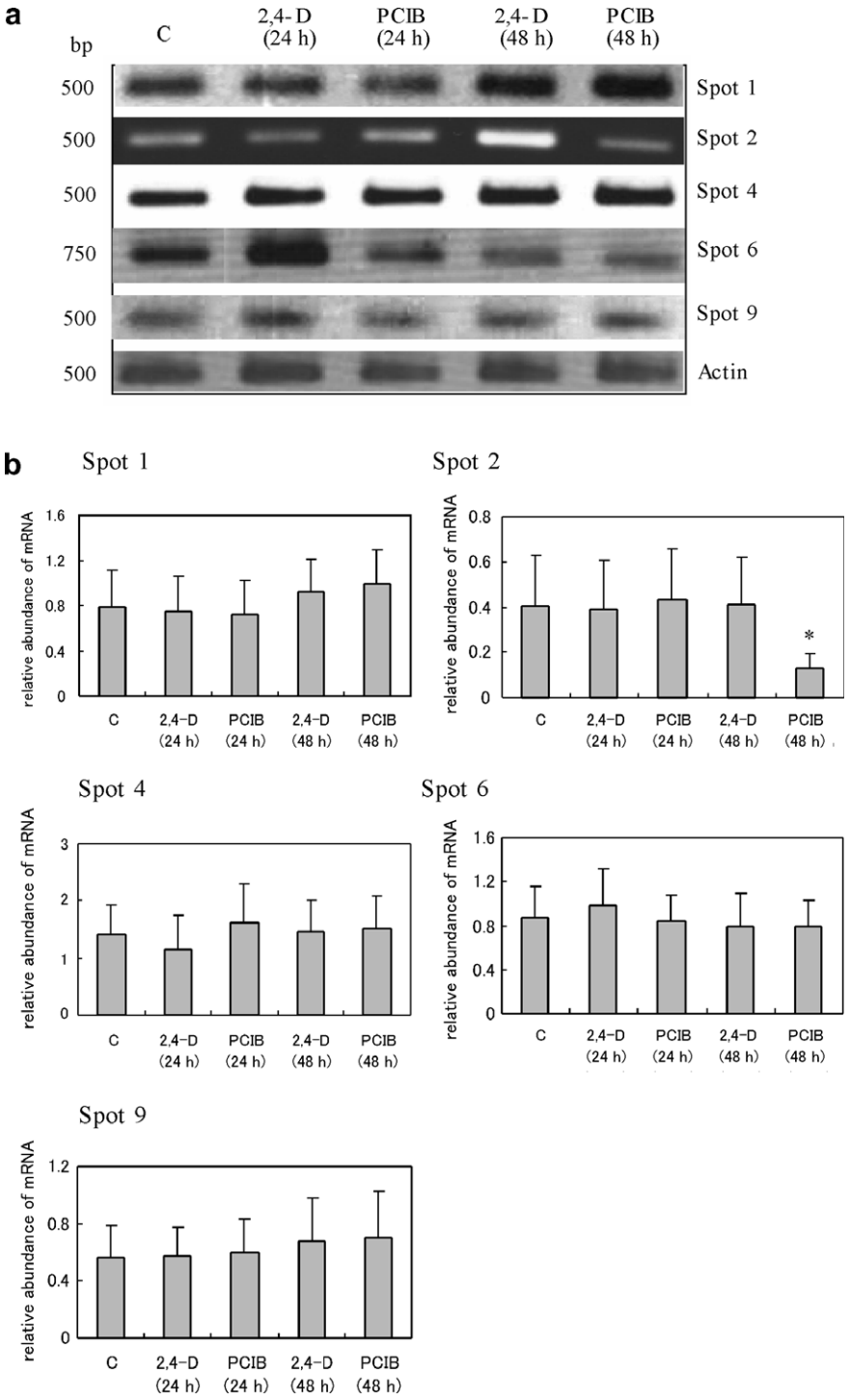

Fig. 4. Expression analysis of the five genes at the transcript abundance level after treatment with 2,4-D and PCIB at 24 h and 48 h, respectively. Five genes whose protein abundance was up-regulated by 2,4-D and down-regulated by PCIB were subjected to RT-PCR. Total RNAs were isolated from the basal parts of leaf sheaths of two-week-old seedlings treated with 0.45 μM 2,4-D or 60 μM PCIB for 24 h and 48 h. After reverse transcription, cDNAs were used as templates for semi-quantitative RT-PCR. Actin was used as an internal control to normalize the amount of cDNA template. Expression of the five genes was quantified with ImageQuant software accompanied by Typhoon scanner. (a) The band images of RT-PCR for five genes. (b) The relative abundance of expression of the five genes. Lane 1 shows non-treatment, lanes 2 and 3 show 24 h treatment of 2,4-D and PCIB, lanes 4 and 5 show 48 h treatment of 2,4-D and PCIB, respectively. Data are shown as the average of product volumes from three RT-PCR experiments ± SE.

protein synthesis. Thus genes are considered to be primary auxin-regulated genes, and ARF and Aux/IAA is direct target of the auxin signaling pathway (Sato et al., 2001; Reed, 2001). In this study, secondary auxin-regulated protein expression events were described using proteomics technique after long-term auxin treatment. To enhance our understanding of auxin's role in regulating lateral root formation in rice, a proteomic approach in combination

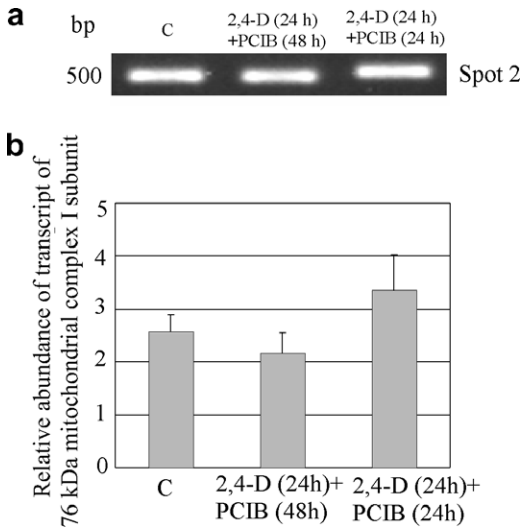

Fig. 5. Expression analysis of the 76 kDa mitochondrial complex I subunit (Spot 2) gene at the transcript abundance level after simultaneous treatment with 2,4-D and PCIB. The 76 kDa mitochondrial complex I subunit gene was subjected to semi-quantitative RT-PCR. Total RNA was isolated from the basal parts of leaf sheaths of two-week-old seedlings treated simultaneously with 0.45 μM 2,4-D and 60 μM PCIB. After reverse transcription, cDNAs were used as templates for PCR. Actin was used as an internal control to normalize the amount of cDNA template. Expression was quantified with ImageQuant software developed for the Typhoon scanner. (a) The band image of RT-PCR. (b) The relative abundance of expression. Lane 1 shows non-treatment, lane 2 shows 24 h treatment of PCIB followed by simultaneous 24 h 2,4-D and PCIB treatment, lane 3 shows 24 h simultaneous treatment of 2,4-D and PCIB. Data are shown as the average of product volumes of three RT-PCR experiments ± SE.

with transcript abundance analysis. From these results, five genes were determined on two-week-old seedlings treated for 24–48 h with 2,4-D and PCIB. Not all five proteins analyzed here contribute to lateral root formation, even though all of them except aldehyde dehydrogenase respond to 2,4-D. The protein and transcript abundance expression data suggest that the 76 kDa mitochondrial complex I subunit, myosin heavy chain and EF-1β′ are involved in auxin's promotion of lateral root formation, whereas aldehyde dehydrogenase and mitochondrial [Mn]SOD are likely the secondary products of auxin inducing lateral root formation. Our results indicate the 76 kDa mitochondrial complex I subunit is a downstream protein of 2,4-D and PCIB and contributes to auxin-induced lateral root formation.

4. Experimental

4.1. Plant materials and chemicals

Rice (*Oryza sativa* L. cv Nipponbare) was grown in the granulated nutrient soil (Kureha Chemical, Tokyo, Japan) under white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h light period/day) at 25 °C and 75% relative humidity in a growth chamber. Treatment was done by soil drenching. 2,4-D (Wako, Osaka, Japan) and PCIB (Frinton Laboratories, Vineland, NJ, USA) were solubilized with EtOH and control treatment contained the same amount of EtOH (0.1% final concentration). The sources of the chemicals used in the present study were as follows: Urea (MP biomedicals, Ohio, USA), ampholine (GE Healthcare, Piscataway, NJ, USA), polyvinyl pyrrolidene-40 (PVP-40), thiourea, dithiothreitol (DTT) (Sigma–Aldrich, MO, USA), and CHAPS, iodoacetamide (IAM), MOPS, dimethylformamide (DMF) (Wako, Osaka, Japan).

4.2. Preparation of protein extract

A portion (100 mg) of the basal part of leaf sheaths of two-week-old rice seedlings was removed and homogenized with 200 μl lysis buffer containing 8 M urea, 2% NP-40, 2% ampholine (pH 3.5–10.0), 5% 2-mercaptoethanol and 5% PVP-40 (O'Farrell, 1975). The homogenate was centrifuged at 15,000*g* for 5 min and the supernatant was further centrifuged at 15,000*g* for 5 min. The supernatant was used as the protein extract and precipitated with 2-D Clean-Up kit (GE Healthcare). The protein pellet was then solubilized in the 25 μl of 7 M urea, 2 M thiourea, 4% CHAPS, 6.5 mM DTT, and 0.5% ampholine (pH 3.5–10.0). The solubilized proteins were labeled with fluorescent dyes.

4.3. Protein labeling with Cy3 and Cy5 fluorescent dyes

The sample was prepared by 150 μg protein from each of the sample prior to labeling. Cyanine dyes were reconstituted in 99.8% anhydrous DMF and added to labeling reactions in a ratio of 400 pmol CyDye: 50 μg protein. Protein labeling was achieved by incubation in the dark for 30 min. The reaction was stopped by the addition of 10 mM lysine (1 μl per 400 pmol dye) followed by incubation on ice for 10 min. These labeled samples were then mixed for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis.

4.4. Two-dimensional polyacrylamide gel electrophoresis

Prepared protein samples were separated in the first-dimension by isoelectric focusing (IEF) tube gel and in the second-dimension by SDS–PAGE (O'Farrell, 1975). An IEF tube gel of 11 cm length and 3 cm diameter was prepared, which consisted of 8 M urea, 3.5% polyacrylamide, 2% NP-40, 2% ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium persulfate and TEMED. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. The electrophoresis was performed at room temperature. After electrophoresis of the first-dimension, SDS–PAGE as the second dimension was carried out using 15% polyacrylamide gel. The isoelectric point (p_I) and relative molecular mass (M_r) of each protein were determined using 2D-PAGE markers (Bio-Rad, Hercules, CA, USA).

4.5. Image acquisition and data analysis

The 2D-PAGE gels on which Cy3 and Cy5-labeled proteins were separated were scanned on the Typhoon 8610 (GE Healthcare) using the optimal wavelength for each fluor: Cy3 (532/580 nm), and Cy5 (633/670 nm). Each image was analyzed with DeCyder (version 5.01) (GE Healthcare) software. Spot detection and relative quantitation of matched gels were performed by the differential in-gel analysis module. Coomassie brilliant blue (CBB)-stained 2D-PAGE gels were scanned in a scanner, with images analyzed using Image Master 2D-Elite software (version 2.0, GE Healthcare). The amount of a protein spot was expressed as the volume of the spot, which was defined as sum of the intensities of all the pixels that make up the spot.

4.6. Amino acid sequence analysis

Following separation by 2D-PAGE, the peptides were electroblotted onto a polyvinylidene difuloride (PVDF) membrane (Pall, Port Washington, NY, USA) using a semidry transfer blotter (Nippon Eido, Tokyo, Japan), and detected by CBB staining. The stained protein bands were excised from the PVDF membrane and applied to a gas-phase protein sequencer (Procise 494, Applied Biosystems, Foster City, CA, USA). The amino acid sequences obtained were compared with those of known proteins in the Swiss-Prot, PIR, Genpept and PDB databases with Web-accessible search program FastA.

4.7. ESI-Q-TOF MS/MS and MALDI-TOF MS analysis

Protein spots stained with CBB were excised from gels, and destained with 50 mM NH_4HCO_3 in MeOH-H_2O (1:1, v/v) for 1 h at 40 °C. Proteins were reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 55 °C and incubated with 40 mM IAM in 100 mM NH_4HCO_3 for 30 min at room temperature. The gel pieces were homogenized and allowed to dry and then rehydrated in 100 mM NH_4HCO_3 with 1 pmol of trypsin (Wako) at 37 °C for 10 h. The digested peptides were extracted from gels slices with 0.1% trifluoroacetic acid in CH_3CN-H_2O (1:1, v/v) three times. The peptide solution was concentrated, loaded into the high performance liquid chromatography (HPLC) (Waters, Milford, MA, USA), and analyzed by electrospray ionization-quadrupole-time-of-flight mass spectrometry (ESI-Q-TOF MS, Q-TOF Micro, Micromass, Manchester, UK). For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis (Voyager, Applied Biosystems, Framingham, MA, USA), the peptide solution was dried and reconstituted with 30 μl of 0.1% trifluoroacetic acid in CH_3CN-H_2O (5:95, v/v) and then desalted with Nutip (M&S Instrument, Osaka, Japan). The above peptide solution was mixed with matrix solution saturated with α-cyano-4-hydroxycinnamic acid, and then air-dried on the flat surface of carbon plate. The mass spectra were subjected to sequence database search using Mascot software (Matrix Science Ltd., London, UK). Genbank (http://www.ncbi.nlm.nih.gov/Entrez/) and KOME (http://cdna01.dna.affrc.go.jp/cDNA/) were used to the Mascot search.

4.8. Isolation of total RNA and semi-quantitative RT-PCR

Total RNA was isolated from basal parts of leaf sheaths of two-week-old rice seedling treated by 2,4-D or PCIB independently for one day and two days using RNeasy Plant Mini kit (Qiagen, Tokyo, Japan). cDNA was generated by ReverTra-Plus- RT-PCR kit (Toyobo, Tokyo, Japan) and was used as templates for semi-quantitative RT-PCR. To reflect truly the transcripts abundance of five genes whose proteins were upregulated by 2,4-D, actin was used as internal marker to normalize the cDNA templates. The PCR amplification was conducted for 20 μl reaction including 1 μl cDNA, 1x Ex Taq buffer, 0.2 mM dNTP, 10 μM forward and reverse primers, and 0.5 U Ex Taq polymerase. PCR running program is 94 °C for 5 min, followed by 19–25 cycles of 94 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min depending on primers used. The primer pairs for five genes were shown in Table 1. The relative

Table 1
The sequences of primers pairs for five genes used in the study

Spots	Genes	Primer pairs	Band size (bp)
1	Myosin heavy chain	5′-TGAAACTGACTCTGCAGGGATC-3′ 5′-CGAGCTCAGACTCCGCAAA-3′	500
2	76 kDa mitochondrial complex I subunit	5′-AGGTGTTCGTGGACGGGCAC-3′5′-TCTTCACCACTGCCACGACCTAAC-3′	>1350
4	Aldehyde dehydrogenase	5′-CACTGGAGCTAGGAGGCAAG-3′ 5′-CGTGTTCAGGTTGTTGGTGAAG-3′	500
6	EF-1β′[a]	5′-AGGGGATGTTGCTACTTATTC-3′ 5′-TGGACGGATGTACTTTTGACATA-3′	750
9	Mitochondrial [Mn]SOD	5′-ACCTACGTCGCCAACTACAAC-3′5′-CCTGCGTATTTCCAGTTCATCAC-3′	500

EF-1β′: elongation-factor-1β′, [Mn]SOD: manganese-superoxide dismutase.
[a] The primer pairs for amplifying elongation factor -1β′ was the one used in Yang et al. (2005).

Table 2
Characterization of proteins regulated by 2,4-D and PCIB in basal parts of rice seedlings

Spot no.	p_I	M_r (kDa)	Homologous protein	Methods	Accession no	P	C	S
1	4.8	63.0	Myosin heavy chain	Q	AY224548	16	16	58
2	5.7	62.0	76 kDa mitochondrial complex I subunit	M	AK071307	7	14	60
3	5.9	39.0	RuBisCO large subunit	M	AK105600	10	14	60
4	5.9	38.1	Aldehyde dehydrogenase	M	AK073079	10	20	68
5	5.9	37.1	Disulfide-isomerase	Ed	AK098931	–	100	–
6	4.7	26.4	Elongation factor-1β′	Ed	AK121942	–	100	–
7	5.5	24.0	Ribosomal protein L27	M	AK061690	6	52	58
8	5.9	23.0	0S proteasome β subunit B	M	AK103126	5	18	61
9	5.8	22.5	Mitochondrial [Mn]SOD	M	AK070528	6	38	38

Q: ESI-Q-TOF MS/MS, M: MALDI-TOF MS, Ed, Edman sequencing.
EF-1β′: elongation factor-1β′, [Mn]SOD: manganese-superoxide dismutase.
P: peptide numbers, C: sequence coverage, S: score.

abundance of transcripts of five genes was quantified with ImageQuant software accompanied by Typhoon scanner. The expression level for each gene was analyzed three times.

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