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Chromium stress response effect on signal transduction and expression of signaling genes in rice

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Hexavalent chromium [Cr(VI)] is a non-essential metal for normal plants and is toxic to plants at high concentrations. However, signaling pathways and molecular mechanisms of its action on cell function and gene expression remain elusive. In this study, we found that Cr(VI) induced endogenous reactive oxygen species (ROS) generation and Ca^{2+} accumulation and activated NADPH oxidase and calcium-dependent protein kinase. We investigated global transcriptional changes in rice roots by microarray analysis. Gene expression profiling indicated activation of abscisic acid-, ethylene- and jasmonic acid-mediated signaling and inactivation of gibberellic acid-related pathways in Cr(VI) stress-treated rice roots. Genes encoding signaling components such as the protein kinases domain of unknown function 26, receptor-like cytoplasmic kinase, LRK10-like kinase type 2 and protein phosphatase 2C, as well as transcription factors *WRKY* and apetala2/ethylene response factor were predominant during Cr(VI) stress. Genes involved in vesicle trafficking were subjected to functional characterization. Pretreating rice roots with a vesicle trafficking inhibitor, brefeldin A, effectively reduced Cr(VI)-induced ROS production. Suppression of the vesicle trafficking gene, *Exo70*, by virus-induced gene silencing strategies revealed that vesicle trafficking is required for mediation of Cr(VI)-induced ROS production. Taken together, these findings shed light on the molecular mechanisms in signaling pathways and transcriptional regulation in response to Cr stress in plants.

Abbreviations – ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylate; AP2/ERF, APETALA2/ethylene response factor; BFA, brefeldin A; CaM, calmodulin; CAMK, CaM-dependent protein kinase; CBL, calcineurin B-like; CDPK, Ca^{2+}-dependent protein kinase; CM-H_2DCF-DA, 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; CRK, cysteine-rich RLK; DAB, 3,3′-diaminobenzidine; DPI, diphenylene iodonium; DTT, dithiothreitol; FDR, false discovery rate; FW, fresh weight; GA, gibberellin; GO, gene ontology; HSF, heat shock factor; IRAK, interleukin-1 receptor-associated kinase; JA, jasmonic acid; KOME, the Knowledge-based Oryza Molecular Biological Encyclopedia; MES, 2-(N-morpholino) ethanesulfonic acid; MS, Murashige and Skoog; NBT, nitroblue tetrazolium; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; qRT-PCR, quantitative real-time polymerase chain reaction; RLCK, receptor-like cytoplasmic kinases; RLK, receptor-like kinase; RMOP, regeneration media of plants; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TFs, transcription factors; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing; XTT, sodium,3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate.

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Introduction

Toxic metal pollution in soil and water is a major source of environmental hazard to plants. Toxic heavy metals at high levels create a stressful environment. Chromium is the seventh most abundant metal on earth and the 21st in the crustal rocks and one of the most important environmental contaminant released mainly from industries using Cr (Cervantes et al. 2001). Cr contamination of soil and water is of recent concern. Relatively high levels of Cr concentration have been reported at some severely contaminated sites. For example, in some sites of OR, USA, Cr concentrations were as high as 14 600 and 25 900 mg kg^{-1} in ground water and soil, respectively (Shanker et al. 2005). In Taiwan, concentrations of Cr in the surface sediments of the Canon river estuary were between 27 and 192 mg kg^{-1} (Chen et al. 2012).

The toxic effect of Cr to plants depends on its valence state. Hexavalent chromium [Cr(VI)] is more toxic than the trivalent form. In plants, physiological processes such as photosynthesis, water relations and mineral nutrition might be affected by Cr stress (Shanker et al. 2005). The uptake of plant nutrients from soil in crop plants, such as maize, soybean and tomato, was affected by Cr stress (Moral et al. 1995, Khan 2001). Proteomics was used to assess proteins involved in response to high concentration of Cr (340–1019 μ*M*) in maize (Labra et al. 2006). Based on the effect of Cr exposure on roots of developing rice seedlings, previous studies reported that induction of oxidative stress was the main biological process underlying Cr toxicity in plants (Panda 2007, Zeng et al. 2012). Although plant responses to heavy metal exposure have been widely studied, the effect(s) of excess Cr(VI) on the plant is still poorly understood and little is known about how the plant responds to Cr(VI) stress at the gene expression level. In addition, plant may reveal defense-related genes in response to excess heavy metal treatment (Sudo et al. 2008). Thus, a deep investigation into transcript profile in response to Cr(VI) stress in rice is important to clarify how higher plants adapt to heavy metal stress.

In plants, signal transduction and transcriptional regulation are essential for plants to respond to biotic and abiotic stresses. After the initial recognition of the stress, a cellular signal transduction pathway is triggered. The stress signal is transduced in the cell to switch on the stress-responsive genes for generating the initial stress response (Grennan 2006). Calcium is an important second messenger in intracellular signal transduction pathways. The action of Ca is mediated by a group of Ca^{2+}-binding proteins including Ca^{2+}-dependent protein kinases (CDPKs), calmodulin (CaM) and CaM-binding protein kinase (CBK) (Huang and Huang 2008). CDPK-mediated cascade is crucial for transduction of extracellular stimuli into intracellular responses in plant cells (Sangwan et al. 2002).

The aim of this study was to investigate the molecular mechanisms of the signaling pathways and early transcriptome profiles regulated by Cr(VI) stress in rice roots. We demonstrated the role of reactive oxygen species (ROS) and Ca in signal transduction pathways under Cr(VI) stress. Specifically, we used a microarray to analyze early Cr(VI)-induced changes of gene expression in rice roots. We report the detailed analysis of genome-wide transcriptome profiling. This investigation into global gene expression profiles will greatly expand our knowledge about cellular responses to Cr(VI) stress and identify candidate signaling components regulated in response to Cr stress in crops.

Materials and methods

Plant material

The rice seeds (*Oryza sativa* L. cv. TN-67) were germinated in a growth chamber at 37°C in darkness (Huang and Huang 2008). Rice roots were treated with 200 μ*M* K_2CrO_4 (Sigma-Aldrich, St. Louis, MO) for 15–240 min. After the treatment, rice roots were taken to determine ROS production, Ca accumulation, in-gel kinase activity, immunoblot, in-gel enzymatic activity and microarray assays. *Nicotiana benthamiana* plants were grown in a growth chamber at 27°C with cool white fluorescent light (100 μmol s^{-1} m^2 light intensity) under long day condition (16 h white light/8 h dark).

Root length determination

The germinated rice seeds with 0.2 cm long root were treated with different concentrations (0–400 μ*M*) of K_2CrO_4. After 3 days of growth followed by Cr(VI) treatment at 26°C in darkness, root length was measured. Mean root length was obtained from 15 individual rice seedlings from at least three separate experiments.

Detection of ROS and Ca^{2+} in rice roots

Six-day-old rice seedlings with 3 cm long root were used to determine ROS generation and Ca^{2+} accumulation during Cr(VI) stress. Rice roots treated with 10 μ*M* 5-(and-6)-chlormethyl-2′,7′-dichlordi-hydrofluorescein diacetate, acetyl ester (CM-H_2DCF-DA, Molecular Probes, Invitrogen, Carlsbad, CA) or 10 μ*M* Oregon Green 488 BAPTA-1 for 30 min were exposed to 200 μ*M* Cr(VI) for 15 min. Fluorescence signals in rice roots were observed with a Leica MPS60 (Leica, Wetzlar, Germany) fluorescent microscope using a green fluorescent protein filter (excitation 450–490 nm,

emission 500–530 nm). Fluorescence and bright-field images were taken with a CoolSNAP Cooled CCD Camera (CoolSNAP 5.0, North Reading, MA).

Protein extraction from rice roots

After 1 h of treatment with 200 μ*M* Cr(VI), protein of rice roots was extracted. Approximately 1 cm of rice root tips cut from 10 rice seedlings was ground in protein extract buffer [50 m*M* Tris–HCl, pH 7.4, 250 m*M* sucrose, 10 m*M* NaF, 10 m*M* Na_3VO_4, 1 m*M* sodium-tartrate, 10% v/v glycerol, 50 m*M* $Na_2S_2O_5$, 1% sodium dodecyl sulfate (SDS) and 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. Homogenized root tissue was centrifuged at 15 700 g for 10 min at room temperature and the supernatant was collected. Protein content was determined by the BioRad Dc Protein Assay (Bio-Rad, Hercules, CA) at OD_{750}.

In-gel kinase activity assay

The root extracts containing 10 μg of proteins were separated by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (PAGE) embedded with histone III-S in the separating gel as a substrate for the kinases. After electrophoresis, the gel was rinsed several times with the washing buffer [25 m*M* Tris, pH 7.5, 0.5 m*M* dithiothreitol (DTT), 5 m*M* NaF, 0.1 m*M* Na_3VO_4, 0.5 mg ml^{-1} bovine serum albumin and 0.1% Triton X-100 (v/v)] to remove SDS. Proteins were allowed to renature overnight at 4°C in the buffer containing 25 m*M* Tris, pH 7.5, 1 m*M* DTT, 0.1 m*M* Na_3VO_4 and 5 m*M* NaF with several changes. The gel was soaked in a 90 ml reaction buffer [40 m*M* Tris, pH 7.5, 0.2 m*M* $CaCl_2$, 12 m*M* $MgCl_2$, 2 m*M* DTT and 0.1 m*M* Na_3VO_4] for 30 min at room temperature to detect CDPK activity. Phosphorylation was carried out in 90 ml of the same reaction buffer with addition of 200 n*M* ATP and 50 μCi γ-^{32}P-ATP (3000 Ci $mmol^{-1}$). The reaction was stopped by placing the gel in 5% trichloroacetic acid (w/v)–1% sodium pyrophosphate (w/v). The gel was washed several times in the same solution to remove the unincorporated γ-^{32}P-ATP. The gel was dried and subjected to exposure with a Kodak BioMax MR (Kodak, Rochester, NY) autoradiographic film. Prestained size markers (Bio-Rad, Cat. No. 161-0305) were used to detect the size of kinases. All kinase activity assays were repeated at least twice with nearly identical results.

NADPH oxidase activity assays

Six-day-old rice seedlings pretreated with 10 μ*M* diphenylene iodonium (DPI) for 1 h were treated with 200 μ*M* Cr(VI) for an additional hour. Approximately 1 cm of rice root tips was cut from 25 rice seedlings and ground in 0.05 *M* potassium phosphate buffer, pH 5.8, to extract NADPH oxidase. Homogenized root tissue was centrifuged at 13 400 g for 20 min at 4°C. The supernatant was collected and used to detect NADPH oxidase activity by in-gel activity assay and sodium,3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) assay. The in-gel activity assay was carried out as described (Sagi and Fluhr 2001). Extracted root proteins (100 μg) were separated on 10% (w/v) native PAGE for 1 h. After washing several times with distilled water, the gel was soaked in the dark with 0.2 m*M* nitroblue tetrazolium (NBT) for 20 min and transferred to a solution with 0.2 m*M* NADPH until blue formazan bands were observed. The reaction was stopped by immersion of the gel in distilled water. The XTT assay mixture of 1 ml consisted 50 m*M* Tris–HCl buffer (pH 7.5), 0.5 m*M* XTT and 100 μ*M* NADPH. The reaction was started by the addition of NADPH. The reduction of XTT was determined by measurement of absorbance at 470 nm (Jiang and Zhang 2002).

In situ detection of ROS

Superoxide anion radicals and H_2O_2 levels in rice roots during Cr(VI) treatment were detected by staining with NBT and 3,3′-diaminobenzidine (DAB), respectively. Rice roots pretreated with 10 μ*M* brefeldin A (BFA) for 30 min were treated with 200 μ*M* Cr(VI) for 0–3 h. For superoxide anion radical staining, rice roots were incubated in 10 m*M* potassium phosphate buffer, pH 7.8, supplemented with 0.5 mg ml^{-1} NBT (Sigma-Aldrich) and 10 m*M* NaN_3. For H_2O_2 staining, roots were soaked in 10 m*M* 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 3.8, with 1 mg ml^{-1} DAB for 8 h in darkness. The stained roots were observed using a Leica MZ12.5 stereomicroscope (Leica, Wetzlar, Germany).

In situ detection of lipid peroxidation

Lipid peroxidation in rice roots exposed to Cr(VI) was detected by the use of Schiff's reagent (Pompella et al. 1987, Yamamoto et al. 2001). Freshly harvested rice roots were incubated with Schiff's reagent for 60 min in dark to detect aldehydes originating from lipid peroxides. The reaction was stopped by immersion of the roots in potassium sulfite solution [0.5% (w/v) $K_2S_2O_5$ prepared in 0.05 *M* HCl]. The stained roots were soaked in the sulfite solution to retain the staining color.

Isolation and purification of total RNA

Total RNA was extracted from 6-day-old rice roots treated with 200 μ*M* Cr(VI) after 1–3 h by use of the

QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany), with some modifications. The yield and purity of total RNA were detected by a spectrophotometer (NanodropND 2000; Nanodrop technologies, Wilmington, DE). RNA samples of 2 μg μl^{-1} and high purity ($OD_{260/280}$ and $OD_{260/230} > 2$) were used for microarray analysis, semi-quantitative real-time polymerase chain reaction (RT-PCR) and quantitative RT-PCR.

Microarray preparation and analysis

Roots of 6-day-old rice seedlings treated with 200 μ*M* Cr(VI) for 1 and 3 h were harvested and total RNA was extracted from the roots. The Agilent Rice Oligo microarray system, containing 44K RAP-DB (4x44K, custom-made; Agilent Technologies, Palo Alto, CA), was used to identify Cr(VI)-regulated genes. Three biological replications were performed to obtain statistically reliable data. Total RNA (0.5 μg) from each pooled sample was amplified by use of a Fluorescent Linear Amplification Kit (Agilent Technologies). The Cr(VI)-treated RNA was labeled with Cy5-CTP and the control RNA was labeled with Cy3-CTP (CyDye; PerkinElmer, Waltham, MA); all reactions were done in the dark. The Cy-labeled cRNA (0.825 μg) was fragmented to an average size of about 50–100 nt by incubation with fragmentation buffer (Agilent Technologies) at 60°C for 30 min. The fragmented labeled cRNA was pooled and hybridized to the Rice Oligo DNA Microarray 44K RAP-DB (G2519F#15241; Agilent Technologies) at 60°C for 17 h. After a washing and blow-drying with a nitrogen gun, microarrays were scanned with use of an Agilent microarray scanner (Agilent Technologies) at 535 and 625 nm for Cy3 and Cy5, respectively.

Signal intensities were analyzed by use of Feature Extraction 9.5.3 (Agilent Technologies). For statistical analysis, we excluded genes with signal intensities of <100 in all experiments after correction for the dye effect by averaging the two color swaps. Statistical significance was assessed by *t* test with use of GeneSpringGX11 (Agilent Technologies). The false discovery rate (FDR) method (Benjamini and Hochberg 1995) was used to calculate corrected *P* values for multiple testing. The fold change in expression of each probe after Cr(VI) treatment was averaged on the basis of three biological replicates. The genes with >2-fold change in expression with Cr(VI) treatment (FDR cut off < 0.1) were extracted.

Cr(VI)-responsive genes were annotated by use of the Rice Annotation Project Database [RAP-DB; http://rapdb.lab.nig.ac.jp/ (Rice Annotation Project 2007, 2008)] and the TIGR Rice Genome Annot ation Resource (http://www.tigr.org/tdb/e2k1/osa1/index.shtml) (Ouyang et al. 2007) and classified into functional categories by AgriGO functional enrichment analysis (Du et al. 2010). Annotation and functional information for signaling and transcription factor (TF)-related gene was retrieved from the Rice Kinase Database (Dardick et al. 2007) and the Database of Rice TFs (Gao et al. 2006), respectively. The microarray data in this study have been deposited in Gene Expression Omnibus and are accessible by the series accession number [GEO: GSE33376] (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33376) (Edgar et al. 2002).

Expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from Cr(VI)-treated rice roots as described. Cr-regulated genes with significant fold change were selected and subjected to semi-quantitative RT-PCR. Oligonucleotide primers are listed in Table S1. A gene encoding α-tubulin (Os03g0726100) was used as a reference gene. Amplicons were analyzed by agarose gel electrophoresis (2%) and PCR products were sequenced. For each gene expression analysis, two or three biological replicates were performed and showed nearly identical results. One of the representative data was shown.

RT-PCR analysis

The cDNA was synthesized from 1 μg of total RNA in a 20 μg reaction volume according to the manufacturer's instructions (Promega, Madison, WI). Quantitative RT-PCR (qRT-PCR) reactions were performed to quantify the transcript levels of the selected genes using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) and iQ™ SYBR® Green Supermix (Bio-Rad). Primers for RT-PCR reaction were designed and analyzed using PRIMER EXPRESS 3.0 Software (Applied Biosystems, Carlsbad, CA). Primer sequences are described in Table S2. The thermal cycles included an initial 95°C for 10 min, followed by 40 cycles each at 95°C for 15 s and 60°C for 1 min. Following PCR, a melting curve analysis was carried out. Relative quantification of specific mRNA levels was analyzed using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). All reactions were performed in triplicate from three independent samples. The relative expression levels were normalized using *α-tubulin* (Os03g0726100) as the reference gene. All statistical analyses to determine the significant difference of relative expression of individual genes between control and Cr-treated samples were carried out by one-way ANOVA followed by Duncan's Multiple Range test, using the SPSS software (version 11.5 for Windows; SPSS Inc., Chicago, IL). Means with different letters are significantly different at $P < 0.05$ level.

VIGS in *N. benthamiana*

To isolate the full-length cDNA sequence of *NbExo70* in *N. benthamiana*, we performed a homology search using an *N. benthamiana* BLAST search of the database Sol Genomics Network, Boyce Thompson Institute for Plant Research (http://solgenomics.net/tools/blast/index.pl). The expressed sequence tags with accession number Niben.v0.3.Scf25258075 in *N. benthamiana* were obtained by using *Arabidopsis AtExo70* as the query nucleotide sequence. A 568 bp *NbExo70* cDNA fragment, annealing between positions 81 and 650, was cloned into *Eco*RI site of pTRV2 (pYL156) empty vector to generate pTRV2-*NbExo70*. For virus-induced gene silencing (VIGS) experiment, the *tobacco rattle virus* plasmid 1 (pTRV1; pYL192), pTRV2-empty and pTRV2-*NbExo70* were transformed separately into *Agrobacterium tumefaciens* GV3101. *Agrobacterium tumefaciens* was grown at 28°C in Luria–Bertani liquid medium supplemented with 50 μg ml^{-1} kanamycin to a stationary phase. Bacteria were collected by centrifugation at 1730 g for 10 min at room temperature and resuspended in 10 m*M* 2-(N-morpholino) ethanesulfonic acid buffer, pH 5.2, 10 m*M* $MgCl_2$ and 200 μ*M* acetosyringone. A mixture of *Agrobacterium* cultures containing pTRV1 and pTRV2-*NbExo70* constructs in a 1:1 ratio was infiltrated into the lower leaf epidermis of plants at the four-leaf stage by use of a 1-ml needleless syringe (Liu et al. 2002). Agrodrench-mediated VIGS in *N. benthamiana* roots was performed as described by Ryu et al. (2004). *Agrobacterium* strains containing pTRV1 and pTRV2-*NbExo70* were mixed in a 1:1 ratio. A volume of 3 ml *Agrobacterium* suspension was drenched into the crown part of each 15-day-old seedlings on pots containing vermiculite. VIGS plants were kept in a growth chamber at 22°C under long day condition (16 h white light/8 h dark) for 2 days. Plants were then cultivated at 27°C for phenotype analysis. Twenty days after infiltration or Agrodrench, *N. benthamiana* plants were treated with 200 μ*M* K_2CrO_4. At 5 days after Cr treatment, leaves and roots were collected for in situ detection of ROS. Superoxide anion radicals and hydrogen peroxide accumulation in leave and roots were detected as described above. Subsequently, treated leaves were decolorized by boiling ethanol.

Leaf disks Cr(VI) bioassay

NbExo70-silenced *N. benthamiana* plants and TRV control plants (5-week-old) were used for Cr(VI) bioassay. Leaf disks with 9-mm diameter were placed in regeneration media of plants (RMOP) amended K_2CrO_4 to a final concentration of 0–400 μ*M*. The RMOP consists of Murashige and Skoog (MS) salts, 1 mg l^{-1} N6-benzyladenine, 0.1 mg l^{-1} 1-naphthaleneacetic acid, 1 mg l^{-1} thiamine, 100 mg l^{-1} inositol, 10 g l^{-1} agar and 30 g l^{-1} sucrose at pH 5.8. The leaf disks were incubated for 5 days in growth chamber. Growth chamber conditions were kept at 27°C and 70% humidity under long day regime (16 h white light/8 h dark). The effect of Cr(VI) on leaf disks was evaluated by quantifying the chlorophyll content and fresh weight (FW) from 10 leaf disks per TRV control plants and *NbExo70*-silenced *N. benthamiana* plants. The leaf disks were frozen by immersion in liquid nitrogen and ground using the TissueLyser LT (QIAGEN). Chlorophyll was extracted from plant material by homogenizing leaf disks in 1 ml aliquots of 80% (v/v) chilled acetone, and the extract absorbance was the measured. The significance of differences between the samples at $P < 0.05$ was assessed by ANOVA using SPSS version 11.5 (SPSS Inc.).

Results

Cr(VI)-induced ROS generation and Ca^{2+} accumulation in rice roots

The effect of Cr(VI) toxicity on root growth was assessed by dose-dependent analysis. At 3 days after treatment with 0, 25, 50, 100, 200 or 400 μ*M* of Cr(VI), the lengths of root elongation were determined. Compared to control plants, plants treated with 50 μ*M* Cr(VI) showed clear reduction in root growth. At 200 μ*M* Cr(VI), growth was strongly inhibited (Fig. 1A).

To determine whether Cr(VI) treatment induced ROS generation and Ca^{2+} accumulation, rice roots were labeled with a ROS-sensitive dye, CM-H_2DCF-DA or a Ca^{2+} indicator, Oregon green 488 BAPTA-1. CM-H_2DCF-DA, a non-fluorescent compound, is able to react with intracellular ROS to generate fluorescent DCF. Rice roots were treated with 200 μ*M* Cr(VI) for 15 min. The levels of DCF and Ca^{2+} green fluorescence were higher in rice roots with Cr(VI) than control treatment (Fig. 1B, C). In addition, it was observed that Cr(VI) had profound effects on ROS-induced lipid peroxidation in rice roots (Fig. S1). These results suggest that Cr(VI) treatment triggered ROS generation and Ca^{2+} accumulation in rice roots.

Cr(VI)-induced NADPH oxidase and CDPK

To determine whether NADPH oxidase could play an important role in Cr(VI)-induced ROS generation, the effect of Cr(VI) treatment on NADPH oxidase activity in rice roots was characterized using an in-gel assay and XTT assay. The results showed that NADPH oxidase-like activities were strongly induced under treatment with

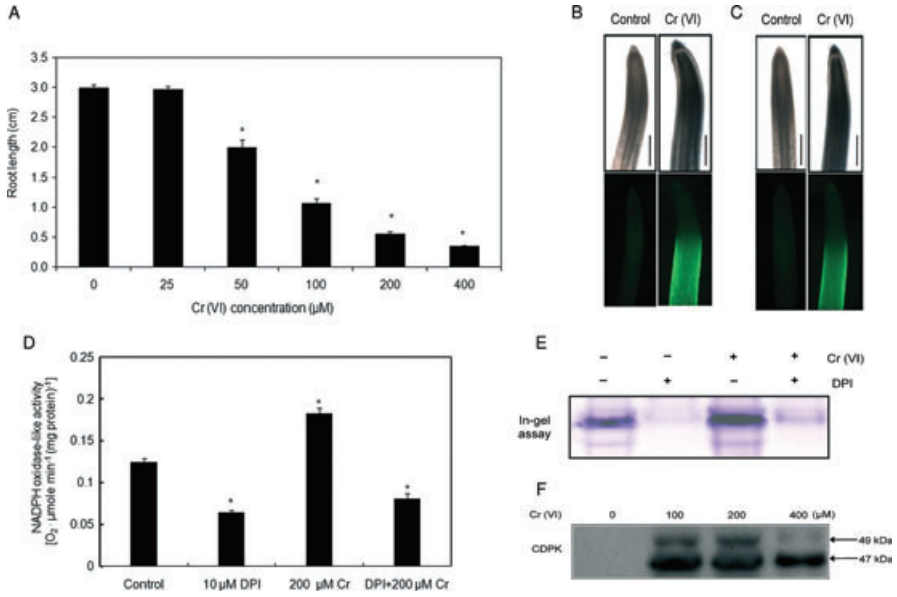

Fig. 1. Effect of Cr(VI) treatment on growth of rice seedlings, ROS production, Ca accumulation and NADPH oxidase and CDPK activity. (A) Rice roots were exposed to different concentrations of Cr(VI) (0, 25, 50, 100, 200, 400 μ*M*) for 3 days, and root lengths were measured. Data are mean ± SD. *Significantly different from the control at $P < 0.05$ by paired *t* test. (B) Rice root were treated with 10 μ*M* CM-H2DCF-DA for 30 min and exposed to 200 μ*M* Cr(VI) for 15 min. Green fluorescence DCF signals indicates ROS in rice roots. Black bars, 500 μ*M*. (C) Root samples (n = 10/each biological replicate) were treated with 10 μ*M* Oregon Green 488 BAPTA-1 for 30 min, and then exposed to 200 μ*M* Cr(VI) for 15 min. Black bars, 500 μ*M*. (D and E) Cr(VI) induced NADPH oxidase activity in rice roots. NADPH oxidase-like activities in protein extracts from rice roots were analyzed by XTT assay (D) and in-gel assay (E). Data for NADPH oxidase-like activity are mean ± SE. (F) Cr(VI) induced CDPK activity in rice roots treated with 0–400 μ*M* Cr(VI) for 1 h. Arrows indicate kinase-active bands.

Cr(VI) at 15 min and were inhibited by DPI, an NADPH oxidase inhibitor (Fig. 1D, E).

Furthermore, we used SDS-PAGE and in-gel kinase assay to assess whether Cr(VI) induced CDPK activation. These results indicated that 47- and 49-kDa CDPK-like kinase activities were significantly increased during Cr(VI) treatment (Fig. 1F).

Microarray-based expression profiling

To elucidate the change in transcriptome of the rice response to excess Cr(VI), we used DNA microarray analysis of RNA from rice roots treated with 200 μ*M* Cr(VI) for 1 and 3 h. We identified 1261 upregulated genes with FDR <0.1 and fold change ≥2. In contrast, 267 transcripts were downregulated in response to 200 μ*M* Cr(VI) (Table S3). We used AgriGO for gene ontology (GO) category enrichment analysis of the 1261 upregulated genes (Table 1, Table S4) (Werner 2008). In total, 1146 genes with changed expression were related to biological processes, such as signal transduction, metabolic process, cellular localization, response to stress, hormone-mediated signaling pathway, phosphorylation, dephosphorylation and regulation of gene expression. In total, 1019 upregulated genes were related to molecular functions such as kinase activity, transferase activity, TF activity, phosphatase activity and calcium ion binding.

The GO category-based data were further confirmed by using MapMan ontology analysis (Usadel et al. 2005). We observed induction of several genes involved in phytohormones (Fig. S2).

Expression profiles of genes encoding for protein kinase and phosphatase

Microarray assay revealed that Cr(VI) induced several components of signaling pathways, such as protein kinase, protein phosphatase and phytohormone-related genes. The rice genome consists of at least six protein kinase groups with approximately 1464 protein-coding genes. These include groups of *GMGC* (including *CDK*, *MAPK*, *GSK3* and *CLK* families), calcium/CaM-dependent protein kinase (*CAMK*), casein kinase 1 (*CK1*), tyrosine kinase-like [*TKL*; including interleukin-1 receptor-associated kinase (IRAK) family and both receptor and cytoplasmic kinases], *AGC* (*PKA*, *PKG* and *PKC*) and *STE* (homologs of yeast sterile 7, sterile 11 and sterile 20 kinases) (Manning et al. 2002, Shiu et al. 2004, Dardick and Ronald 2006).

Table 1. GO analysis of 1261 genes upregulated with Cr(VI) stress by AgriGO functional enrichment analysis. GO terms with *P*-value < 0.001 and FDR ≤ 0.05 were regarded as overrepresented terms. [a]Query item number in *Oryza sativa* (KOME, FL cDNA) genome version 2008-01-24. [b]Query item number in 1261 upregulated genes in rice root in Cr(VI) stress. [c]Determined by Fisher exact test. [d]Determined by Benjamini–Hochberg–Yekutieli procedure.

GO ID	Background item[a]	Query item[b]	*P*-value[c]	FDR[d]	GO term
Biological process					
Metabolic process					
GO:0044267	374	113	4.80E-85	2.70E-83	Cellular protein metabolic process
GO:0006629	81	24	1.70E-19	2.50E-18	Lipid metabolic process
GO:0034641	32	30	3.50E-46	6.80E-45	Cellular nitrogen compound metabolic process
GO:0042180	86	40	1.40E-40	2.40E-39	Cellular ketone metabolic process
GO:0009698	11	6	9.20E-08	8.90E-07	Phenylpropanoid metabolic process
GO:0005984	10	5	2.00E-06	1.70E-05	Disaccharide metabolic process
GO:0016101	35	5	0.0016	0.0094	Diterpenoid metabolic process
GO:0044036	21	5	0.00013	0.00084	Cell wall macromolecule metabolic process
GO:0009150	17	5	4.30E-05	0.0003	Purine ribonucleotide metabolic process
GO:0009311	10	5	2.00E-06	1.70E-05	Oligosaccharide metabolic process
GO:0006511	13	5	9.80E-06	9.80E-06	Ubiquitin-dependent protein catabolic process
GO:0016310	258	79	5.80E-61	1.30E-59	Phosphorylation
GO:0016311	14	6	5.60E-07	5.10E-06	Dephosphorylation
Regulation of biological process					
GO:0010468	324	102	3.80E-79	1.50E-77	Regulation of gene expression
GO:0051171	323	101	4.40E-78	1.40E-76	Regulation of nitrogen compound metabolic process
GO:0050789	429	121	7.70E-87	5.50E-85	Regulation of biological process
GO:0042592	7	7	5.50E-12	6.30E-11	Homeostatic process
GO:0007165	106	14	3.90E-07	3.60E-06	Signal transduction
Response to stimulus					
GO:0006950	103	50	2.00E-51	3.90E-50	Response to stress
GO:0070887	43	9	8.80E-07	7.70E-06	Cellular response to chemical stimulus
GO:0009755	42	7	6.90E-05	0.00045	Hormone-mediated signaling pathway
GO:0009734	39	5	0.0026	0.015	Auxin-mediated signaling pathway
Cellular process					
GO:0006810	232	53	1.50E-34	2.50E-33	Transport
GO:0051641	21	6	8.70E-06	6.90E-05	Cellular localization
GO:0008219	124	13	1.30E-05	9.70E-05	Cell death
Molecular function					
Catalytic activity					
GO:0016740	507	179	3.1E-145	3.90E-143	Transferase activity
GO:0016301	261	88	1.10E-71	3.30E-70	Kinase activity
GO:0004674	204	71	1.60E-59	3.80E-58	Protein serine/threonine kinase activity
GO:0016817	44	31	7.10E-40	1.10E-38	Hydrolase activity, acting on acid anhydrides
GO:0015036	10	5	2.00E-06	1.50E-05	Disulfide oxidoreductase activity
GO:0004568	21	6	8.70E-06	5.90E-05	Chitinase activity
GO:0004842	17	5	4.30E-05	0.00029	Ubiquitin-protein ligase activity
GO:0016791	16	12	8.40E-17	9.90E-16	Phosphatase activity
GO:0019787	19	5	7.80E-05	0.00048	Small conjugating protein ligase activity
Binding					
GO:0046914	132	132	8.40E-210	1.40E-207	Transition metal ion binding
GO:0030554	467	129	4.90E-91	2.70E-89	Adenyl nucleotide binding
GO:0005524	465	120	3.10E-81	1.20E-79	ATP binding
GO:0005509	39	27	1.40E-34	2.00E-33	Calcium ion binding
Transcription regulator activity					
GO:0003700	116	50	3.80E-48	7.50E-47	TF activity
Enzyme regulator activity					
GO:0004867	37	6	0.00027	0.0016	Serine-type endopeptidase inhibitor activity
Transporter activity					
GO:0005215	173	28	5.10E-15	5.80E-14	Transporter activity
GO:0022892	85	13	1.80E-07	1.30E-06	Substrate-specific transporter activity
GO:0004871	32	19	7.60E-23	1.00E-21	Signal transducer activity
GO:0043492	17	9	6.80E-11	6.60E-10	ATPase activity, coupled to movement of substances

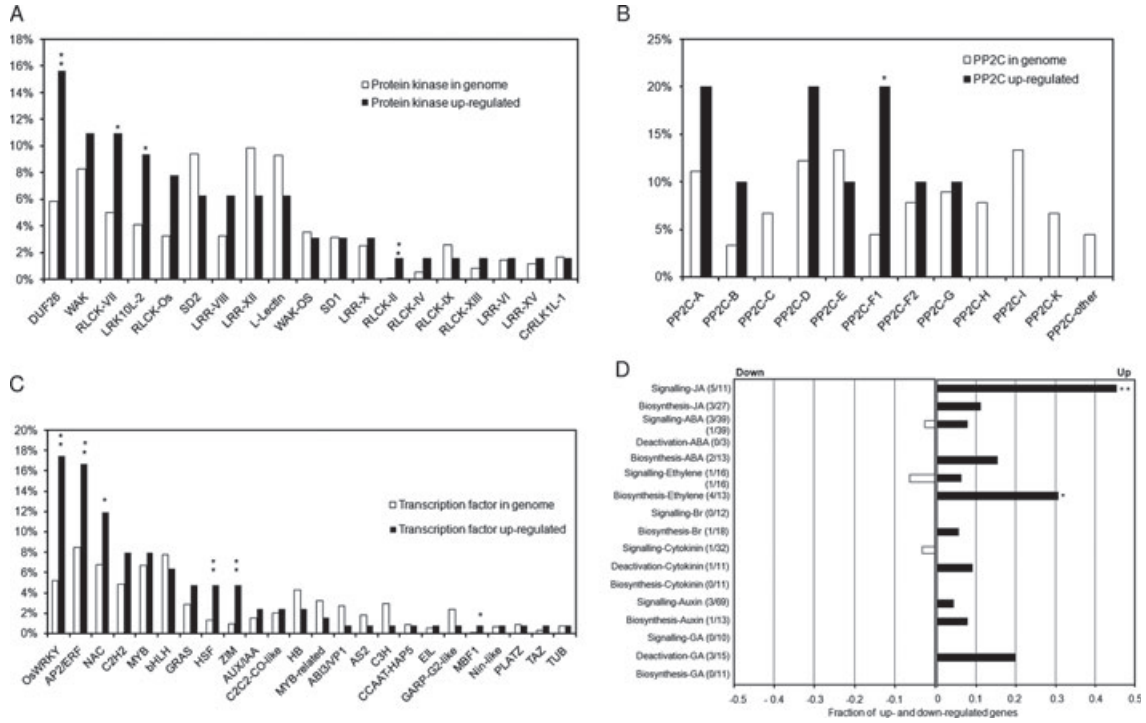

Fig. 2. Protein kinase families (A), protein phosphatase 2C families (B), TF families (C) and phytohormone families (D) of Cr(VI) stress response genes. In (A), (B) and (C), there are 19 protein kinase families, 12 protein phosphatase 2C families and 24 TF families, respectively. White bars indicate total number of genes within the functional category/total number of genes in the rice genome. *Black bars* indicate total number of upregulated genes within the functional category/number of all genes within the functional category. The data are percentages. Protein kinase families, protein phosphatase 2C families and TF families that are significantly overrepresented in the functional category are shown with ** ($P < 0.01$) or * ($P < 0.05$). In (D), the number in parentheses is the number of differentially expressed genes in each category. Positive and negative values indicate up-and down-regulated genes, respectively. The numbers of differentially expressed genes in relation to phytohormone-related pathways are plotted as a percentage. $^{**}P < 0.01$ and $^{*}P < 0.05$ are significantly overrepresented (Chi-square test).

Of the 1169 protein kinase genes represented on our microarray data, we identified 80 transcripts that were upregulated with Cr(VI) treatment and 6 downregulated ones (Table S5). Within IRAK family, the transcriptional regulation of domain of unknown function 26 (*DUF26*), receptor-like cytoplasmic kinase (*RLCK*) and LRK10-like kinase type 2 (*LRK10L-2*) significantly increased during Cr(VI) stress (Fig. 2A). In addition, our microarray revealed a number of calcium regulation-related genes belonging to *CAMK* group, such as *OsCDPKs* and *OsCMLs* (Table S6). Transcripts for two *CDPK* genes, *OsCPK4* (Os02g0126400) and *OsCPK13* (Os04g0584600), were significantly increased by Cr(VI) treatment. In addition, four *OsCML* genes were regulated by Cr(VI); especially transcript level of *OsCML31* (Os01g0955100) was strongly increased (Table 2).

Signal extinction will, in part, involve the subsequent association of phosphatases to dephosphorylate the protein. Protein phosphatases play important roles in this inactivation process. The rice genome encodes 132 rice protein phosphatase-coding genes phylogenetically categorized into five classes: *PP2A*, *PP2C*, *PTP*, *DSP* and *LMWP* (Singh et al. 2010). *PP2C* is the major class of protein phosphatase, including 90 genes (Table S5). Our arrays indicated that 10 *PP2C* genes were upregulated with Cr(VI) treatment (Fig. 2B, Table 3).

Analysis of genes related to TFs

The rice genome encodes approximately 1930 TF genes divided into 63 families. Our microarray data indicated that 126 TF genes were upregulated with Cr(VI) treatment. These belong to the *WRKY*, *AP2/ERF* (APETALA2/ethylene response factor), *bHLH*, *C2H2*, *MYB*, *NAC*, heat shock factor (*HSF*) and *ZIM* families (Fig. 2C, Table S7). More than 30% of the genes encoding *WRKY* and *HSF* TFs were induced by Cr(VI) stress. In addition, 22 TF genes (*bHLH* and *OsbZIP* families) were downregulated with Cr(VI). Transcript level of 21 *AP2/ERF* TFs was increased more than twofold with Cr(VI) treatment (Table S7). Among the 22

Table 2. Cr(VI)-responsive genes related to signal transduction. Data are number of genes showing significant differences in transcript abundance (FDR < 0.1).

Functional categories	In genome	On array	Detected	Increased	Decreased
Calcium signaling cascades					
CaM	37	34	27	4	1
CBL	10	9	9	0	0
CIPK	29	29	21	0	0
CDPK	30	29	25	2	0
				Os02g0126400	
				Os04g0584600	
IQD	28	27	24	0	0
MAPK cascades					
MAPK	16	16	16	1	0
MAPKK	8	6	6	0	0
MAPKKK	75	71	64	9	0

Table 3. Cr(VI)-responsive genes related to protein phosphatase. Data are number of genes showing significant differences in transcript abundance (FDR <0.1).

Functional categories	In genome	On array	Detected	Increase	Decrease
OsLMWP	1	1	1	0	0
OsDSP	22	22	20	0	0
OsPP2A	17	17	17	0	0
OsPTP	2	2	2	0	0
OsPP2C	90	89	67	10	0
				Os02g0799000	
				Os03g0207400	
				Os03g0761100	
				Os04g0451900	
				Os04g0609600	
				Os05g0457200	
				Os06g0698300	
				Os09g0325700	
				Os11g0109000	
				Os12g0198200	

WRKY upregulated genes, the transcripts of 11 were increased by at least fivefold.

Expression pattern of genes involved in phytohormone pathways

Phytohormones play an important role in signaling. The rice genome encodes 324 phytohormone-related genes classified into seven families. To analyze whether phytohormone-related gene transcript level changed in rice roots exposed to Cr(VI), we analyzed transcript expression level changes of seven phytohormone-related gene families (Table S8). Among 299 phytohormone-related genes presented on our microarray data, 26 and 3 phytohormone-related genes were up- and down-regulated with Cr(VI) treatment, respectively. Transcripts putatively related to gibberellins (GAs), ethylene, abscisic acid (ABA) and jasmonic acid (JA) metabolism were affected by Cr(VI) (Fig. 2D). In addition, almost 30% of JA-related genes were induced by Cr(VI). Specifically, transcripts for two genes encoding enzymes for ABA biosynthesis (Os07g0154100) and JA biosynthesis (Os08g0143600) increased in abundance by approximately 69- and 50-fold, respectively, with Cr(VI) stress (Table S8).

Expression profiles of vesicle trafficking-related genes

A strong effect of Cr on cellular localization genes can be seen in the AgriGO analysis (Table 1). Cr(VI) strongly induced a number of genes involved in vesicle trafficking pathway, including five *OsExo70* genes (Os01g0763700, Os06g0255900, Os01g0905300, Os01g0905200 and Os11g0649900) and one *Tom1* gene (Os05g0475300) (Table S9). The rice genome contains 41 *OsExo70* genes classified into nine groups, A–I (Synek et al. 2006, Chong et al. 2010). *OsExo70* upregulated genes belong to the groups E, F and H (Table 4).

We next tested the role of vesicle trafficking pathway in Cr(VI)-induced ROS production. The superoxide anion

Table 4. Cr(VI)-responsive genes related to Exo70 family. Data are number of genes showing significant differences in transcript abundance (FDR < 0.1).

Functional categories	In genome	On array	Detected	Increased	Decreased
Total Exo70 family	41	39	22	5	0
Exo70.1	1	1	0	0	0
Exo70.2	1	0	0	0	0
Exo70A	4	4	3	0	0
Exo70B	3	3	2	0	0
Exo70C	2	2	2	0	0
Exo70D	2	2	2	0	0
Exo70E	1	1	1	1	0
				Os01g0763700	
Exo70F	20	19	9	3	0
				Os06g0255900	
				Os01g0905300	
				Os01g0905200	
Exo70G	2	2	2	0	0
Exo70H	4	4	1	1	0
				Os11g0649900	
Exo70I	1	1	0	0	0

was distributed in root tips of Cr(VI)-treated roots. H_2O_2 was produced in both root tips and vascular tissues (Fig. 3). Rice seedling roots were pretreated with BFA, and ROS generation was assayed in rice roots immediately after exposure to Cr(VI) stress. BFA markedly suppressed ROS production during Cr(VI) stress (Fig. 3).

Suppression of endogenous transcripts of *NbExo70* by VIGS

In order to gain more insight into the role of *Exo70* in response to Cr(VI) stress, we extended the transcriptomic and physiological studies to examine its functions in *N. benthamiana* by VIGS strategies. A full-length cDNA clone of 1797 nt was isolated and termed *NbExo70*. The nucleotide sequence of the *NbExo70* cDNA contained an open reading frame (ORF) that would encode a predicted polypeptide of 598 amino acids (Fig. S3). The molecular weight of the predicted polypeptide was 68.45 kDa. The amino acid sequence of NbExo70 showed significant homology to *Solanum lycopersicum* SlExo70, *Vitis vinifera* VvExo70, *Arabidopsis thaliana* AtExo70, *O. sativa* OsExo70 with 93, 75, 69 and 60% identity, respectively. pTRV-*NbExo70* containing partial sequence of ORF were inoculated into *N. benthamiana* young leaves by leaf infiltration and into roots by Agrodrench. After 3 weeks of inoculation with the gene silencing constructs pTRV-*NbExo70* and pTRV-empty vector as a control, the effect of VIGS on endogenous level of *NbExo70* mRNA in Cr(VI) stress response was examined by semi-quantitative RT-PCR. Expression of the housekeeping gene elongation factor 1-α (*NbEF1-*α) was an equal loading control and remained constant in all tested plants. The Cr(VI)-induced *NbExo70* gene expression was significantly reduced in *NbExo70*-silenced plant in comparison with the wild-type and TRV control plants (Fig. 4A). The morphology of *NbExo70*-silenced plants was not different from the TRV control plants (Fig. 4B). After 5 days of Cr(VI) treatment, the leaves of TRV control plant began to wilt and necrotize, whereas all leaves of *NbExo70*-silenced plants remained vigorous and intact (Fig. 4B). In addition, ROS staining with NBT and DAB for in situ detection of ROS in leaves and roots showed that *NbExo70*-silenced plants with Cr(VI) treatment inhibited the Cr(VI)-induced ROS production as compared with TRV control plants (Fig. 4C). This result suggested that vesicle trafficking-related *NbExo70* may relate to Cr(VI)-induced ROS production. The silencing of *NbExo70* may decrease the oxidative burst in Cr(VI) stress response.

Cr(VI) resistance bioassay

The leaf disks collected from *NbExo70*-silenced plants and TRV control plants were maintained in the RMOP containing various Cr(VI) concentrations for 5 days. TRV control plant disks developed severe chlorosis and eventually died, whereas *NbExo70*-silenced plants were highly resistant to 200 μM Cr(VI), even at 400 μM Cr(VI) (Fig. 4D). Measurements of chlorophyll content and FW showed that *NbExo70*-silenced plants had higher chlorophyll content and FW than TRV control plants (Fig. 4D). These results suggested that the silencing of *NbExo70* may increase Cr(VI) stress tolerance in *N. benthamiana*.

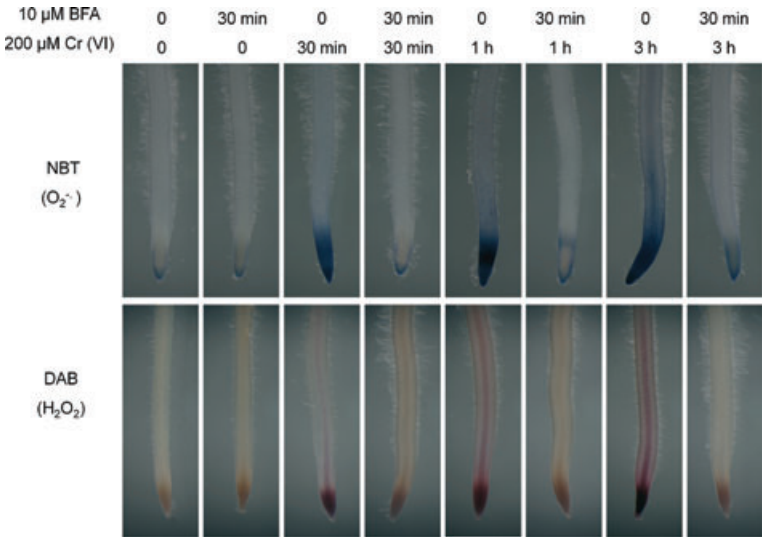

Fig. 3. Effect of BFA treatment on 200 μ*M* Cr(VI)-induced ROS accumulation in rice roots. Root samples pretreated with 10 μ*M* BFA for 30 min were exposed to 200 μ*M* Cr(VI) for 0–3 h. Superoxide anion radicals and H_2O_2 were determined by staining roots with NBT and DAB, respectively.

Verification of microarray data by semi-quantitative RT-PCR and qRT-PCR

The selection of Cr-regulated genes from the microarray data such as two signal transduction-related genes (*OsMPK3*, *OsCML31*), three protein kinase-related genes (*OsWAKL-Os*, *OsLRK10L-2*, *OsDUF26-If*), two TF-related genes (*OsWRKY26*, *OsAP2/ERF-130*) and two vesicle trafficking-related genes (*OsExo70Fx14*, *OsExo70Fx15*) were subjected to semi-quantitative RT-PCR. The results obtained from all nine genes tested exhibited similar expression profiles in response to Cr(VI), which compares with our microarray data and indicates the reliability of our results (Fig. 5). Transcript levels for the selected genes increased and peaked at 3 h with Cr(VI) treatment.

In order to provide further evaluation of our microarray data, qRT-PCR analysis was carried out to quantify the changes of mRNA expression level. We selected 10 candidate genes representing different functional categories, including four protein kinase genes (*RLCK-Os4*, *RLCK-VIIa*, *DUF26-If* and *LRK10L-2*), one protein phosphatase gene (*OsPP2C68*), three phytohormone genes (*OsNCED2*, *OsGA2ox1* and *OsACO5*) and two vesicle trafficking genes (*OsExo70Fx14* and *OsExo70Fx15*), obtained by microarray analysis. In a dose-response experiment, rice roots were treated with different concentrations of Cr(VI) ranging from 0 to 300 μ*M* for 3 h (Fig. 6A). The expression of all 10 genes was upregulated under Cr(VI) treatment. *LRK10L-2*, *OsPP2C68*, *OsExo70Fx14* and *OsExo70Fx15* showed a similar dosage-dependent expression pattern with maximum transcript level induced by 300 μ*M*. Cr(VI) increased their expression by about 8-, 35-, 13- and 10-fold relative to untreated controls, respectively. The expression of *RLCK-VIIa* and *OsACO5* showed similar levels at 100, 200 and 300 μ*M*. The expression of *RLCK-VIIa* and *OsACO5* was increased by 15- and 7-fold, respectively. In comparison, *RLCK-Os4*, *DUF26-If*, *OsNCED2* and *OsGA2ox1* had the stimulatory peak at 200 μ*M*. Cr(VI) stress increased their expression by 67-, 23-, 54- and 11.5-fold, respectively.

We next compared Cr(VI) treatment with Cd-, Cu- and As-treatment to analyze the expression characteristics of the candidate genes (Fig. 6B). Six-day-old rice seedlings were treated with different concentrations of metals [50 μ*M* K_2CrO_4, 200 μ*M* K_2CrO_4, 50 μ*M* $CuCl_2$, 50 μ*M* $CdCl_2$ and 50 μ*M* $Na_2HAsO_4.7H_2O$] for 3 h. The expression level of all candidate genes was significantly increased in Cr(VI)-treated rice roots as compared with Cd, Cu and As.

Further, we selected 200 μ*M* Cr(VI) to treat rice roots for different time periods ranging from 0 to 24 h (Fig. S4). As in period experiment, all the 11 genes were upregulated in response to Cr(VI) treatment. Dynamic changes in the transcript abundance of the candidate genes with early Cr(VI) treatment (3 h) were determined. The transcript level of three protein kinases (*RLCK-VIIa*, *DUF26-If* and *LRK10L-2*), one protein phosphatase (*OsPP2C68*), three phytohormones (*OsNCED2*, *OsGA2ox1* and *OsACO5*) and two vesicle trafficking genes (*OsExo70Fx14* and *OsExo70Fx15*) were strongly increased soon after Cr(VI) treatment, which declined at 12 h exposure.

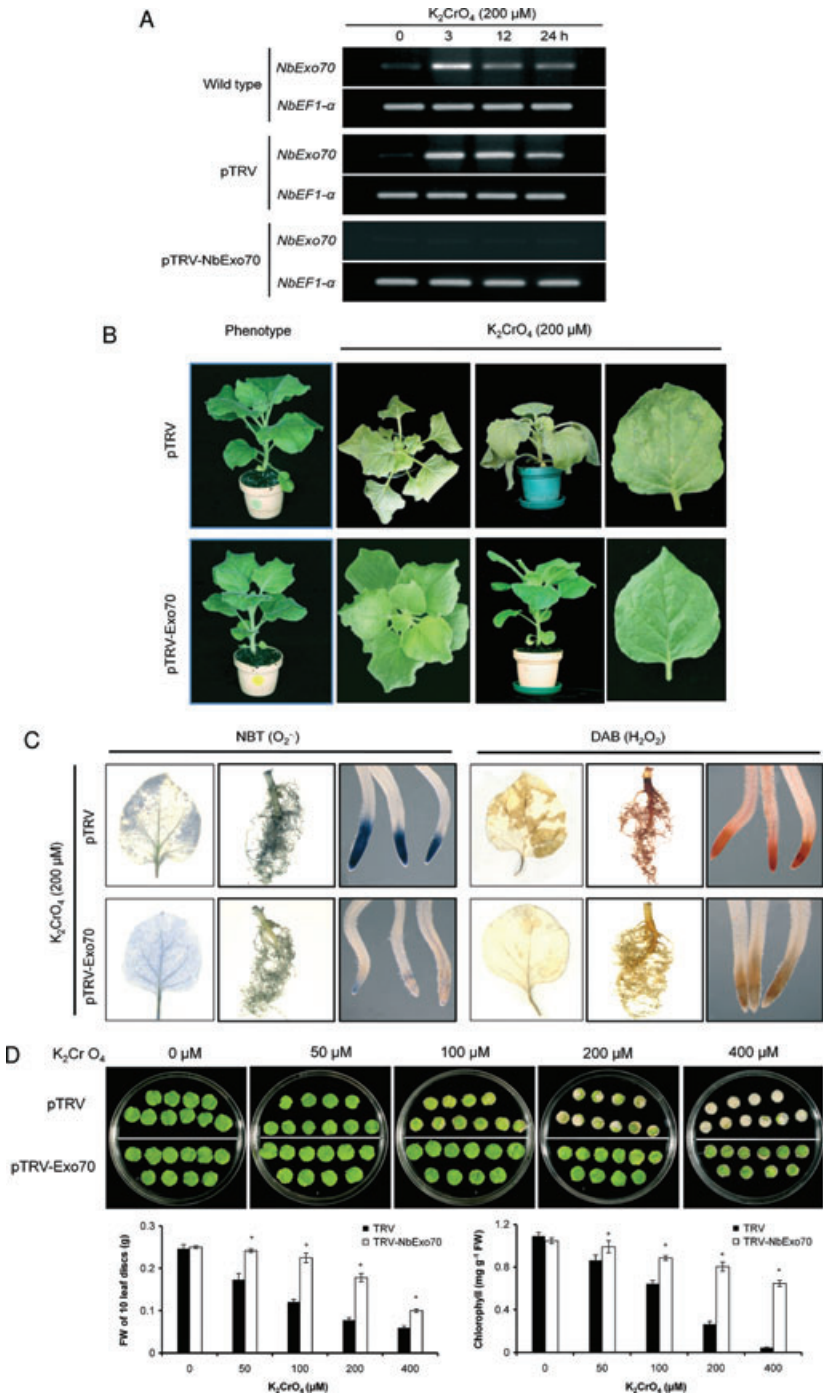

Fig. 4. Functional analysis of *Exo70* in *Nicotiana benthamiana* in Cr(VI) stress response by VIGS strategies. (A) Effect of VIGS on *NbExo70* transcript levels in *N. benthamiana* in response to Cr(VI) treatment. The total RNA was extracted from leaf tissue from wild-type plant, TRV control plants and *NbExo70*-silenced plants treated with 200 μ*M* Cr(VI) at 0, 3, 12 and 24 h. The transcript level of the genes was analyzed by semi-quantitative RT-PCR. *NbEF1-α* mRNA levels served as a control. (B) Silencing of *NbExo70* improved Cr tolerance in *N. benthamiana* in response to 200 μ*M* Cr(VI) at 5 days post-treatment. TRV control plant showed necrosis and wilt in leaves in response to Cr(VI) treatment, whereas all leaves of *NbExo70*-silenced plants remained vigorous and intact. (C) Reduce of ROS accumulation in *NbExo70*-silenced plants after 200 μ*M* Cr(VI) treatment for 5 days. Detached leaves were stained with NBT and DAB solution as described previously. (D) Effect of Cr(VI) treatment on cellular integrity, FW and chlorophyll content of TRV control and TRV-*NbExo70* plant leaf tissue. Asterisks indicate significance at 0.05 levels (ANOVA).

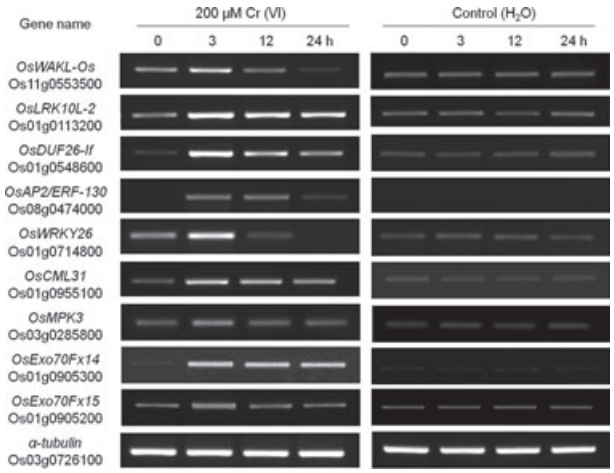

Fig. 5. Validation of microarray-based gene expression profiles of rice roots in response to 200 μ*M* Cr(VI) treatment for 0, 3, 12 and 24 h by semi-quantitative RT-PCR analysis. *α-tubulin* was an internal control.

Discussion

Cr is toxic and detrimental to plant development, growth and reproduction (Cervantes et al. 2001). Dubey et al. (2010) analyzed late transcriptome profiles of rice roots treated with Cr(VI) for 24 h, whereby, genes upregulated in rice roots under Cr(VI) stress were involved in membrane transport and signal transduction, biosynthesis of secondary metabolites and xenobiotics and amino acid metabolism. In contrast, downregulated genes were related to cell growth and death and energy metabolism. A complex network of regulatory pathways-related TF and *cis*-acting elements may modulate Cr(VI)-response of rice was suggested. However, no mechanism signaling transduction pathways specific and gene expressions to early Cr(VI) treatment was identified.

In this study, microarray analysis revealed that the phytohormone-, protein kinase-, protein phosphatase-, vesicle trafficking- and TF-related genes may play important roles in response to Cr(VI) stress in rice roots. In order to gain a deeper and more comprehensive insight into the role of the Cr(VI)-response genes, transcriptional changes of protein kinase-, protein phosphatase-, phytohormone- and vesicle trafficking-related genes in the dosages and time courses of Cr(VI) treatment were validated with qRT-PCR (Fig. 6A, Fig. S4). The results showed the early dynamic response and dosage-dependent response of the selected genes (*RLCK-Os4*, *RLCK-VIIa*, *DUF26-lf*, *LRK10L-2*, *OsPP2C68*, *OsNCED2*, *OsGA2ox1*, *OsACO5*, *OsExo70Fx14* and *OsExo70Fx15*) during Cr(VI) stress in rice roots. As compared with other metals (Cu, Cd and As), Cr(VI) was significantly able to upregulate these selected genes at both 50 and 200 μ*M* concentrations (Fig. 6B). However, *RLCK-Os4* and *OsNCED2* genes were also induced by Cu and As stress, respectively. The results suggest that the two early responsive genes are not only regulated by Cr but also by other heavy metal stresses. In this study, *OsExo70Fx14*, *OsExo70Fx15* and *OsGA2ox1* are Cr(VI)-specific responsive genes.

In plants, stress responses and growth are regulated by phytohormones (Bogatek and Gniazdowska 2007). In this study, the transcript levels of *OsNCED2* and *OsNCED3*, two genes encoding key enzymes of ABA biosynthesis, were strongly increased under Cr(VI) stress (Table S8). In addition, three ABA signaling genes were upregulated by Cr(VI) stress in rice roots (Table S8). Beaudoin et al. (2000) and Ghassemian et al. (2000) indicated that ABA inhibits root growth in *Arabidopsis* by signaling through the ethylene response 1 (ETR1) pathway. Furthermore, eight genes involved in biosynthesis and signaling of JA were induced by Cr(VI) treatment (Table S8). It was found that heavy metal stresses can induce the JA pathway lead to a fast increase of JA content in *O. sativa*, *Arabidopsis* and bean plants (Rakwal et al. 1996, Kruzmane et al. 2002, Maksymiec et al. 2005). Exogenous treatment of JA significantly reduced the *Arabidopsis* seedling growth in terms of root length and root FWs (Staswick et al. 1992). Recently, heavy metal stress was found to induce ethylene production (Mattoo et al. 1992, Blechert et al. 1995, Vassilev et al. 2004). The root growth inhibition by ethylene treatment and the increase in root elongation by treatment with ethylene perception inhibitors have been reported (Gallie et al. 2009). In this study, we found transcripts for two *OsACS* and two *OsACO* genes (*OsACS1*, *OsACS2*, *OsACO4* and *OsACO5*, respectively) involved in 1-aminocyclopropane-1-carboxylate (ACC) synthase biosynthesis, which encode key enzymes in the biosynthesis of ethylene, strongly induced by Cr(VI) (Table S8). In addition, one ethylene signaling gene (*OsEIN3;4*) was upregulated in Cr(VI) stress response. The catabolic enzyme GA2-oxidase (GA2ox) is found to be activated by stress to reduce GA levels and to inhibit growth (Achard et al. 2008, Hedden and Thomas 2012). In our microarray data, we indicated that transcript level of three *OsGA2ox* genes (*OsGA2ox1*, *OsGA2ox3* and *OsGA2ox9*) was strongly increased by Cr(VI) treatment (Table S8). Our MapMan analysis found the particular increase of ABA, JA and GA genes involved in Cr(VI) response (Fig. S1). Therefore, ABA, JA, GA and ethylene may potentially modulate Cr(VI)-induced inhibition of root growth in rice seedlings.

Protein kinases play critical roles in signaling pathways responding to heavy metal stress in plants. By perceiving or sensing the extracellular signals,

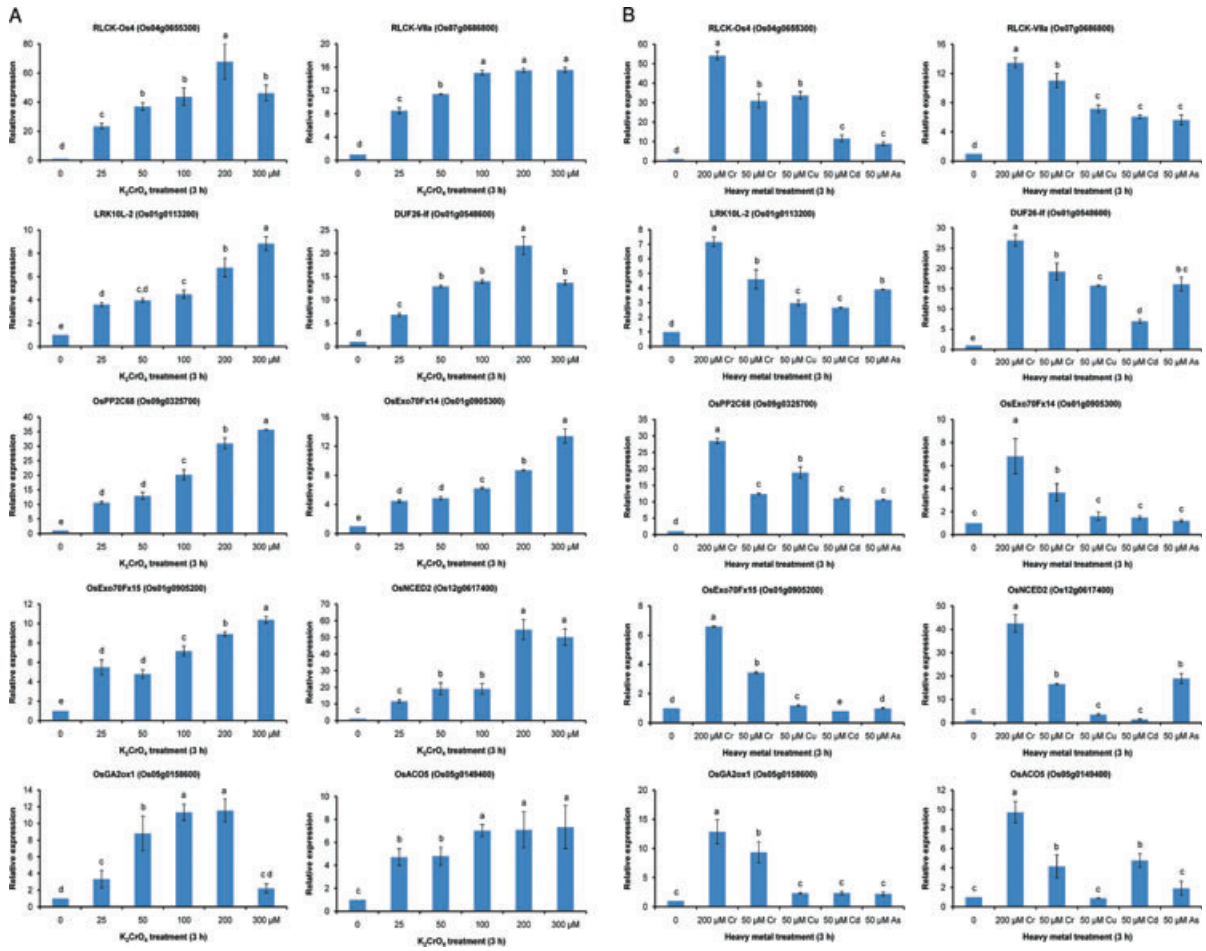

Fig. 6. Dosage of Cr(VI) (A) and metal stresses (B) effect on expression of protein kinase-, protein phosphatase-, phytohormone- and vesicle trafficking-related genes. Relative expression level of the transcript was quantified via the Livak method ($2^{-\Delta\Delta Ct}$). Data are showed as mean relative expression ± SD for three replicate RT-PCR reactions from three independent samples. Means with different letters are significantly different at $P < 0.05$ level (ANOVA).

receptor-like kinase (RLK) activates the downstream signaling pathway by phosphorylating specific targets (Becraft 2002). Among 70 regulated Cr(VI)-responsive *RLK* genes, *DUF26*, *RLCK* and *LRK10-L* were found to be significantly regulated and participated in transcriptional regulation (Table S5). Recent studies have indicated *RLCK-VII*, *RLCK-Os* and *RLCK-IV* playing roles in stress responses (Vij et al. 2008, Lehti-Shiu et al. 2009). In our microarray data, only cysteine-rich RLK (*CRK*) and *LRK10-L* were upregulated under Cr(VI) stress rice roots. The *DUF26 RLKs*, also known as *CRKs*, are largest subgroup of the *RLK* family, with more than 40 members (Shiu and Bleecker 2003). Wrzaczek et al. (2010) reported that DUF26 plays an important role in response to O_3, light stress, herbicides paraquat and hormone treatment. At least 26 and 8 *DUF26* genes were upregulated in response to O_3 and light stress, respectively. In addition, *LRK10-2*, also known as pathogenesis-related 5-like receptor kinase (PR5K) in *Arabidopsis*, has about 40 members in the rice genome (Morillo and Tax 2006, Dardick et al. 2012). In our study, we found six *LRK10L-2* genes with upregulated expression with Cr(VI) treatment in rice roots. *LRK10L-2* genes showed increased expression in response to UV-B and osmotic stress (Lehti-Shiu et al. 2009). Thus, differential expression of genes encoding transmembrane receptor kinases with Cr treatment revealed that multiple receptor kinases belonging to different families may have unique regulatory mechanisms.

Ca and protein kinases play important roles in signaling pathways in response to environmental stress in plants. Recent studies have indicated that Ca-regulated proteins and kinases (Das and Pandey 2010) such as *CaM* (Yang and Poovaiah 2003) and calcineurin B-like

(*CBL*) protein (Gu et al. 2008), *CDPK* genes and CBL-interacting protein kinase (*CIPK*) (Ray et al. 2007) are related to abiotic stress response in plants. In this work, we found that Cr(VI) stress induced Ca^{2+} accumulation in rice roots (Fig. 1C). In addition, the expression levels of four *CaM* genes and two *CDPK* genes (*OsCPK4* and *OsCPK13*) were increased by Cr(VI) stress (Table S6). Different stresses induce *CDPK* transcript levels, coupled with their increased kinase activity. Previous studies found that *CDPKs* were upregulated in rice roots under cold or salt stress. In transgenic rice lines, overexpression of *OsCPK13* (Os04g0584600) enhanced tolerance to cold, drought and salt stress (Saijo et al. 2000). *OsCPK4* was induced in rice roots under cold stress (Ray et al. 2007). In addition, our previous study showed that the activity of 47- and 49-kDa CDPK-like kinases was increased with heavy metal treatment (Yeh et al. 2007). In this study, activity of CDPK-like kinase was induced in response to Cr(VI) treatment. Taken together, these results implied that the transcriptional activation and posttranslation phosphorylation can regulate the activity of plant Ca sensor during Cr(VI) stress. Furthermore, two CDPKs (StCDPK4 and StCDPK5) could phosphorylate NADPH oxidases and thereby positively regulate the generation of ROS in plasma membrane (Kobayashi et al. 2007). We found NADPH oxidase-like activities induced by 200 μ*M* Cr(VI). CDPKs and NADPH oxidase may regulate ROS production during Cr(VI) treatment.

Protein kinases and protein phosphatases are essential for regulating reversible phosphorylation of protein in signal transduction and response to environmental stimuli in plants (Chernoff 1999, Iten et al. 1999, Luan 2000). The rice genome encodes approximately 132 rice protein phosphatase genes phylogenetically classified into five classes: *PP2A*, *PP2C*, *PTP*, *DSP* and *LMWP*. With our Cr(VI) stress treatment, all phosphatase transcripts with changed expression belonged to the *PP2C* class. Among them, genes were involved in Cr(VI) stress response, most of them belonging to *PP2C-A*, *PP2C-D* and *PP2C-F* subclasses. Most genes in the *PP2C-A* subclass play primary roles in stress (salt, mannitol and cold stress) tolerance in rice (Xue et al. 2008). Members of *PP2C-D* subclass may represent positive regulators in ABA-mediated signaling pathways. Some of the members of the *PP2C-F* subclass could be involved in mitochondrial stress signaling (Fujii and Toriyama 2008). In addition, PP2C inhibits the activity of CDPK (Geiger et al. 2011). Thus, Cr-induced PP2Cs, PP2C-A, PP2C-D and PP2C-F1, may switch off CDPK signaling cascades in response to Cr(VI) treatment in rice roots.

The TFs interact with *cis*-elements in the promoter regions of stress-related genes and induce the expression of downstream target genes, leading to abiotic stress tolerance (Agarwal and Jha 2010). TFs of WRKY family play a key role in the plant response to abiotic and oxidative stress (Eulgem and Somssich 2007, Lee et al. 2007). The microarray analysis of Cr(VI)-inhibited rice roots led us to identify 22 *WRKY* genes, indicating their participation in response to Cr(VI) stress in rice (Table S7). In addition, involvement of AP2/ERF in the response to abiotic stresses has been reported (Park et al. 2001, Yamaguchi-Shinozaki and Shinozaki 2006, Hinz et al. 2010). We found 21 *AP2/ERF* genes upregulated by Cr(VI) stress. In this study, the Cr-induced *WRKY* and *AP2/ERF* TF genes demonstrate their possible involvement in coping with the metal stress.

Vesicle trafficking machinery is involved in modulation of plant responses to environmental stresses. By regulating the rate of trafficking from and to the plasma membrane, the cell can adjust the effect of stresses (Levine 2002). Traffic-associated signal transduction pathways, membrane recycling or improved delivery of essential materials to its destination may work in concert to combat stresses (Levine et al. 2001, Surpin and Raikhel 2004, Sutter et al. 2006). Furthermore, the exocyst complex may function in Golgi-to-plasma membrane vesicle trafficking (Hsu et al. 2004). In plants, the exocyst complex consists of eight subunits: Exocyst (Exo)70p and Exo84p, Secretory (Sec)3p, Sec5p, Sec6p, Sec8p, Sec10p and Sec15p. *Oryza sativa* and *A. thaliana* are predicted to have 41 and 23 *Exo70* genes, respectively (Chong et al. 2010). Increased expression of *Exo70* genes was found in birch (*Betula pendula*) in response to Cu stress (Keinänen et al. 2007). Our previous study (Lin et al. 2013) revealed that vesicle trafficking-related genes were upregulated in early response (3 h) to Cu treatment. *AtExo70H1*, *H2*, *H7* and *B2* mRNA levels were strongly upregulated by elicitor peptide elf18 treatment (Pečenková et al. 2011) and loss of *AtExo70B2* and *AtExo70H1* functions in *Arabidopsis* enhanced the susceptibility to pathogens. The transcript level of *AtExo70A3* was increased by hormones such as ABA, zeatin and methyl jasmonate and ACC. In *Arabidopsis*, *Exo70* genes (*AtExo70A2*, *AtExo70B2*, *At Exo70 H1* and *AtExo70H4*) had increased expression levels in response to abiotic stress treatments (salt, cold, drought, UV- and oxidative) (Sottosanto et al. 2004, Li et al. 2010). Cr(VI)-induced vesicle trafficking-related gene expression was found in roots of willow (*Salix alba*) (Quaggiotti et al. 2007). In this study, Cr(VI) induced five *Exo70* genes and one VHS domain-containing protein (Table S9).The Cr(VI)-induced expression of exocytosis-related genes such as *OsExo70FX14* and *OsExo70FX15* was further validated in rice roots (Figs 5 and 6A). These results implied the potential involvement of *Exo70* genes in rice roots responding to Cr stress.

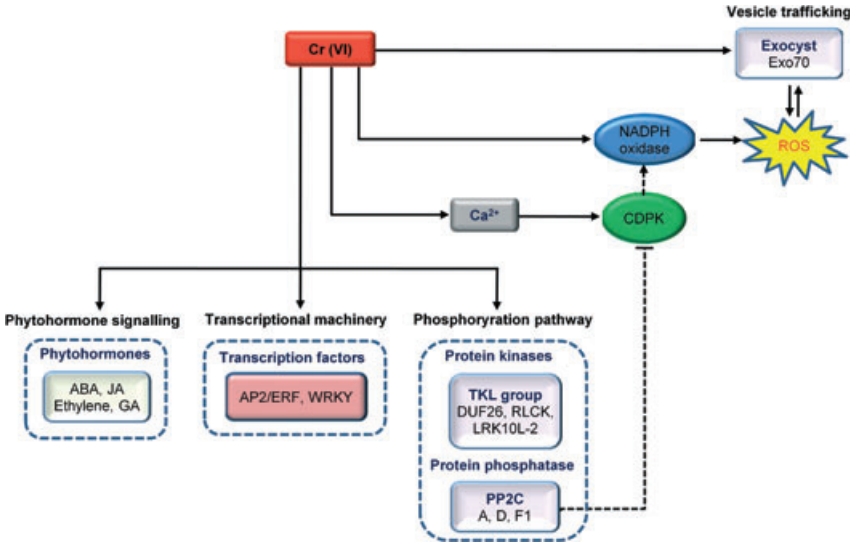

Fig. 7. Model of signaling pathways responding to Cr(VI) stress and the relation of genes identified in this expression analysis of rice root. Cr(VI) stress induces ROS production by NADPH oxidase in rice roots. Cr(VI) stress induces Ca accumulation. Increasing concentrations of Ca activate CDPK-like phosphorylation cascades. Cr(VI)-induced and ROS generation-mediated stress has different effects on transcriptional regulation in terms of signaling transcriptional machinery, toxicity mechanism and vesicle trafficking in rice roots. Vesicle trafficking is required for mediation of Cr(VI)-induced ROS generation in rice roots.

Endogenous generation of ROS is responsible for the intracellular communication system that regulates the response to environmental stresses (Fedoroff 2006). When the plants are under salinity stress, the traffic destination of the ROS is regulated by vesicle trafficking complexes (Sanderfoot et al. 2000). Leshem et al. (2007) reported markedly decreased ROS accumulation in roots treated with BFA, a general vesicle trafficking inhibitor, during salt stress, so inhibition of membrane trafficking can inhibit ROS accumulation during salt stress. Moreover, the vesicle trafficking leads to the intracellular activation of NADPH oxidase to generate ROS that acts in signaling of the salt tolerance responses (Leshem et al. 2006). In previous study, we found that vesicle trafficking is required for mediation of Cu-induced ROS generation in root tissues (Lin et al. 2013). It was found in this study that Cr(VI) treatment induced the expression of *Exo70*, which plays a central role in vesicle trafficking. We also showed that BFA strongly suppressed ROS production during Cr stress. Silencing of *NbExo70* decreased the Cr(VI)-induced ROS production and oxidative burst in the leaves and roots. In addition, the silencing of *NbExo70* exhibited an increased tolerance to Cr(VI) stress. Therefore, the Exo70 may play important roles in regulating ROS homeostasis, which may trigger downstream signal transduction in response to the heavy metal stress.

Comparison of our data with recent study (Dubey et al. 2010) showed little agreement. Only 57 (about 5%) of the 1138 upregulated genes (twofold change) on long-term 100 μ*M* Cr(VI) treatment were represented in our set of 1261 Cr(VI)-induced genes (Table S10). The previous study by Dubey et al. (2010) revealed upregulation of 36 TF genes belonging to *MYB* (30.5%), Zinc finger protein (36.1%) and *WRKY* (22.2%) families. However, our microarray data identified 126 Cr-regulated TF genes (*WRKY*, 17.5%; *AP2/ERF*, 16.7%; *NAC*, 11.9%) (Table S11). The reason for the differences could be that Dubey et al. (2010) treated rice roots with Cr(VI) for a longer period of time than we used (24 h vs 1–3 h).

In conclusion, we demonstrated that CDPK and NADPH oxidase are activated in response to Cr(VI) via the accumulation of calcium and generation of ROS, respectively. Our microarray analysis suggests that Cr may have a significant effect on inhibiting root growth in rice through ethylene, ABA, JA and GA. In addition, proteins involved in signal transduction, including protein kinases and vesicle trafficking, have important roles in response to the Cr(VI) stress in rice (Fig. 7).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The forward and reverse primer sequences used in semi-quantitative RT-PCR for detecting gene expression.

Table S2. The forward and reverse primer sequences used in qRT-PCR for detecting gene expression.

Table S3. List of regulated genes with expression responding to 200 μ*M* Cr(VI).

Table S4. GO analysis (AgriGO) of 1261 genes upregulated by Cr(VI) stress.

Table S5. Expression profiles of protein kinase and protein phosphatase genes induced by Cr(VI) stress.

Table S6. Expression profiles of genes associated with signal transduction induced by Cr(VI) stress.

Table S7. Expression profiles of TF genes induced by Cr(VI) stress.

Table S8. Expression profiles of phytohormone-related genes induced by Cr(VI) stress.

Table S9. GO terms (AgriGO) of cellular localization in Cr(VI) stress.

Table S10. Comparison of Trinh et al. microarray data to Dubey et al. (2010) microarray data.

Table S11. Comparison of Trinh et al. and Dubey et al. (2010) for TF families significantly upregulated from Cr(VI)-treated rice roots.

Fig. S1. Lipid peroxidation detected in rice roots during Cr(VI) treatment. Histochemical detection of lipid peroxidation caused by Cr(VI) in rice roots. Rice roots were treated with 200 μ*M* Cr(VI) for 3h. The roots were stained with Schiff's reagent.

Fig. S2. Phytohormone genes up- or down-regulated with Cr(VI) treatment in rice roots. MapMan was used to visualize the phytohormones. Each BIN is represented as a block, with each transcript displayed as a square, in red for upregulation and blue for downregulation.

Fig. S3. Alignment of the amino acid sequences of NbExo70 with representative Exo70 members from other plant species. The protein sequences are deposited in GenBank under the following accession numbers: *Solanum lycopersicum* SlExo70 (XP_004241875), *Vitis vinifera* VvExo70 (XP_002272396), *Arabidopsis thaliana* AtExo70 (NP_177391) and *Oryza sativa* OsExo70 (NP_001063276). The black boxes indicate identical residues and gray boxes indicate conservative substitutions. Hyphens indicate gaps introduced to optimize alignments. The numbers on the left indicate the amino acid residues in NbExo70 protein sequence. The alignments were generated at the BOXSHADE web site (http://www.ch.embnet.org/software/BOX_form.html).

Fig. S4. Time course of Cr(VI) effect on expression of protein kinase-, protein phosphatase-, phytohormone- and vesicle trafficking-related genes. Relative mRNA expression was calculated via the Livak method ($2^{-\Delta\Delta Ct}$). Data are presented as mean relative expression ± SD for three replicate real-time reactions from three independent samples. Means with different letters are significantly different at $P < 0.05$ level.

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