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Proteomics to identify pathogenesis-related proteins in rice roots under water deficit

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Abstract: Upland and lowland rice (*Oryza sativa* L.) showed different mechanisms of water stress resistance. Hydroponically grown 3-week-old seedlings of a lowland variety IR64 and an upland variety were exposed to 15% polyethylene glycol (PEG-6000). After 7 d of treatment, IR64 maintained high relative water content and developed a well-branched root. Therefore, IR64 had better water-deficit tolerance than Azucena under PEG treatment. To identify water-deficit-responsive proteins associated with the tolerance differences between two ecotypes, a comparative proteomic analysis of roots was conducted. Out of 700 proteins reproducibly detected on two-dimensional electrophoresis gels, 65 proteins exhibited significant changes in at least one ecotype at 48 h of water deficit. Only 15 proteins showed different responses to water deficit between the two ecotypes. Twelve proteins were identified by matrix-assisted laser desorption/ionization-time of flight/time of flight-mass spectrometry, which involved in energy and metabolism, protein processing and degradation, detoxification and pathogen-related (PR) proteins, i.e. PR-1a, RSOsPR10 and JIOsPR10. All three PR proteins were induced more strongly in IR64 than in Azucena by water deficit at both protein and mRNA level. The results suggested that PR-1a, RSOsPR10 and JIOsPR10 may play important roles in protecting root cells against water deficit in rice.

Key words: rice; water deficit; two-dimensional electrophoresis; pathogen-related protein.

Abbreviations: ABA, abscisic acid; CRT, C-repeat; 2–DE, two-dimensional electrophoresis; DRE, dehydration-responsive element; JA, jasmonic acid; MALDI TOF/TOF, matrix-assisted laser desorption/ionization-time of flight/time of flight; PEG, polyethylene glycol; PR, pathogen-related; RWC, relative water content; SA, salicylic acid; SAR, systemically acquired resistance.

Introduction

Rice (*Oryza sativa* L.) is used as a staple food by more than half of the world's population. Water deficit is the major abiotic stress that has adverse effects on growth and yield of rice. The increasing worldwide water shortage and uneven rainfall distribution limit rice production (Rabello et al. 2008). It is therefore necessary to develop rice varieties with increasing drought tolerance and high yield. But so far only limited progress has been made in the area (Price et al. 2002).

Rice is a highly diverse species. The cultivars suitable for each type of soil moisture regimes are developed by a long period of natural and human selection under different water conditions (Price et al. 2002; Wang et al. 2007). Upland rice is usually grown under rain-fed, naturally well-drained soils without surface water accumulation, while lowland rice is cultivated in paddy fields (Wang et al. 2007). These two ecotypes of cultivars differ not only somewhat in plant architecture, but also greatly in drought resistance (Rabello et al. 2008). The possession of a deep thick root system which allows access to water deep in the soil profile is considered crucially important in determining drought resistance in upland rice. This trait may be less important in rain-fed lowland rice, where hardpans may severely restrict root growth. Upland rice and lowland rice showed different mechanisms of drought resistance, and upland varieties generally had better drought tolerance (Price et al. 2002). The determination of the mechanisms directly involved in drought tolerance remains a challenging task since drought is a complex trait that involves several metabolic pathways (Rabello et al. 2008). The root is an important component in the sensing and signaling of environmental cues to the whole plant, but it is intrinsically difficult to study, particularly in the natural environment (Price et al. 2002). Achieving drought tolerance in rice root will require a deeper understanding of the possible physiological and molecular mechanisms available for water deficit tolerance.

Proteomics offers a powerful approach to discover the proteins and pathways that are crucial for stress responsiveness and tolerance (Yan et al. 2006; Yu et al. 2008). In recent years, several proteomic studies have been carried out on water stress on rice (Salekdeh et al. 2002; Rabello et al. 2008; Choudhary et al. 2009). However, there are still no reports on the effects of water deficit on contrasting upland and lowland rice ecotypes. In the present study, a comparative proteomic analysis of rice roots subjected to water deficit has been adopted

Table 1. PCR primers, annealing temperature, cycle numbers and PCR expected length of four genes and actin.

Genes	Forward primer 5'-3'	Reverse primer 5'-3'	Temperature	No. of cycles	Expected length
PR-1a	GGGTGTCGGAGAAGCAGTGGTA	GCGAGTAGTTGCAGGTGATGAAGA	55 ℃	30	171 bp
JIOsPR-10	CAGTTCAACTTCACCTCAGCCAT	GCAAAACCAACAGGTAGATGCT	55 ℃	30	535 bp
RSOsPR-10	GTATCTAGCTAAGCAGTGGT	GCAATACGGAGATGGATGTA	58 ℃	30	586 bp
SAMS	CTTAACCCATCTGGTCGCTTC	TGCCAGTGCCGTATGTGTC	55 ℃	25	286 bp
β-actin	GAACTGGTATGGTCAAGGCTG	ACACGGAGCTCGTTGTAGAAG	54 ℃	25	250 bp

to identify the important responsive or tolerant proteins.

Material and methods

Plant material

Experiments were performed using an upland tropical *japonica* rice (*Oryza sativa* L.) cultivar Azucena and a lowland *indica* rice cultivar IR64. Rice seeds were germinated in the dark at 37 ℃ for 48 h. Uniform seedlings were then transferred to a plastic tray with nutrient solution (Yoshida et al. 1976), covered by a PVC sheet with nylon screen attached holes. The seedlings were grown at 28/25 ℃ (day/night) with a 16 h photoperiod under an irradiance of 350–400 μmol m^{-2} s^{-1} and a relative humidity of 60–80% in a growth chamber. Three-week-old seedlings were treated with 15% polyethylene glycol (PEG)-6000 for 7 d.

Root relative water content (RWC)

At least five roots were taken from each control and treated group at 2 d and 7 d of treatment, respectively. RWC = (fresh weight – dry weight) / (turgid weight – dry weight) × 100%.

Growth measurement of root

At the 7 d of treatment, the seminal root length was measured with a ruler, and adventitious roots and lateral roots on the seminal root of more than 1 cm in length were counted. Then root and shoot were separated. Dry weight of root was determined after drying the samples at 80 ℃ for 72 h.

Protein extraction and electrophoresis

Roots were harvested at the 12 h, 24 h and 48 h time points, frozen in liquid nitrogen and kept at –70 ℃. The root proteins were extracted using trichloroacetic acid/acetone method (Yan et al. 2005). Two-dimensional electrophoresis (2–DE) was performed as described by Yu et al. (2008). At least triplicate gels were performed for each sample.

Image analysis

The silver-stained gels were scanned at a resolution of 300 dots per inch on a UMAX Power Look 2100XL scanner (Maxium Tech., Taipei, China), and were analyzed as previously described (Yu et al. 2008). Only spots with significant and reproducible changes were considered to be differentially expressed proteins.

Protein identification

The silver-stained protein spots were excised from the gels, and rinsed twice with ddH_2O, then destained in a 1:1 solution mix of 30 mM potassium ferricyanide and 100 mM ammonium bicarbonate (pH 8.0). After hydrating with acetonitrile and drying in a SpeedVac, gel samples were rehydrated in a minimal volume of sequencing-grade porcine trypsin (Promega, Madison, WI, USA) solution (20 μg/mL in 25 mM NH_4HCO_3) and incubated at 37 ℃ overnight. The supernatants were transferred into a 200 μL tube and the remainings was further extracted with 1% trifluoroacetic acid in 67% acetonitrile twice. All extracted fluid was pooled with the trypsin supernatant and completely dried in a SpeedVac centrifuge.

Protein digestion extracts were resuspended with 5 μL of 0.1% trifluoroacetic acid, and mixed in 1:1 ratio with an α-cyano-4-hydroxy-trans-cinnamic acid saturated solution in 50% acetonitrile and 1% trifluoroacetic acid. Aliquots of 0.8 μL were spotted onto stainless steel sample target plates. Peptide mass spectra were obtained by a 4800 matrix-assisted laser desorption /ionization-time of flight/time of flight (MALDI TOF/TOF) mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). Data were acquired in positive MS reflector using a CalMix5 standard to calibrate the instrument (ABI 4700 Calibration Mixture). Mass spectra were obtained from each sample spot by accumulation of 900 laser shots in an 800–3,500 mass range. For MS/MS spectra, the five most abundant precursor ions per sample were selected for subsequent fragmentation and 2,000 laser shots were accumulated per precursor ion. The criterion for precursor selection was a minimum S/N of 50. The interpretation of both the MS and MS/MS data were carried out by using the GPS Explorer software (V3.6, Applied Biosystems, Foster City, CA, USA), which acts as an interface between the Oracle database containing raw spectra and a local copy of the MASCOT search engine (V2.1, Matrix Science, London, UK). Peptide mass fingerprints obtained from MS analysis were used for protein identification in the NCBI non-redundant database. Total of 50,346 sequences in the database were actually searched. All peptides mass values were considered monoisotopic and mass tolerance was set at 60 p.p.m. One missed cleavage site was allowed, cysteines were considered carboamidomethylated, methionine was assumed to be oxidized. Only peptides with confidence interval value no less than 95% were considered as being identified.

RT-PCR analysis

Total RNA was isolated from the root, stem, and leaf of control and treated plants using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) at 12 h, 24 h and 48 h time points. Reverse transcription was performed using an oligo(dT)18 primer and Superscript II kit (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction. First-strand cDNA were used as templates for RT-PCR. Gene-specific primers and numbers of amplification cycles are listed in Table 1. The rice actin gene was amplified as a housekeeping gene (*OsActin*1: 5'-GAACTGGTATGGTCAAGGCTG-3'; *OsActin*2: 5'-ACACGGAGCTCGTTGTAGAAG-3').

Table 2. Effect of water deficit on the growth of rice roots.[a]

Varieties	Seminal root length (cm)	Lateral root number	Adventitious root number	Root dry weight (g)
IR64 control	16.07 ± 0.50	10.3 ± 0.6	32.7 ± 2.1	12.6 ± 0.9
Treated	16.53 ± 0.55	19.3 ± 0.6**	17.0 ± 1.0**	14.9 ± 1.3*
Azucena control	19.97 ± 0.45	4.3 ± 0.6	17.7 ± 0.6	13.6 ± 2.1
Treated	23.77 ± 0.29**	6.3 ± 0.6*	3.00 ± 1.7**	11.0 ± 0.68*

[a] * and ** show significant differences at $p < 0.05$ and $p < 0.01$ from control, respectively.

Table 3. Differentially expressed proteins identified by MALDI-TOF-TOF MS.

Spot No.	Fold change[a] IR64	Azucena	NCBI Acc. No.	Protein name	Mowse score	SC (%)[b]	Theoretical Mr	Theoretical pI
Pathogen-related protein								
1	↑ 5.00 ± 0.09	↓ 2.37 ± 0.15	gi|117655417	PR-1 type pathogenesis-related protein PR-1a	193	14.9	17,523	4.55
5	↑ 4.74 ± 0.04	↑ 2.38 ± 0.03	gi|18539471	JIOsPR10	122	13.8	17,163	5.85
9	On	↑ 8.37 ± 0.13	gi|38678114	Root-specific pathogenesis-related protein 10 (RSOsPR10)	190	19.4	16,890	4.88
Detoxification								
10	↑ 1.20 ± 0.02	↑ 3.25 ± 0.04	gi|34910930	Putative glyoxalase II	62	2.7	44,460	9.42
Protein processing and degradation								
2	↑ 3.30 ± 0.08	↓ 1.65 ± 0.07	gi|34907258	Putative nascent polypeptide associated complex α chain	135	6.4	22,079	4.39
8	↑ 2.16 ± 0.17	↑ 9.72 ± 0.12	gi|3138799	β-6 subunit of 20S proteasome	117	10.9	24,266	6.43
Energy and metabolism								
6	↑ 3.75 ± 0.10	↑ 1.27 ± 0.04	gi|450549	S-adenosyl methionine synthetase	224	8.6	43,193	5.74
4	↑ 1.2 ± 0.07	Off	gi|20358	Cytosolic glutamine syntethase	47	3.6	39,234	5.73
11	↓ 4.02 ± 0.06	↓ 6.04 ± 0.08	gi|34901780	Putative ATP synthase δ chain mitochondrial precursor	62	9.3	21,200	5.72
12	↓ 2.00 ± 0.07	↓ 3.00 ± 0.21	gi|553107	Triosephosphate isomerase	304	16.9	27,588	6.60
Unclassified								
3	↑ 3.04 ± 0.15	↓ 1.65 ± 0.04	gi|303835	21 kDa polypeptide	74	5.4	18,934	4.51
13	↓ 1.5 ± 0.18	↓ 2.28 ± 0.25	gi|50932159	Universal stress protein	66	7.8	17,992	5.22

[a] Relative fold change compared with the control (mean±SE). ↑, up-regulated; ↓, down-regulated; On, expressing only in the treated sample; Off, expressing only in the control sample.
[b] SC, amino acid sequence coverage.

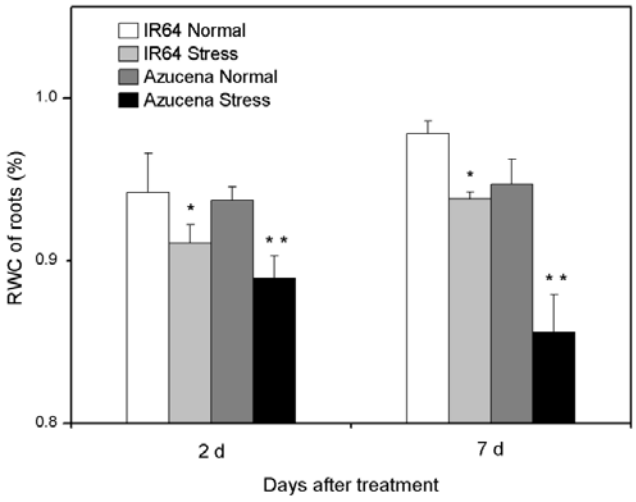

Fig. 1. Effects of water deficit on the relative water content of the root.

Results

Different response to water deficit in upland and lowland rice roots

In order to compare the tolerance between the two rice genotypes, three-week-old seedlings were subjected to water deficit for 7 d. RWC is considered to be the best integrated measure of plant water status (Choudhary et al. 2009). The lowland rice IR64 showed the smaller decline in RWC as compared to the upland rice Azucena at the 2 d and 7 d of treatment (Fig. 1). The maintenance of higher RWC in IR64 can be attributed to osmotic adjustment at lowered water potential, which might help in maintaining the metabolic activities and physiological processes (Choudhary et al. 2009). This could be substantiated by the observation of the growth of the seedling roots. After 7 d of treatment, there was a significant change in root architecture. The seminal root length and lateral root number on seminal root were increased, whereas adventitious root number was decreased significantly by water deficit in both genotypes (Table 2). Under water deficit, IR64 seedlings developed a greater number of lateral roots and adventitious roots, and thereby increasing significantly its root dry weight (Table 2). In contrast, the total root dry weight of Azucena plants, which although had longer seminal roots, was decreased significantly by water deficit (Table 2). These results indicated that IR64 maintained a higher root RWC and developed a well-branched root system with a larger surface area for water absorption. Therefore, lowland rice IR64 had better water-deficit tolerance than upland rice Azucena under PEG treatment. But the above results are not in agreement with those obtained from the pot experiments (Price et al.

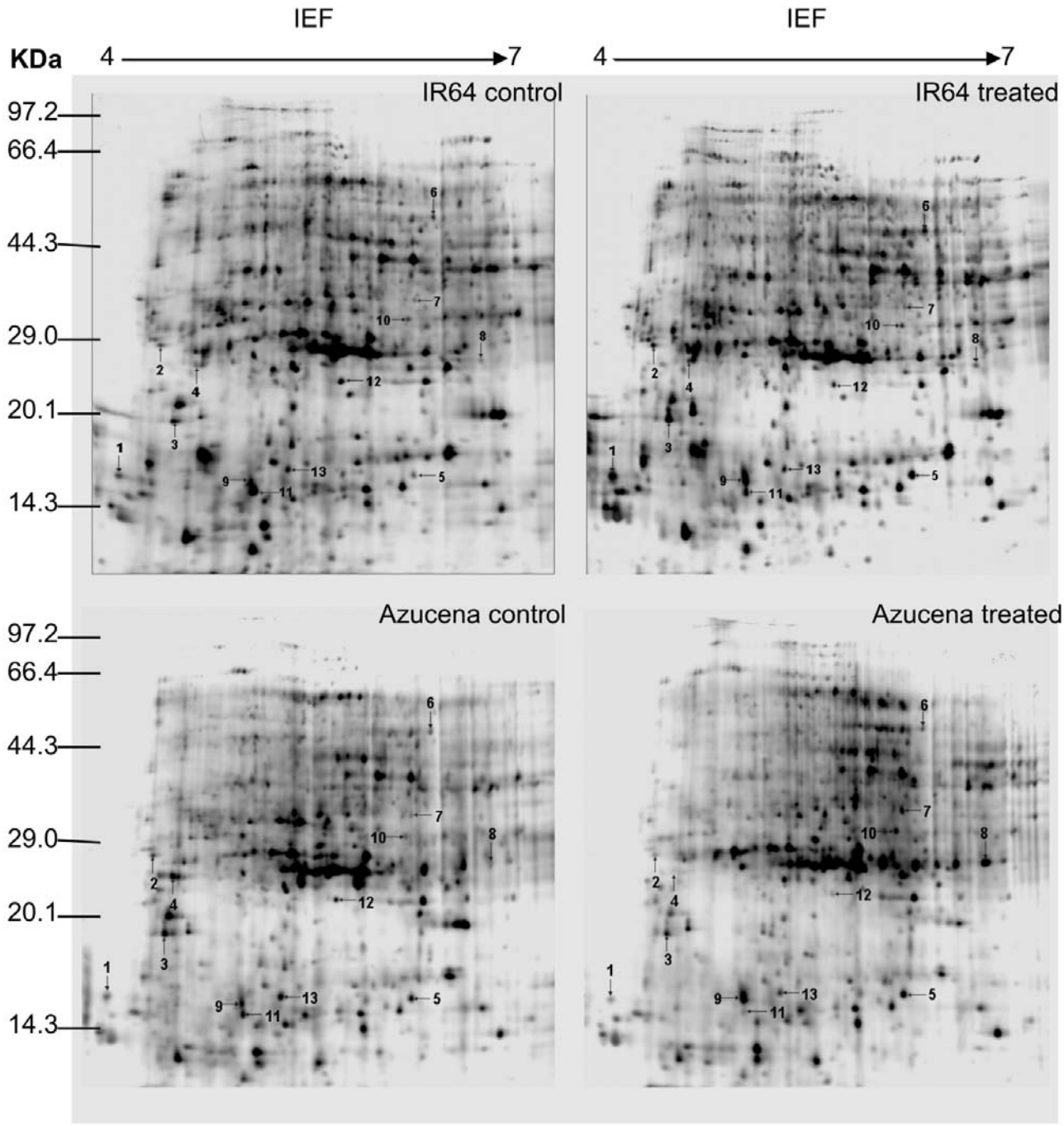

Fig. 2. Two-dimensional electrophoresis gels of rice root proteins at 48 h of water deficit treatment.

2002; Wang et al. 2007). The main reason may be that PEG solution treatment was used in this experiment.

Root proteomic responses to water deficit in upland rice and lowland rice

To compare early responses of two rice genotypes to water deficit at protein level, roots were harvested at 12, 24, and 48 h after treatment. In order to distinguish tolerant responses from developmental changes in protein expression, both control and treated roots were harvested at the same time. The overall protein spot number (about 700) reproducibly detected was similar among the samples of Azucena and IR64. There were more differentially expressed protein spots at 48 h than at other time point, suggesting that 48 h may be the pivotal time point for the rice root to response at protein level. Thus, 48 h time point was selected for analysis of expression of root proteins. The representative gel images stained by silver nitrate are presented in Figure 2. A total of 65 spots exhibited more than 1.5-fold differences in the intensity in response to water deficit in at least one genotype. Twenty-five protein spots were responsive to water deficit in IR64 only, while 11 in Azucena only. Twenty-nine spots were differentially expressed under water deficit in both genotypes. But only 15 proteins showed significantly different responses to

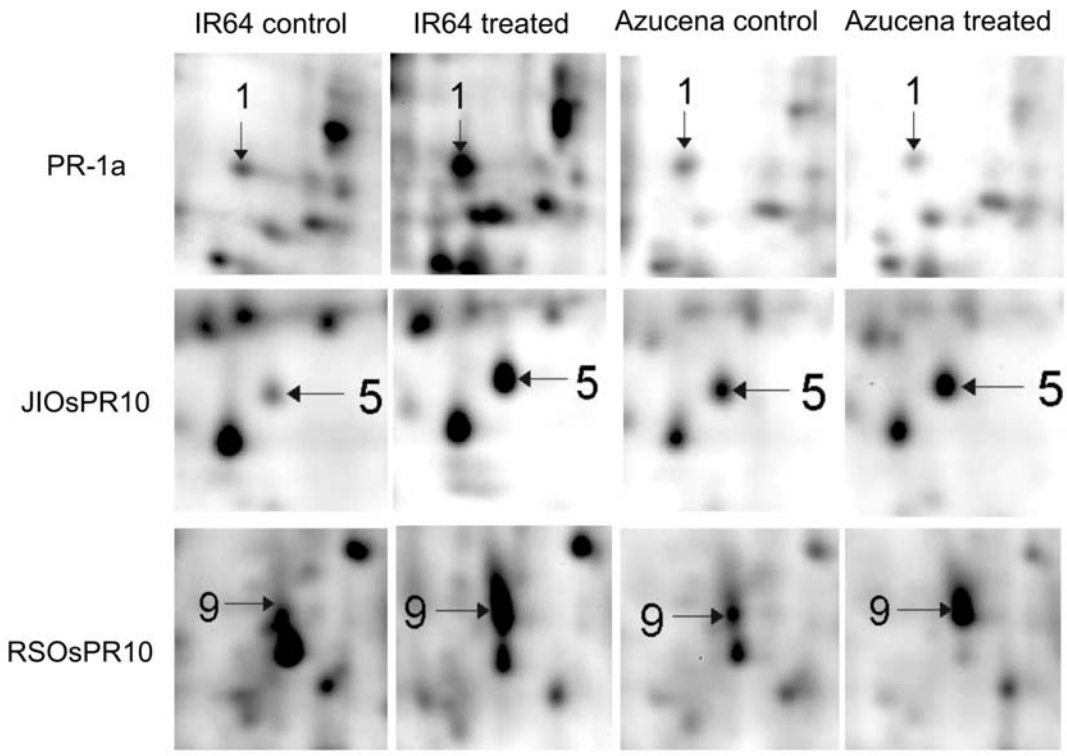

Fig. 3. Expression of three pathogen-related proteins in rice roots of the two ecotypes under 48 h of water deficit.

water deficit between the two genotypes. Among them, five (spots 1, 2, 3, 4 and 14) were up-regulated in IR64 and down-regulated in Azucena. Six protein spots (spots 5, 6, 7, 8, 9 and 10) were up-regulated and four (spots 11, 12, 13 and 15) were down-regulated in both two genotypes, but expression differences between the two genotypes were more than 1.5-fold (Table 3, Fig. 2). These 15 proteins were selected for identification.

Analysis of the differential responsive proteins in two genotypes

Among 15 proteins, 12 were identified (Table 3). Twelve identified proteins can be classified into five functional categories: energy and metabolism, protein processing and degradation, detoxification, pathogen defense, and proteins with unknown function (Table 3). Here, we focused on three pathogen-related (PR) proteins (i.e. PR-1a, JIOsPR10 and RSOsPR10) (Table 3). As shown in Figure 4, three PR genes were induced more strongly in IR64 than in Azucena by water deficit.

Discussion

An important self-defense mechanism of plants responding to invading pathogen is to produce or accumulate PR proteins. The major families of PR proteins have been grouped at least into 14 different classes, primarily on the basis of their amino acid sequence identity (Hashimoto et al. 2004). PR-1 was the first identified and the most dominant group among the PR proteins, and PR-1a was the acidic-type subclass of it (Kim et al. 2001). A salicylic acid (SA) dependent pathway was found to activate PR-1a, which could regulate the enzymatic activity of extracellular β-(1→3)-glucanases at the onset of tobacco defense reactions (Riviere et al. 2008). In addition to pathogen infection and chemical SAR (systemically acquired resistance) activators, H_2O_2 and $CuSO_4$ also induced the expression of *PR-1a* (Kim et al. 2001).

PR10 proteins are small, primarily acid intracellular proteins of about 16 kDa. The gene *RSOsPR10* was rapidly induced almost exclusively in roots (named *root specific rice PR10*) by salt, desiccation and jasmonic acid (JA) (Hashimoto et al. 2004). JIOsPR10 belongs to a different type of PR10 protein, which was also shown to be induced by JA and SA at the mRNA level (Jwa et al. 2001), and up-regulated by some abiotic stresses at protein level (Kim et al. 2008). Immunohistochemical techniques revealed that JIOsPR10 was localized to the palea of flower, in the exodermis and inner part of the endodermis of the root. JIOsPR10 was found to possess ribonuclease activity, but did not exhibit direct antifungal activity. The disulfide bonding between cysteine residues of the protein may play a role in constitutive self-defense mechanisms in plants against biotic and abiotic stresses (Kim et al. 2008).

The expression of PR-1 protein has been reported to be up-regulated by drought stress in tolerant upland rice roots and repressed in susceptible rice roots (Rabello et al. 2008). RSOsPR10 exhibited a significant increase in Nipponbare root after 15 h air-drying treatment, and its transcription was strongly up-regulated within 3 h (Hashimoto et al. 2004). JIOsPR10 was rapidly induced by air-dried treatment in the stem, but not in the leaves (Kim et al. 2008), while its expression change in rice root was not examined. In this case,

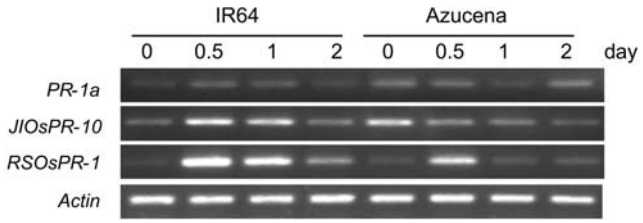

Fig. 4. RT-PCR analysis of three genes encoding pathogen-related proteins in the roots of IR64 and Azucena.

PR-1a (spot 1) was up-regulated in IR64 and down-regulated in Azucena, while both RSOsPR10 (spot 9) and JIOsPR10 (spot 5) proteins were induced more strongly in IR64 than in Azucena by water deficit at protein level (Table 3, Fig. 3).

At the mRNA level, the expression of all three PR genes was also up-regulated to a greater degree in the tolerant IR64 root than in the sensitive Azucena root (Fig. 4). Taken together, we strongly suggest that these three PR proteins may play roles in root development under water deficit other than disease resistance response (Kim et al. 2001, 2008; Hashimoto et al. 2004). Concerning the induction mechanisms of PR proteins expression, especially in relation to the cross-talk between various biotic and abiotic stresses, there were only a few reports (Kim et al. 2008). The complex regulatory and interaction network occurring between hormone-signaling pathways were considered to allow the plant to activate the responses to different types of stimuli (Si et al. 2009). Abscisic acid (ABA) regulates interacting signaling pathways involved in plant responses to several abiotic stresses as well as plant growth and development (Huang et al. 2008; Si et al. 2009). SA not only induces the production of PR proteins and activates local and SAR and thereby involved in plant defense responses (Hashimoto et al. 2004), but also modulates redox balance and protects rice plant from oxidative stress caused by biotic and abiotic stress (Huang et al. 2008; Si et al. 2009). JA and its various metabolites can alter gene expression positively or negatively in regulatory networks with synergistic and antagonistic effects in relation to SA and ABA (Huang et al. 2008). The 3 kb promoter regions of three genes encoding PR proteins were analyzed using the Motif Sampler algorithms, which can be accessed through the PlantCARE database website (Lescot et al. 2002). All three genes contained dehydration-responsive element (DRE), DRE/CRT (C-repeat) and MYBR (v-myb avian myeloblastosis viral oncogene homolog-target) motifs as well as ABA-responsive elements in their promoters. W-boxes for the SA induction were also identified (data not shown). The results suggested that expression of *PR-1a*, *RSOsPR10* and *JIOsPR10* under water-deficient stress may be regulated by ABA and SA signals. Detailed functional characterization of the three PR proteins in the tolerance to water deficit is required to be explained by further research. Transgenic overexpression and RNA interference of the three corresponding genes are being carried out.

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