KKU Res. J. 2013; 18(5): 749-758
http : //resjournal.kku.ac.th

Analysis of Gene Expression in Roots of Rice Seedlings: Differing Responses of Three Cultivars to Salt-Stress

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Abstract

A semi-quantitative RT-PCR technique was used to analyze the expression pattern of genes encoding 8 salt-responsive proteins in the roots of seedlings of three rice cultivars: Pokkali (salt-tolerant), KDML105 (salt-sensitive) and IR64 (moderately salt-sensitive). Rice seedlings were grown for 21 days in hydroponic solutions and then transferred to saline solutions for various times. The expression levels of genes that encode proteins with metabolic, redox reaction and housekeeping functions were analyzed in the roots of control and salt-treated plants of the three rice cultivars. It was found that in the roots of all three rice cultivars, salt stress produced only small changes in levels of transcripts of most of the genes studied, relative to controls; however transcripts of genes encoding fructose-bisphosphate aldolase, phosphoglycolate phosphatase, thioredoxin and cyclophilin-like protein, were higher in roots of both KDML105 and IR64 after salt stress.

Keywords: gene expression, salt-stress, salt responsive gene, rice seedling

1. Introduction

Rice yields around the world are negatively affected by salinisation resulting from over-irrigation. Many locally important rice cultivars with valuable flavour characteristics are particularly susceptible to salt-stress, which hinders their growth and development by inhibiting various metabolic activities, by reducing cell expansion, and in some cases by triggering programmed cell death (1). By reducing the water uptake of roots, salts cause ionic, osmotic and nutrient stresses. Cellular responses to these stresses, and to subsequent secondary stresses such as oxidative stress, add to the complexity of the salt tolerance trait. Molecular studies have revealed that salt tolerance is controlled by interactions between several independently regulated but temporally and spatially coordinated processes (2).

Transcriptomic approaches were used to investigate the response of rice (*Oryza sativa* L.) to salt-stress. Previously identified salt stress-responsive proteins include some involved in major metabolic processes such as photosynthetic carbon dioxide assimilation, photorespiration and photosynthetic oxygen evolution and also stress-responsive proteins such as heat shock proteins, ascorbate peroxidase, catalase and ferritin (3, 4). DNA microarrays have also been used to monitor changes in the steady-state abundance of salt-stress regulated transcripts (5, 6). These studies

have identified large numbers of differentially expressed genes.

In a previous report, proteomic analysis was employed to detect and identify proteins differentially expressed in the leaf lamina of the salt-sensitive rice cultivar KDML105 and the salt-tolerant cultivar Pokkali, grown in a hydroponic solution for 21 days and then exposed to high salinity (120 mM NaCl) for 7 days (7). In leaves of KDML105 two proteins, identified as the 23 kDa polypeptide of photosystem II (PSII23) and Rubisco activase (RCA), increased after exposure to salinity, while levels of the Rubisco large subunit (Rubisco) decreased. Six proteins that increased in Pokkali functioned in photosynthesis and photorespiration, i.e. PSII23, putative oxygen evolving complex protein (OEC18), fructose-bisphosphate aldolase chloroplast precursor (FBA), sedoheptulose-1,7-bisphosphatase precursor (SBP), Rubisco and phosphoglycolate phosphatase (PGP). Two further differentially expressed proteins were identified in Pokkali: 2-Cys peroxiredoxin chloroplast precursor protein (Prx) and thioredoxin type H (Trx), both of which are involved in protection of chloroplasts from oxidative stress damage. The cyclophilin-like protein (CyP), which has important functions in the repair of damaged chloroplast proteins, was also identified in Pokkali and reported as a salt-inducible protein in rice for the first time (8). The levels of mRNA transcripts for these proteins in leaves have also been investigated (9). In Pokkali, up-regulation of the identified salt-induced proteins was related to an increase in the abundance of the respective mRNA transcripts. In contrast, although mRNA transcripts encoding all identified proteins could be detected in KDML105, only three differential proteins spots were detected by proteomic analysis. This suggests that, although KDML105 contains elevated levels of gene transcripts needed for salt tolerance, the posttranscriptional mechanisms controlling protein expression levels were not as efficient as in Pokkali, indicating targets for future genetic improvement (9).

The next objective was to extend this work to the transcription profiling of genes encoding salt-responsive proteins in the roots of rice seedlings exposed to high salinity, compared with unstressed seedlings, using semi-quantitative RT-PCR analysis and to identify genes that are co-regulated during the acute phases and in the later phases of the salt-stress response.

2. Materials and Methods

Plant materials

Seeds of rice (*O. sativa* L.) cvs. Pokkali (salt-tolerant), KDML 105 (salt-sensitive) and IR64 (moderately salt-sensitive) used in this study were kindly provided by Pathumthani Rice Research Institute, Thailand. The methods of growing plants and salinization of the nutrient solution were modified from Gregorio et al (10). Rice seeds were surface-sterilized by treatment with 1.5% (w/v) calcium hypochlorite for 30 min, thoroughly washed and germinated in distilled water. The uniformly germinated seeds were transferred to a plastic grid placed over a 6-liter container filled with distilled water. After 5 days, when seedlings were well established, distilled water was replaced by nutrient solution (11). The pH was adjusted to 5.0 with 1 N KOH or 1 N HCl and renewed every week. When seedlings were 21 days old, the culture solution was replaced by solution containing 120 mM NaCl. After treatment of the seedlings with NaCl for 2h, 5h, 24h (acute phase) and 3 days, 7 days (later phase), the roots were harvested, frozen in liquid nitrogen and stored at -80 °C until further use. Experiments were performed in triplicate to obtain three independent samples for RNA analysis.

RNA extraction and reverse transcriptase PCR (RT-PCR) assay of gene transcripts

RNA was isolated from roots of 28-day-old seedlings of rice cvs. Pokkali and KDML 105 which had been subjected to salt stress for 7 days, using the RNeasy plant mini kit (Qiagen, USA). Extracted RNA was measured and used for cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen, USA) according to the manufacturer's protocol. RNA purity was determined by 1.2% formaldehyde agarose gel electrophoresis. The cDNA was synthesized using oligo-(dT) as a primer and the reverse transcription reaction mixture was incubated at 42°C for 1.30 hrs in a total volume of 20 µl. The cDNA products were used as templates for PCR amplification of gene transcripts. The following conditions were used for the PCR reactions: pre-denaturation at 94°C (1 min) followed by 20-35 cycles consisting of 30 s at 95°C (denaturing), 30 s at 55°C (annealing), 1 min at 72°C (extension) and a final extension for 7 min at 72°C. Nine pairs of forward and reverse primers were used (Table1). The primers were designed from the nucleotide sequences of genes encoding ten proteins identified in this study, one house-keeping gene, ubiquitin5 (*UBQ5*) (12) and a previously reported salt-regulated gene (*SalT*) (13) and chloroplast ATP synthase β-subunit (*CF1*β), using the primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cg). The products of RT-PCR amplifications were separated on 3% (w/v) agarose gels and stained with ethidium bromide. Photographic documentation was performed using a gel documentation system (GENEFLASH, Syngene Bio-imaging). For quantitation of relative band intensities, the pixel intensities of the RT-PCR products were analyzed using Metamorph software (Molecular Devices, USA) and normalized relative to the abundance of the *UBQ5* loading control.

Table 1. Primers sequences for RT-PCR analysis and product size

Primer name	Forward primer	Reverse primer	Product size (bp)
Rubisco activase (*RCA*)	TGTGGAGAACATTGGCAAGA	CGCAGAACCGTAGAAGGAAC	223
Fructose-bisphosphate aldolase (*FBA*)	GGGCGGAGACCTTCTTCTAC	GTTCATCGCGTTCAGGTTCT	236
Phosphoglycolate phosphatase (*PGP*)	CGATTTCCCCAAAGACAAGA	ACCCTGGATTCTCACGAATG	242
Thioredoxin Type H (*Trx*)	TGCCGACCTTCCTATTCATC	TCGCATGATATTCGAGGACA	240
2-Cys peroxiredoxin BAS1 (*Prx*)	TGAGACCATGAGGACCCTTC	GATCAGACGAGCACACGGTA	244
cyclophilin-like protein (*CyP*)	GGCACTGAGTAATGGGAGGA	GCTTTGGAGCTACTGCATCC	152
chloroplast ATP synthase β-subunit (*CF1*β)	CGAGACACTGACGGTAAGCA	CTCCCCAAGAACGTTGAAAA	195
salt-regulated genes (*SalT*)	GGAATATGCCATTGGTCCAT	GTCTTGCAGTGGAATGCTGA	214
Ubiquitin 5 (*UBQ5*)	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT	69

3. Results and Discussion

The expression levels of eight genes, *RCA*, *FBA*, *PGP*, *Trx*, *Prx*, *CyP*, *CF1*β and *SalT*, were analyzed in the roots of control and salt-treated plants of the three rice cultivars (Figs. 1-7). The results showed that gene expression levels were similar in all three rice cultivars, the transcript abundance of the genes of interest either remained the same at most time points as in the respective controls or changed only slightly. With the exception of the genes encoding fructose-bisphosphate aldolase, phosphoglycolate phosphatase, thioredoxin and cyclophilin-like protein, for which the transcript abundance was up-regulated in KDML105 and IR64, and *Prx*, *CF1*β, which was down-regulated in IR64. It was previously reported that the abundance of most gene transcripts was high expressed in leaves of rice cultivars (7). The elevated transcript abundance of important photosynthetic and protective genes in even unstressed Pokkali leaves may provide this salt-tolerant rice variety with better innate adaptation to salt stress compared to salt-sensitive KDML 105. A similar observation was made in recent comparative transcriptome mapping of salinity stress response between Pokkali and IR64, a salt-sensitive cultivar (14). This study identified several genes that showed higher transcript abundance even in the absence of stress, and maintained a high level of expression until a late phase of stress. In a similar study of *Thellungiella halophila*, a relative of *Arabidopsis thaliana* that is a true halophyte, it was also found that many genes in similar functional categories showed elevated transcript abundance in the unstressed state (15).

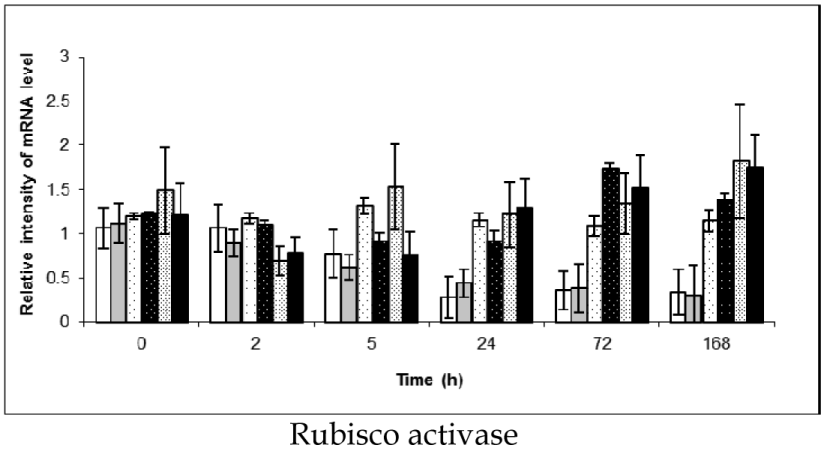
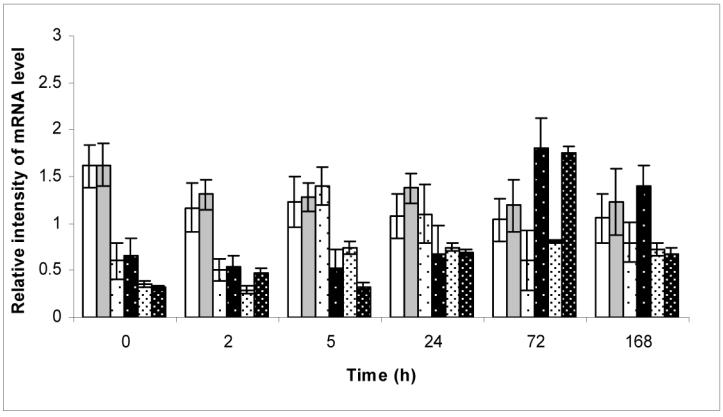


Figure 1. Relative intensities of mRNA levels of *RCA* genes involved in CO_2 assimilation.

control, Pokkali control, KDML105 control, IR64

stress, Pokkali stress, KDML105 stress, IR64

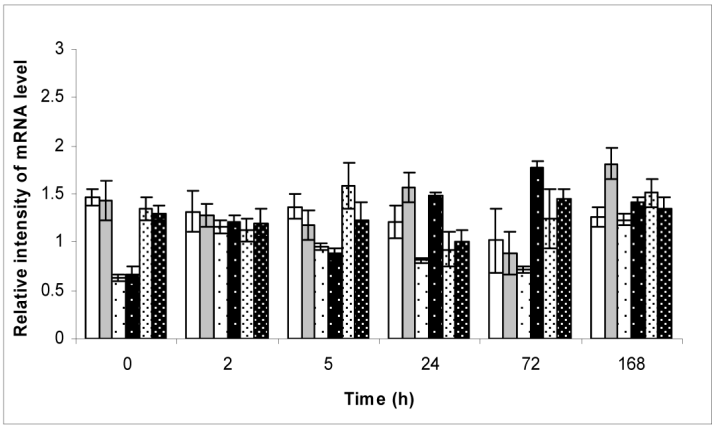


fructose-bisphosphate aldolase

Figure 2. Relative intensities of mRNA levels of *FBA* genes that encoded enzyme in Calvin cycle

control, Pokkali control, KDML105 control, IR64

stress, Pokkali stress, KDML105 stress, IR64

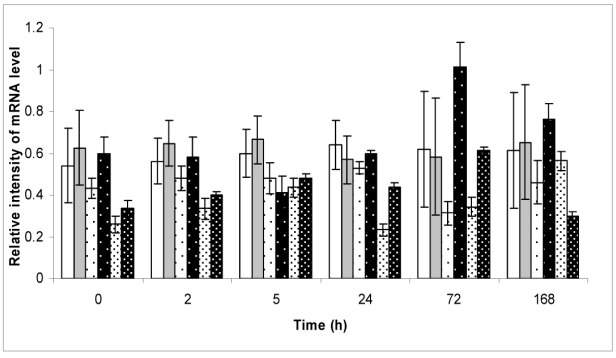


phosphoglycolate phosphatase

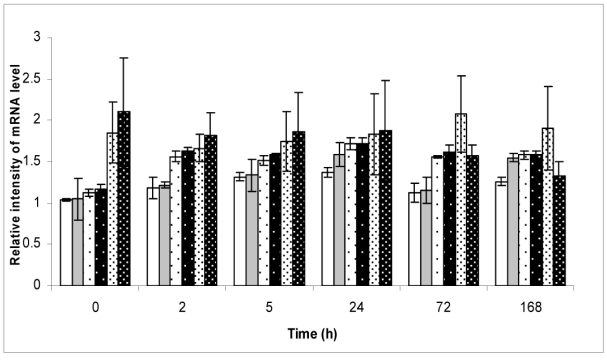
Figure 3. Relative intensities of mRNA levels of *PGP* gene involved in photorespiration.

control, Pokkali control, KDML105 control, IR64

stress, Pokkali stress, KDML105 stress, IR64



thioredoxin type H

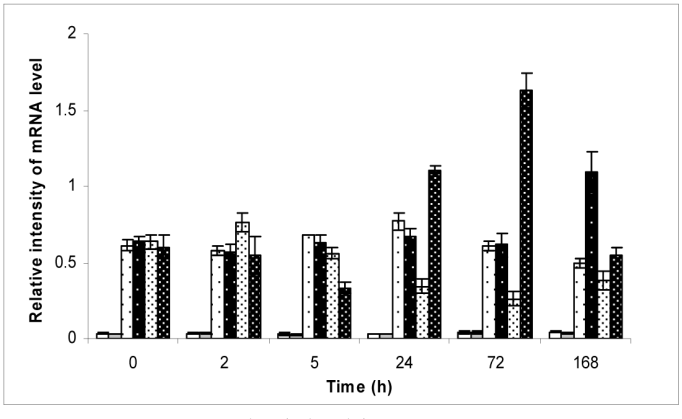


2-Cys peroxiredoxin

Figure 4. Relative intensities of mRNA levels of *Trx*, *Prx* genes encoded redox proteins involved in anti-oxidant defense.

control, Pokkali control, KDML105 control, IR64

stress, Pokkali stress, KDML105 stress, IR64

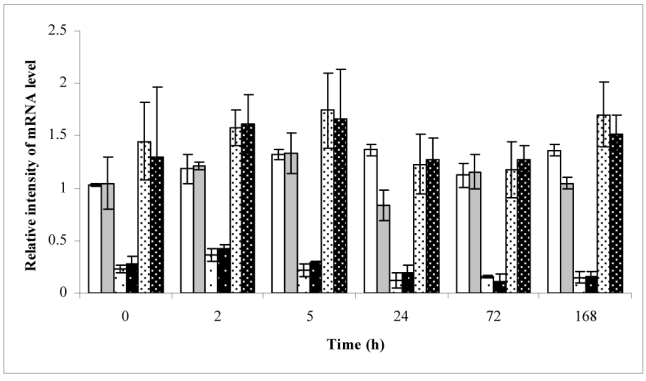


cyclophilin-like protein

Figure 5. Relative intensities of mRNA levels of *CyP* gene involved in protein folding.

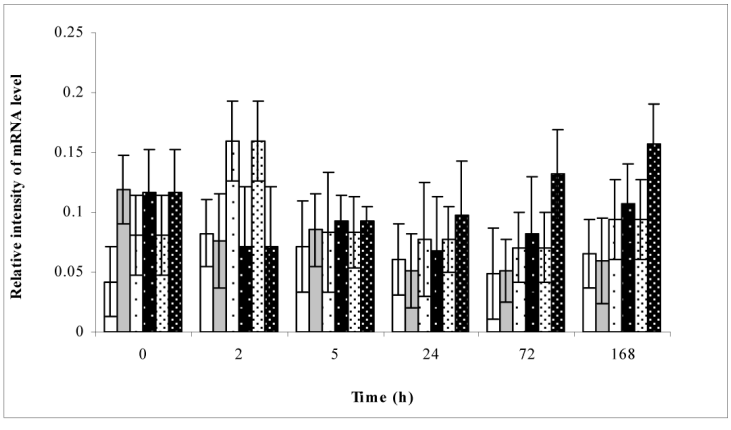
control, Pokkali control, KDML105 control, IR64

stress, Pokkali stress, KDML105 stress, IR64



chloroplast ATP synthase β-subunit

Figure 6. Relative intensities of mRNA levels of *CF1*β genes involved in light reactions of photosynthesis.
control, Pokkali control, KDML105 control, IR64
stress, Pokkali stress, KDML105 stress, IR64



salt-regulated

Figure 7. Relative intensities of mRNA levels of *salT* genes involed in salt-stress regulation.
control, Pokkali control, KDML105 control, IR64
stress, Pokkali stress, KDML105 stress, IR64

The protein product of the *CF1*β gene is a major subunit of chloroplast ATP synthase, with vital functions in energy production. It was observed that transcripts of this gene in all cultivars were changed only slightly or small decreased in root of IR64. Chloroplast ATP synthase *b*-subunit was found to be increased in rice leaves by salinity, drought and heat (16, 17). However roots and leaves respond to stress differently: roots can continue elongation while at the same time shoot elongation is completely inhibited. The consequence of this differential response is that roots can reach water in deeper soil while inhibition of shoot elongation to conserve water consumption. This rationalizes the differential gene expression between tissues in response to stress (18). The decrease in *CF1 b* expression could be associated with transiently increased photosynthetic

rates and up-regulation of the Calvin cycle enzymes.

Compared with other genes that have been investigated, transcripts of the *Trx* and *Prx* genes were relatively more abundant than others even in the non-stressed roots. This indicates that root tissues are normally under oxidative stress and need to synthesize large amounts of thioredoxin and peroxiredoxin. The transcription of Trx gene tended to be induced during the later phase of salt stress, in case of KDML105 and IR64 roots. But *Prx* was up-regulated during the later phase of salt stress. Root tissues synthesize other antioxidative proteins not included in this study, such as superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, glutathione reductase and monodehydroascorbate reductase, the genes for which are expressed in salt-stressed roots (19, 20, 21). The transcripts observed in roots may result from transcription of another member of the *Trx* h gene family (22). Trx h proteins are reduced by NADP-thioredoxin reductase, and have been found to regulate a range of biochemical processes in plants. Thioredoxin plays a central role in the coordination of multiple regulatory steps during the germination of cereals, reducing intramolecular disulfide bonds in enzyme inhibitors, seed storage proteins and enzymes (14). Prx, which has broad substrate specificity and detoxifies various peroxide substrates, may function as a more general antioxidant (23).

In KDML105 and IR64, the level of expression of the *CyP* gene in roots was highly increased during the later phase of stress. The late response of this gene may reflect the constitutive level of CyP proteins being high enough for its function in repairing damaged proteins for several days during salt-stress. Expression of the CyP gene in IR64 was very high during the first and third day of salt treatment, in stressed root tissues. And slightly increased at day 7 of stress. Although mRNA transcripts of the CyP gene in the stressed tissues of both Pokkali and KDML105 were several times higher than those in the control, only in Pokkali was CyP protein detected as being salt-inducible. In case of the expression of the *SalT* gene remained unchanged as found in Pokkali and KDML105 or down-regulated in IR64. Its level of expression remained steady in the roots throughout the whole period of investigation.

4. Conclusion

Analysis of expression of *RCA*, *FBA*, *PGP*, *Trx*, *Prx*, *CyP*, *CF1b* and *SalT* genes under salt stress revealed that the abundance of most gene transcripts was changed only slightly in roots of the three rice cultivars, with the exception of the *FB,A PGP, Trx* and *CyP* genes expressed in KDML105 and IR64. Most research on the effects of salinity on plants has concentrated on the salt-responsiveness of leaf proteins. The behaviour of roots is different, because they are in direct contact with the soil or saline solution. Although there are opportunities to control salt entering leaves at various points along the transpiration stream, the root must perform a crucial function in the management of input and throughput.

5. Acknowledgement

We are grateful to Dr. Meriel G. Jones and Dr. James Hartwell, School of Biological Sciences, University of Liverpool, UK for their guidance, support and intensive training on molecular analysis. We also thank Assoc Prof. Dr. Piyada Theerakulpisut for advice, encouragement, help and correction of this work. This work was supported by funds from the Thailand Commission on Higher Education and the Science and Social Sciences, Burapha University, Sakaeo Campus.

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