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THE POSSIBLE INVOLVEMENT OF MYB, WAK AND RIM2 PROTEINS IN SALT TOLERANCE IN RICE

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SUMMARY

Salinity in plants has two major components: osmotic effects (phase 1) and ionic effects (phase 2). In this research the effects of phase 1 on gene expression in two rice varieties differing in salt tolerance (IR4630 and IR15324) was studied using a modified cDNA-amplified fragment length polymorphism (AFLP) technique. To separate osmotic from ionic effects of salt, mannitol was applied as a non-ionic osmoticum at the equivalent osmotic potential to 50 mM NaCl. mRNA was sampled at 0.5, 6, 24, 48 and 192 hours after salinisation. Several products (AFLP-bands) were detected, which were upregulated in the response to ionic effects of salt in the tolerant line (IR4630) and not expressed in the sensitive line (IR15324) at both phase 1 and 2 of salinisation. Bioinformatic analysis indicated these AFLP-bands to be: 1) a gene (*OsMYB)* encoding a Myb-related protein (for an isolated AFLP-band belonged to salt-stressed shoots of IR4630 at 0.5 h after salinisation); 2) a gene (*OsWAK)* encoding a putative wall-associated protein kinase (for an isolated AFLP-band belonged to salt-stressed shoots of IR4630 at 24 h after salinisation) and 3) a gene (*OsRim2)* encoding a Rim 2 protein (for an isolated AFLP-band belonged to salt-stressed shoots of IR4630 at 192 h after salinisation). The possible roles of the products of candidate genes are discussed.

***Key words*: cDNA-AFLP, gene expression, *Oryza sativa*, salinity.**

INTRODUCTION

The injurious effects of salt (NaCl) on plants can be divided into osmotic and ionic effects (Munns 2002). Munns has hypothesised that plant growth is initially inhibited (phase 1) by cellular responses to the osmotic effects of external salt, i.e. by responses to the decreased availability of soil water. In a later, second response (phase 2), growth is further inhibited by the toxic effects of excessive salt accumulation within the plant (ionic effects of salt). In attempts to separate these phases, the effects of iso-osmotic solutions, such as polyethylene glycol (PEG), mannitol or various concentrations of a mixture of salts, have been compared with the effects of NaCl alone (Yeo *et al*. 1991, Munns 2002, Umezawa *et al.* 2002). It is of critical importance that the early effects of salinity on plants are hypothesised to be a consequence of changed water activity and not due to changes in the activity of the ions bringing about the salinity. For rice, the first phase is rather short-lived in comparison to other cereals (Yeo *et al.* 1991) because of the rapid built-up of Na^+ in the shoots to toxic levels (Yeo 1992).

Earlier studies have shown that gene expression is altered by salinity in rice and other plants. However, in these studies a high concentration of NaCl was used which may cause a salt shock and result in changes in transcription of genes responsive to the osmotic effect of NaCl and not its ionic component. In addition, in some

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molecular investigations the physiological conditions of experiments are not properly considered, which affect the applicability of the results. Moreover, in some published work the main aim has been to identify changes of gene expression in a single species, without considering genes differentially expressed in closely related lines, but differ in sensitivity to salt. The latter would be helpful in plant engineering experiments to transfer genes responsible for salt tolerance between genotypes of a given crop and reduce possible problems due to transformation of 'foreign' genes.

In the present study an attempt was made to identify, using the cDNA-AFLP technique, those genes of IR4630 (the tolerant line) that were induced by the ionic effect of salt, and not expressed in IR15324 (the sensitive line); that is those genes that make IR4630 more tolerant to salinity than IR15324. Several products (AFLP-bands) were detected in this profile, which were upregulated in the response to ionic effects of salt in the tolerant line (IR4630) and not expressed in the sensitive line (IR15324) at both phase 1 and 2 of salinisation. These AFLP-bands were purified from the cDNA-AFLP gel. Subsequent cloning, sub-cloning, sequencing and bioinformatic analysis indicated them to be: 1) a gene (*OsMYB)* encoding a Myb-related protein (for an isolated AFLP-band belonged to salt-stressed shoots of IR4630 at 0.5 h after salinisation); 2) a gene (*OsWAK)* encoding a putative wall-associated protein kinase (for an isolated AFLP-band belonged to salt-stressed shoots of IR4630 at 24 h after salinisation) and 3) a gene (*OsRim2)* encoding a Rim 2 protein (for an isolated AFLP-band belonged to salt-stressed shoots of IR4630 at 192 h after salinisation). An attempt has been made to elucidate the possible role of the products of candidate genes in salt tolerance in rice.

MATERIALS AND METHODS

Caryopses of rice (*Oryza sativa* L.) were obtained from the International Rice Research Institute, Manila, Philippines. These consisted of parents (IR 4630-22-2-5-1-3 and IR 15324-117-3-2-2) of a cross (IR55178-3B-9-3) that has been evaluated for quantitative trait loci associated with salt tolerance (Koyama *et al.* 2001). The two parental lines were used to evaluate effects of salinity on gene expression. Caryopses were soaked for 24 hours in distilled water and transferred to germinate on the surface of nylon mesh over the nutrient solution of Yoshida *et al*. (1976), but modified by reducing the phosphate concentration by 50%. Seedlings were transplanted after seven days into black-painted plastic containers, filled with the same culture solution, each with 20 plants. The conditions in the growth chamber were as follows: day/night air temperature 30/26 ºC, irradiance at plant level 400-500 µmol m^{-2} s^{-1} PAR (photosynthetic active radiation), photoperiod 12 h, relative humidity 50%. Two weeks after germination, when the third leaf was fully expanded, plants were subjected to stress treatment. The seedlings were exposed either to 50 mM NaCl, or to 85 mM mannitol, in order to separate osmotic effects from those due to salinity *per se*. As a control, other plants were grown in normal culture solution, without imposing any stress.

RNA extraction and ds-cDNA synthesis: Fourteen-day-old rice plants (where the third leaf was fully expanded) were harvested 0.5, 6, 24, 48 and 192 hours after the initiation of NaCl (50 mM) and mannitol (85 mM) stress and separated into shoots and roots, control plants were harvested at the same times. Total RNA was isolated from about 150 mg of frozen (liquid nitrogen) shoots or roots using RNeasy plant mini kit (QIAGEN). For isolation of $polyA^+$ mRNA from around 50 µl of total RNA solution, the Oligotex mRNA spin-column protocol (QIAGEN) was used. Double-stranded cDNA was synthesised from mRNA (190 ng) using a cDNA synthesis kit (cDNA synthesis system, Roche).

cDNA-AFLP procedures: For performance of cDNA-AFLP, 160 ng of ds-cDNA was subjected to a standard AFLP template production (Vos *et al*. 1995) applying 2.4 u of EcoRI and 2.5 u of MseI, and then 2.5 pmol of EcoRI and MseI adapters were ligated onto digested cDNA ends as described by Vos *et al*. (1995). The sequence of the adaptors are as follow: EcoRI adapter top strand, 5'-CTCGTAGACTGCGTACC-3'; EcoRI adapter bottom strand, 3'- CATCTGACGCA TGGTTAA-5'; MseI adapter top strand, 5'-GACGATGAGTCCTGAG-3'; MseI adapter bottom strand, 3'- TACTCAGGACTCAT-5'. The reaction for a single sample was: 2.5 pmol of EcoRI adapter, 2.5 pmol of MseI adapter, 1 mM of rATP, 0.6 u of T4 ligase and 10X RL buffer in a final volume of 5 µl. This was added

to each reaction containing the restricted ds-cDNAs. These samples were incubated in the PTC-200 thermocycler at 37°C for 3 hours (or preferably overnight). Then, they were diluted 1:10 in 1X TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to use for the next step (pre-amplification step). For pre-amplification, 13µl of 1:10 dilution from the restriction-ligation product was used as a template for carrying out a reaction with a total volume of 50 µl per sample. For a single reaction the reagents were: 75 ng of each E00 and M00 primers, 0.2 mM of dNTPs, 1 u of *Taq* DNA polymerase. The core sequences of primers for pre-amplification and selective amplification were as: E00 primer, 5'-GACTGCGTACCAATTC –3'; M00 primer, 5'-GATGAG TCCTGA G TAA -3'; (all primers were obtained from Sigma-genosys). The cycling regime was: 30 cycles of 94°C-30 s (denaturation); 56°C-60 s (annealing); 72°C-60 s (polymerization), extra extension step for 5 minutes at 72°C. Then, PCR-products were diluted 1:10 in 1X TE in a flat-bottomed microtitre plate as a template for the next step (selective amplification step). For the selective amplification step, one of the two primers (EcoRI or MseI) had to be radioactively labelled: the EcoRI-primer was chosen (Vos *et al*. 1995). For the selective amplification process, four different primers were considered to find the best banding pattern. They were E00 (applied in the pre-amplification without any selective nucleotides), E (+1) with only one deoxyadenosine (A) as the selective nucleotide, E12 and E15 (primers with 2 selective nucleotides, in turn, AC and CA). To test these four primers, five samples (restricted ds-cDNAs) were used identically. E(AC) and E(CA) selective primers had been already recognised as the most suitable for rice-AFLP experiments by Koyama (Miki Koyama pers. comm.). As a result of the better resolution of AFLP-bands on the radiogram produced using E(AC)- E12- primer, this primer was used to produce cDNA-AFLP profile for all 56 samples. The selective primers were 5'-end-labelled using [γ-^{33}P] ATP and T4 polynucleotide kinase. One labelling reaction consisted of 0.12 u of T4 polynucleotide kinase, 5 ng of the selective primer, 1.2 µl [γ-^{33}P] ATP and 1,4 all buffer in a final volume of 3.7 µl. The incubation of tubes was accomplished at 37 °C in the hot-block for at least 2 hours. For a single sample of selective amplification step, the following ingredients were used: 30 ng of M00 primer, 0.2 mM of dNTPs, and 0.5 u of *Taq* DNA polymerase. 5.0 µl of 1:10-diluted pre amplification product was added separately to each well as a DNA template. The cycling regime was 13 cycles of 94 °C-30 s (denaturation), 65 °C-30 s (annealing), 72 °C-60 s (polymerization), then 23 cycles of 94 °C-30 s, 56 °C-30 s, 72 °C-60 s. The resulting PCR products were separated by polyacrylamide gel electrophoresis under denaturing conditions, and then gels were dried on Whatman 3MM paper using a gel dryer (BIO-RAD model 583) and exposed to Kodak film (type 35x43, X-ray film) for at least 48 hours. Fingerprint patterns were visualised using an automatic developer (Konica SRY-101A).

Preparing the AFLP fragments for cloning: For cloning, those AFLP fragments that appeared only for salt-treated samples (both root and shoot) of IR4630 were chosen. An accurate position of the bands to be excised was determined by superposing the marked film on the gel. The bands of interest were marked on the films and cut from the gel by a sharp blade. For extraction of DNA fragments from the denaturing gel, isolated bands were separately soaked in 1.5 µl Eppendorf tubes containing 50 µl of 1X TE buffer for 5-6 hours. Before cloning, AFLP-bands were recovered by PCR with those primers that were used for the pre-amplification (M00 and E00 primers). The purpose of this PCR was to increase the yield of product and to add a single deoxyadenosine (A) to the 3' ends of PCR products using DNA Taq polymerase (because this enzyme has a non-template dependent terminal transferase). This then produced PCR products suitable for ligation into the TOPO TA plasmid vector, which has a single over-hanging 3' deoxythymidine (T) residue. The total volume for this PCR reaction was 50 µl with following reagents: 0.2 mM dNTPs, 0.15 µg M00 primer, 0.15 µg E00 primer and 0.5 u Taq DNA polymerase. The cycling regime was 30 cycles of 94 °C-60 s (denaturing), 50 °C-60 s (annealing), and 72 °C-60 s (extension). A final step of 72 °C for 7 minutes was used to enable products to extend fully. To determine the purity and confirm the size of isolated cDNA-AFLP bands, 10 µl of the PCR products was run on the 0.8% agarose gel (w/v). After gel visualisation, those samples that had more than one band on the gel were omitted

from the cloning procedure and the products were re-extracted. The entire sequences of cloned DNA fragment were determined with a CEQ-2000 automated sequencer. Nucleotide sequences and translated sequences were analysed for homology to nucleotide or protein sequences in GenBank non-redundant databases using the BLAST program.

RESULTS AND DISCUSSION

Bands from the different genotypes and treatments vary in intensity, but there were only a few bands that were differentially expressed between IR4630 and IR15324. They appeared in the shoots of the tolerant line and not the sensitive line and in salt-treated plants but not in control plants or those treated with mannitol. Thus these were assumed as salt-specific genes expressed in a tolerant. After cloning, sub-cloning, PCR screening, DNA purification, and sequence determination, BLAST analysis through the database 'Genbank' was carried out for these AFLP bands, which appeared differentially in the profile of salt tolerant rice. Table 1 and figures 1-3 show the result of the best homologues in the database for cloned AFLP bands.

Two genotypes of rice (IR4630 and IR15324) with different sensitivity to salt were selected to identify genes that contribute to salt tolerance in the tolerant line. Genes responding specifically to NaCl were sought as an explanation of differences in tolerance. To achieve this aim, cDNA-AFLP was utilised to identify differences between the response of the two genotypes to salt and mannitol.

MYB-related Protein: A large number of genes are known to respond to salt stress at the transcriptional level. To regulate changes in gene expression in response to the environmental stress transcription factors are required. The Arabidopsis thaliana genome contains more than 1500 transcription factors, many of which are specific to plants (Riechmann *et al*. 2000). Several different classes of transcription factors are induced by water-deficit stress (osmotic stress), including bZIP (AREB1), homeodomain (ATHB-6, –7 and –12), AP2 domain (DREB2A), MYB (ATMYB2) and MYC-related factors. These are all likely to be involved in the up-regulation of genes, many of which are signalled through ABA (Bray 2002).

Atmyb2 is a MYB-related protein gene, which was found in *Arabidopsis* by Urao *et al.* (1993). Their RNA gel blot analysis showed that the *Atmyb2* mRNA was induced by dehydration and disappeared upon rehydration. The *Atmyb2* mRNA also accumulated upon salt stress (Quesada *et al*. 2000). The putative protein (ATMYB2) encoded by *Atmyb2* has 274 amino acids, a molecular mass of 32 kD, and a putative DNA binding domain that shows considerable homology to plant MYB-related proteins, such as maize- C1. Urao *et al.* (1996) suggested that the MYB-related transcription factor is involved in the regulation of genes that are responsive to water stress in *Arabidopsis*. In *Arabidopsis*, the induction of a dehydration-responsive gene, *rd22*, is mediated by abscisic acid (ABA) and requires protein biosynthesis for ABA-dependent gene expression (Urao *et al*. 1996). They have analysed the *rd22* promoter in transgenic tobacco plants and found ATMYB2 (MYB)

Table 1. The results of search blast for both AFLP bands.

Clone ID	Best homologue in the database (X BLAST analysis)	Score	E value	Sequence identity
The first AFLP-band (*afb1*) from Shoot, 30 min, salt-specific, E(AC) sample	Myb-related protein	243	6e-59	94%
The second AFLP-band (*afb2*) from Shoot 24h, salt-specific, E(AC) sample	Putative wall-associated protein kinase	82.4	4e-15	46%
The third AFLP-band (*afb3*) from Shoot 192hSalt-specific, E(AC) sample	Rim 2 protein	73.9	8e-13	91%

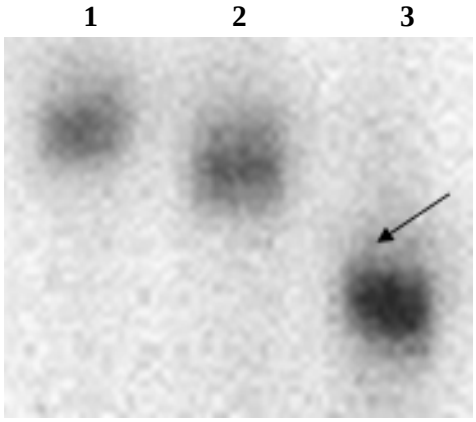

Fig. 1. Visualisation of *afb1*. Lanes 1and 2 derive, in turn, from the control and salinised stressed shoots of IR4630 at the first harvest (0.5 h after stress treatment). The arrow in lane 2 shows the band *afb1* from cDNA-AFLP profile (prepared with E(AC) as a selective primer), which is differentially expressed under salt treatment. Searching through the Genbank database showed 94% identity of this fragment to a gene encoding Myb-related protein.

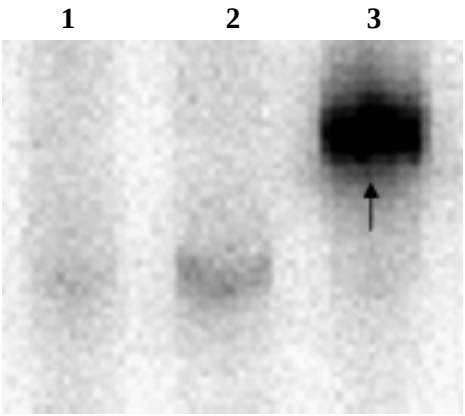

Fig. 2. Lanes 1, 2 and 3 derive, in turn, from the samples of control, mannitol and salinised shoots of IR4630 at 24 h after stress treatment. The arrow shows the position of *afb2*, the second isolated band from cDNA-AFLP profile (prepared with E(AC) as a selective primer), which is apparently expressed differentially in response to salinity. BLASTX results showed 46% identity of that to a putative wall-associated protein kinase.

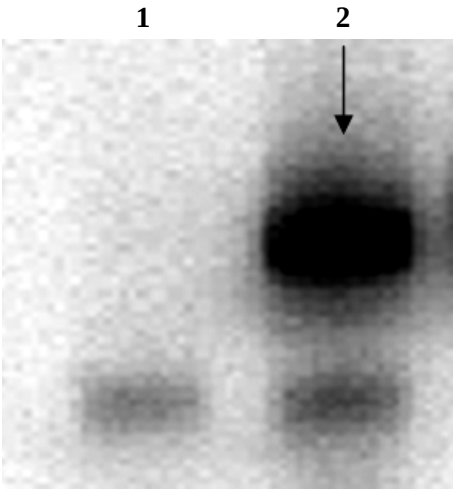

Fig. 3. Lanes 1and 2 are from the samples of control and salinised shoots of IR4630 at the final harvest (192 h after stress treatment). The arrow shows the position of *afb3*, the third isolated band from cDNA-AFLP profile (prepared with E(AC) as a selective primer), which is apparently expressed differentially in response to salinity. BLASTX results showed 91% identity of that to a Rim 2 protein.

proteins functioned as transcriptional activators in the dehydration- and ABA-inducible expression of the *rd22* gene. Moreover, Abe *et al.* (2003) declared that AtMYB2 proteins function as transcriptional activators in all ABA-inducible gene expression under drought stress in plants. In other words, AtMYB2 (MYB) functions as a transcriptional activator in abscisic acid signalling. The appearance of an AFLP-band just in response to ionic effect of salt which has 94% identity to a gene encoding MYB-related protein might exhibit the involvement of this gene in the cross-talk processes of osmotic and salt-specific stress.

Wall-associated protein Kinase: A family of genes with sequence similarity to the cell wall-associated kinase (WAK) genes in *Arabidopsis* has recently been identified (Verica and He 2002). The WAKs represent a unique class of receptor-like kinase genes whose products are suited for exchanging signals between the intracellular and extracellular compartments. Each gene encodes a transmembrane protein with a cytoplasmic serine/threonine kinase domain, which can interact with other components through protein phosphorylation/ dephosphorylation, and an extracellular region with similarity to vertebrate epidermal growth factor (EGF)-like domains. The cell wall is vital for plant development and interactions with the changing environments, yet little is known about the molecular mechanisms of how cell

wall interacts with and regulates intracellular compartments. The WAKs and the WAK-like kinases (WAKLs) are good candidates for signalling molecules that may physically link the cell wall to the cytoplasm of a plant cell. WAK/WAKL proteins thus may play important roles in cell-cell and cell-environment communications. Previous molecular and genetic studies have shown that WAK genes are required for various cellular processes including pathogenesis and cell elongation (Lally *et al*. 2001). Jackson *et al.* (2003) examined the functional roles for one of the newly discovered *WAKL* members, *WAKL14*. Based on their biochemical, molecular and genetic analyses of *WAKL14*, they concluded that the *WAKL14* gene encodes a receptor kinase that may play an important role in *Arabidopsis* to response to various abiotic stresses. They found that the WAKL14 expression in root tips is highly up-regulated when roots are exposed to 80 µM Zn, Ni or Cu. Lally *et al.* (2001) also noted to the expression of WAK members in specific organs and that they were regulated differentially by various biotic and abiotic factors. They have hypothesized that signals from adjacent cells and/or environment can be sensed by WAKs' extracellular domains and be further transduced to downstream components possibly through protein phosphorylation. Studies conducted in several labs have revealed that WAK1 is a pathogenesis-related (PR) protein (Lally *et al*. 2001) and *WAK* members are expressed in specific organs and differentially regulated by various biotic and abiotic factors. So, it is not far from the logical conclusion that some members of *WAKs* genes may be localized in the salt-responsive gene category and serve plants in their strategies for salt tolerance phenomenon.

Rim2 Protein: A rice transcript, Rim2, was identified that accumulated in both incompatible and compatible interactions between rice and *Magnaporthe grisea*, a fungus, (He *et al*. 2000). They suggested that Rim2 might be one component of a large CACTA-like element, whose transcript accumulates in response to attack by pathogens. The availability of huge amounts of rice genome sequence now permits large-scale analysis of the structure and molecular characteristics of this transposase-encoding Rim2 (also called Hipa) element, which is transcriptionally activated by infection with the fungal pathogen *Magnaporthe grisea* and by treatment with the corresponding fungal elicitor (Wang *et al.* 2003). Based on genomic cloning and data mining from 230 Mb of rice genome sequence, 347 Rim2 elements, with an average size of 5.8 kb, were identified. This indicates that an estimated total of 600-700 Rim2 elements are present in the whole genome. Phylogenetic analysis indicates that the putative Rim2 proteins fall into a subgroup distinct from the TNP2-like transposable element subgroup of transposases. Southern hybridization with genomic DNA from monocotyledonous and dicotyledonous plants demonstrates that the Rim2-coding sequence is unique to the *Oryza* genome. Recent results demonstrate that the Rim2 elements from rice belong to a distinct super family of CACTA-like elements with evolutionary diversity.

Regard to healthy appearance of treated and harvested plants for this research, which removes the possibility of fungal infection, and finding an AFLP-band just in response to ionic effect of salt which has 91% identity to a gene encoding Rim2 might indicate the involvement of this gene in response to abiotic stress (in this case: salt stress) as well as biotic stress. Future experiments might try more primer combinations in the selective amplification step of cDNA-AFLP, which would raise the chances of finding gene(s) that specifically are responsive to ionic effect of NaCl and make IR4630 more tolerant to IR15324.

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