Plant Soil (2009) 315:135–147
DOI 10.1007/s11104-008-9738-6

REGULAR ARTICLE

The ionic effects of NaCl on physiology and gene expression in rice genotypes differing in salt tolerance

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Received: 10 July 2008 / Accepted: 23 July 2008 / Published online: 16 August 2008

Abstract The effects of ionic stress on the physiology and gene expression of two rice genotypes (IR4630 and IR15324) that differ in salt tolerance, were investigated by evaluating changes in the biomass, Na^+ and K^+ concentrations and applying the cDNA-AFLP technique to highlight changes in gene expression. Over 8 days of salinisation, the effect of NaCl on the reduction of biomass (dry weight) was apparent from 24 h after salinisation (the first time point), indicating that the consequences of the build up of Na^+ (and Cl^-) in the leaves of both lines was rapid. Furthermore, root growth of IR15324 was much more sensitive to salt than that of IR4630 (the reduction in root dry weight compared to non-salinised plants was three times greater in IR15324 than IR4630). The two rice lines also differed in their Na^+ accumulation in saline conditions, a difference that was more marked in the shoots, particularly at the final harvest, than in the roots. Under salt stress, the K^+ content (μmol/shoot) increased over four successive harvests (24, 48, 96, 192 h) in both lines, but was always greater in IR4630 than in IR15324: differences in Na^+/K^+ ratio appear to be an important determinant of salt tolerance in rice. To separate osmotic from ionic effects of salt, mannitol was applied as a non-ionic osmoticum at an osmotic potential estimated to be equivalent to 50 mM NaCl. Messenger RNA 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). Bioinformatic analysis indicated three of these AFLP-bands have a high-degree of sequence similarity with the genes encoding a proline rich protein, senescence associated protein and heat-shock protein. The data are novel in that they differentially highlight changes induced by the ionic rather than osmotic effects of salt and in a tolerant rather than a sensitive genotype. The possible roles of the products of these genes are discussed.

Responsible Editor: John McPherson Cheeseman.

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Keywords cDNA-AFLP · Gene expression · Ionic effects · Salinity · Rice

Abbreviations

AFLP	Amplified fragment length polymorphism
ATP	adenosine tri-phosphate
cDNA	complementary DNA
dNTP	deoxynucleotide tri-phosphate
ds-cDNA	double stranded cDNA

HSP	heat shock protein
PAR	photosynthetically active radiation
PEG	polyethylene glycol
PRP	proline-rich protein
RT-PCR	reverse-transcription polymerase chain reaction
SAG	senescence associated gene
X-GAL	5-bromo-4-chloro-3-indolyl-B-D-galactoside

Introduction

The injurious effects of salt (NaCl) on plants can be divided into osmotic and ionic effects (Munns 2002). Munns (2002) hypothesized that plant growth is initially inhibited (phase 1) by cellular responses to the osmotic effects of external salt; later in the 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, any first phase is rather short-lived in comparison to other cereals (Yeo et al. 1991) because of the rapid build-up of Na^+ in the shoots to toxic levels (Yeo 1992).

Earlier studies have shown that gene expression is greatly altered by salinity in rice and other plants (e.g.: Pillai and Yanagihara 2001; Saneoka and Ishiguro 2001; Yokoi et al. 2002; Yu and Zhou 2003; Fukuda and Chiba 2004), but most of these studies have been conducted using a high concentration of NaCl alone, a salt shock, which causes changes in transcription of genes responsive to the osmotic effect of NaCl and not its ionic component. In the present study, the cDNA-AFLP technique has been used to identify some genes of IR4630 (a salt tolerant line) that were induced by the ionic effect of salt, and not expressed in IR15324 (a sensitive line): that is AFLP bands that 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. 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) in both the first and second phases of salinisation. These AFLP-bands were purified from the cDNA-AFLP gel and subsequent cloning, sub-cloning, sequencing and bioinformatic analysis were completed to assess their possible function in imparting salinity tolerance.

Material and methods

Plant material and growth conditions

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 germinated on the surface of nylon mesh placed over the modified (reduction in the phosphate concentration by 50%) nutrient solution of Yoshida et al. (1976). Seedlings were transplanted after seven days into black-painted plastic containers, filled with the same nutrient solution, each with 20 plants and kept in a growth chamber with day/night air temperature of 30°/26°C; irradiance of 400–500 μmol m^{-2} s^{-1} PAR (photosynthetic active radiation), photoperiod of 12 h and 50% relative humidity. Two weeks after germination, when the third leaf was fully expanded, plants were subjected to stress treatment. 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*. Plants grown in nutrient solution lacking NaCl and mannitol served as a control.

Growth and salt accumulation

Plants that were 10–11 days old were either subjected to NaCl (50 mM) culture solution or were left in

normal culture solution as a control. At each of four harvests (24, 48, 96 and 192 hours after salinisation), the shoots and roots of ten plants (salinised and control) were harvested and a t-test used to analyse the significance of physiological data. Shoots and roots were dried separately in an oven at 60°C for 48 hours and the dry weights recorded. Plant growth was measured by determining dry weights over a period of 8 days (i.e. during sequential harvests). The slopes of fitted linear regressions were calculated as indicators of the relative growth rate (Hunt et al. 1978). Analysis of the ion content was accomplished after extracting weighed, dried material in acetic acid (100 mM) for at least 2 h at 90°C. K^+ and Na^+ concentrations in control and salinised plants were determined by atomic absorption spectrophotometry (Pye Unicam SP9 919). The shoots and roots were washed several times with deionised water to remove any NaCl from the culture solution. All plants were handled with gloves to avoid transfer of Na^+ from hands to plants.

Determination of isotonic NaCl and mannitol concentrations

In order to separate specific ion effects of NaCl from its osmotic effects (an important aspect of the molecular experiments described later), mannitol was used as a non-ionic osmoticum. To determine the concentration of mannitol that was, for rice, osmotically equivalent to NaCl, the effects of 85 mM and 100 mM mannitol were compared with that of 50 mM of NaCl (−0.22 MPa). For these experiments, leaf growth rate was measured using displacement transducers (ST 200 Linear Variable Differential Transformers; Sangamo, Bognor Regis, W. Sussex, UK) as described by Malone (1992). Fifteen-day-old plants of both lines were used.

RNA extraction and ds-cDNA synthesis

Fourteen-day-old rice seedlings (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 time. Total RNA was isolated from ~150 mg of frozen (liquid nitrogen) shoots or roots using RNeasy plant mini kit (QIAGEN). Oligotex mRNA spin-column protocol (QIAGEN) was used for isolation of $polyA^+$ mRNA. Double-stranded cDNA was synthesized 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 2.5 u of *Eco*RI and 2.5 u of *Mse*I, and then 2.5 pmol of *Eco*RI and *Mse*I adapters were ligated to the digested cDNA ends as described by Vos et al. (1995). The sequence of the adaptors was as follows: *Eco*RI adapter top strand, 5′-CTCGTAGACTGCGTACC-3′; *Eco*RI adapter bottom strand, 3′- CATCTGACGCATGGTTAA-5′; *Mse*I adapter top strand, 5′-GACGATGAGTCCT GAG-3′; *Mse*I adapter bottom strand, 3′- TACT CAGGACTCAT-5′. The reaction for a single sample was: 2.5 pmol of *Eco*RI adapter, 2.5 pmol of *Mse*I adapter, 1 mM of rATP (adenine ribose triphosphate nucleotide used as a cofactor), 0.6 u of T4 ligase and 10X RL (a ready-to-use buffer for its corresponding enzyme, which purchased from the company) buffer in a final volume of 5 µl. This was added to each reaction containing the restricted ds-cDNAs These samples were incubated in a PTC-200 thermocycler at 37°C for 3 hours (or overnight)., The samples were then diluted 1:10 in 1X TE (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) for use in the next, pre-amplification, step. For pre-amplification, 13 µl of 1:10 dilution from the restriction-ligation product was used as a template in a reaction with a total volume of 50 µl per sample. For a single reaction 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: E00 primer, 5′-GACTGCG TACCAATTC-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. PCR-products were diluted 1:10 in 1X TE in a flat-bottomed microtitre plate and used as a template for the next, selective amplification, step. For the selective amplification, the *Eco*RI-primer (rather than *Mse*I) was radioactively labelled (Vos et al. 1995). Four different primers were compared for optimal banding patterns: 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 recognized as the most suitable for rice-AFLP experiments (M. Koyama, personal communication). 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 the 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 [$\gamma-^{33}$P] ATP and 1,4 all buffer in a final volume of 3.7 µl. The tubes were incubated (37°C) in the hot-block for at least 2 hours. For a single sample of selective amplification, the following 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), and 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 3 MM paper using a gel dryer (BIO-RAD model 583) and exposed to Kodak film (type 35×43, X-ray film) for at least 48 hours. Fingerprint patterns were visualized using an automatic developer (Konica SRY-101A).

Preparing the AFLP fragments for cloning

For cloning, those AFLP fragments that appeared only in salt-treated samples of IR4630 (the salt tolerant line) (both root and shoot) 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 ml 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 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 step led to production of PCR products suitable for ligation into the TOPO TA plasmid vector, which has a single over-hanging 3′ deoxythymidine (T) residue. Total volume for the 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) and a final extension step of 72°C for 7 minutes. 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 visualization, 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 analyzed for homology to nucleotide or protein sequences in GenBank non-redundant databases using the BLAST program.

Validation of cDNA-AFLP experiments by RT-PCR

To verify that the products isolated via the cDNA-AFLP analysis were truly differentially transcribed, RT-PCR analysis was carried out using the original cDNA samples with the specific primers. Although it is difficult to be quantitative using this approach, large differences in the amount of product between samples confirms that the product sequenced does relate to the differentially transcribed cDNA-AFLP fragment. PCR reactions were carried out in a total volume of 20 µl containing: 2 mM Mg^{2+} , 0.3 mM dNTPs, 0.25 µM forward primer, 0.25 µM reverse primer and 1u *Taq* DNA polymerase. Primers were constructed, according to the sequence of candidate genes found in the Genbank databases (i.e. *PRP* of rice, *SAG* of pea and *HSPCO25* of rice): Sense primer: 5′-CATCTCCA CCACCATACGTG-3′, antisense primer: 5′-GACGG GGAGGTAGATGTTGA-3′ for searching A gene of rice similar to *Zea mays PRP* gene for *afb1* (the first isolated AFLP band); Sense primer:5′-ACCCT TTTGTTCCACACGAG-3′, antisense primer: 5′-TAT CCAGGGAAACCAACAGC-3′ for a senescence-

associated gene of *Pisum sativum* for *afb2* (the second isolated AFLP band); Sense primer: 5′-GCACC CAGCTGAAAGAAAAG-3′, antisense primer: 5′-GTTGCCTCATCCACTTCCAT-3′ for a gene of *Homo sapience* HSPCO25-like protein of rice for *afb3* (the third isolated AFLP band). The cycling regime was 30 cycles of 94°C–60 s (denaturing), 55°C–60 s (annealing) and 72°C–60 s (extension), and a final extension step of 72 °C for 7 minutes. PCR products were run on a 2% agarose gel (w/v) to evaluate the abundance of the products.

Results

Growth changes of IR15324 plants under salinity

Comparison of shoot dry weights of control and salinised plants of IR15324 showed that after the second day of salinisation, the growth of stressed plants was less than that of control plants (Fig. 1): relative growth rates (RGRs) calculated from the slopes of fitted linear regressions (Hunt 1978) were 0.27 d^{-1} for control shoot and 0.14 d^{-1} for salinised shoots. From the second to the final harvest (from day 2 to day 8 of salinisation), this dissimilarity increased, so that after 96 hours of salinity the dry weight of stressed plants was about 60% of control, and 36% by 192 hours. Roots responded similarly to the shoots to the presence of 50 mM NaCl, although the growth of roots was more sensitive to salt than that of shoots under salinity stress (Fig. 1B; the RGRs for control and salinised roots were 0.23 d^{-1} and 0.06 d^{-1}, respectively).

Growth changes of IR4630 plants under salinity

In IR4630, shoot dry weight was only slightly reduced by salt, particularly over the early harvests (Fig. 1). The RGRs of control and salinised shoots

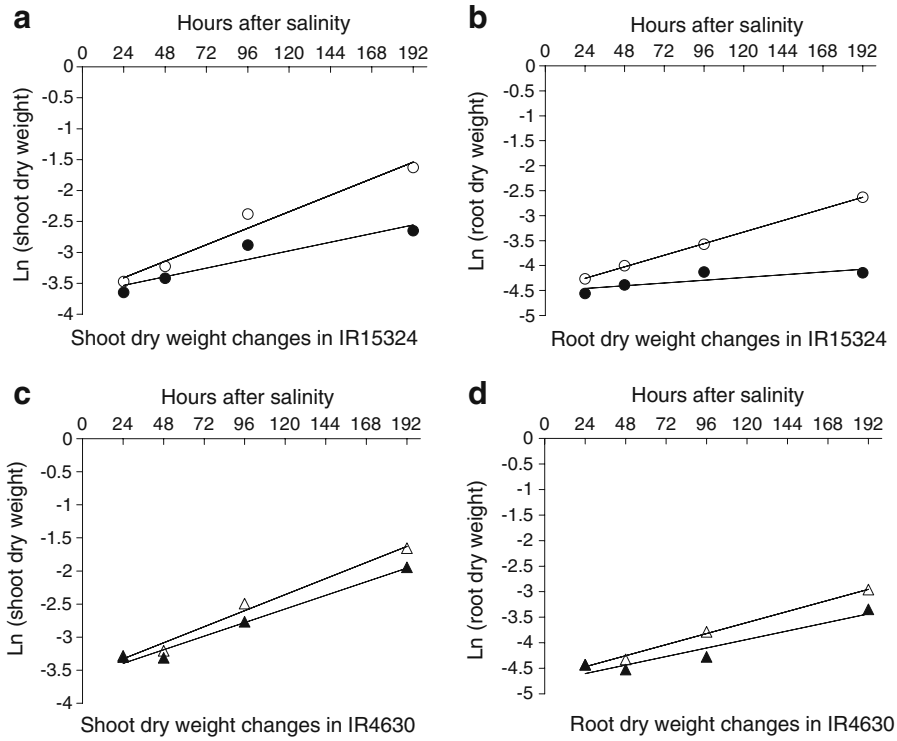

Fig. 1 Changes of shoot and root dry weights in two genotypes of rice, IR4630 and IR15324, after adding sodium chloride (50 mM) to the growth medium in comparison with 'control' plants, which were not subjected to salinity or mannitol. The slopes of the lines are the relative growth rates. (**a** and **b**) The comparison of control and salinised shoot and root dry weights in IR15324, respectively. (**c** and **d**) The comparison of control and salinised shoot and root dry weights in IR4630, respectively. *Hollow circles* Control plant of IR15324; *filled circles* salinised plant of IR15324; *hollow triangles* control plants of IR4630; *filled triangles* salinised plants of IR4630

were 0.24 d^{-1} and 0.21 d^{-1}, respectively: after 8 days in the saline solution, the dry weight of stressed shoots was 74% of the control shoots. With regard to root growth, 24 hours after adding salt to the culture solution, there was no clear difference in the growth of the roots of salinised and the control plants (Fig. 1; the RGR of control roots was 0.22 d^{-1}). The growth of the salinised roots gradually decreased but to a lesser extent than that of IR15324. Averaged over the eight days, the relative growth rates of salinised shoots of IR15324 was 67% of that of salinised shoots of IR4630 (0.21 d^{-1}) and that of salinised roots of IR15324, 33% of that in salinised roots of IR4630 (0.17 d^{-1}). These data all indicate that IR4630 was a more salt-tolerant line than IR15324.

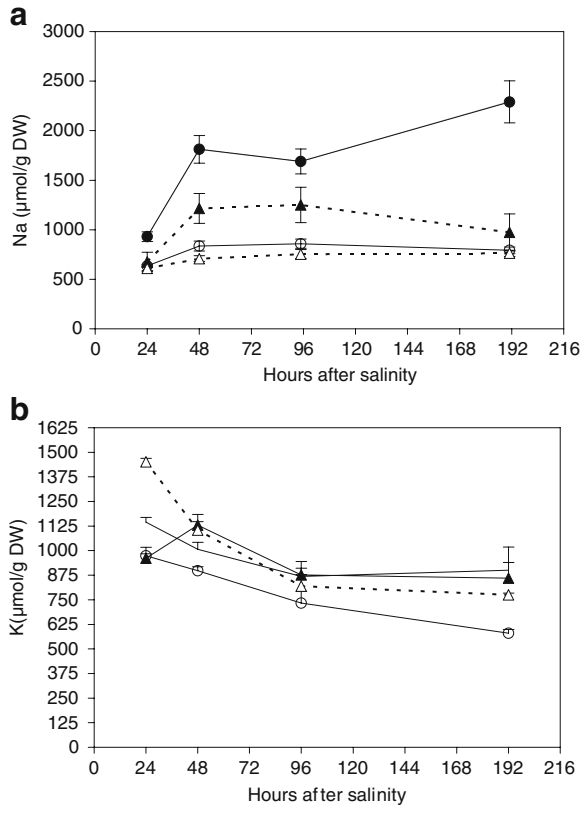

Fig. 2 Na^+ and K^+ concentrations in salinised shoots and roots of two lines of rice, IR4630 (*broken line*) and IR15324 (*solid line*), following salinisation with NaCl (50 mM). (**a**) Na^+ concentration; (**b**) K^+ concentration. *Filled circles* IR15324 shoot; *filled triangles* IR4630 shoot; *hollow circles* IR15324 root; *hollow triangles* IR4630 root. Vertical bars represent ± standard error of the mean

Ion uptake upon salinity

The two rice lines differed in their Na^+ accumulation in saline condition (Fig. 2a). This difference was more marked in the shoots, particularly at the final harvest, than in the roots. By this final harvest, the Na^+ concentration in salinised shoots of IR15324 (2291 µmol g^{-1} dry weight) was over double that of IR4630 (973 µmol g^{-1} dry weight), while there was little difference in Na^+ concentration in the roots of the two lines (Fig. 2a). The Na^+ concentrations at the first (24 h) and last (192 h) harvest in roots of IR15234 were, in turn, 635 and 793 µmol g^{-1} dry weight while these values for IR4630 were 609 and 762 µmol g^{-1} dry weight. The K^+ concentration in the salinised roots of IR15324 was less than that of IR4630 (Fig. 2b – between 68% and 90% of IR4630 at different harvests). Under salt stress, the K^+ content (µmol/shoot) increased over four successive harvests in both lines, but was always greater in IR4630 than in IR15324, so that K^+ content at the final harvest in IR15324 shoots was only about 50% of that in IR4630 (Fig. 3).

Mannitol isotonic to 50 mM NaCl

For identification of specific effects of NaCl on gene expression during the initial (phase 1) response to salinisation, mannitol was used as a non-ionic osmoticum, in order to separate osmotic from specific-ion effects. We used a criterion of the absence of any change in growth rate on initial change of external osmoticum in order to find the mannitol concentration isotonic to

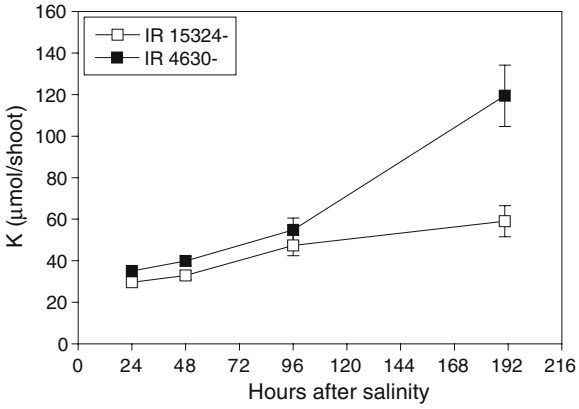

Fig. 3 Total K^+ content in shoots of two lines of rice, IR4630 and IR15324, subject to treatment with NaCl (50 mM). Vertical bars represent ± standard error of the mean

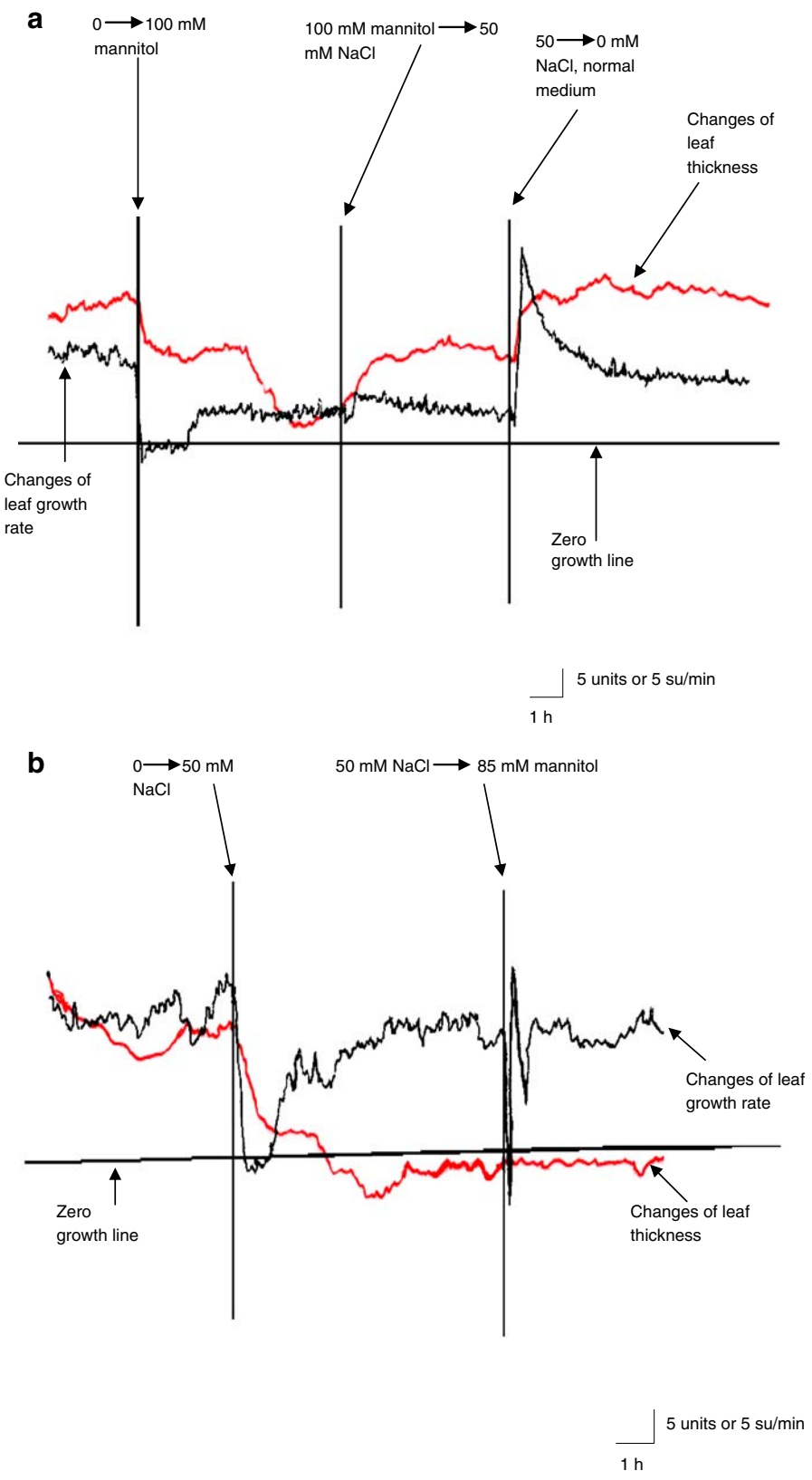

Fig. 4 (**a**) Evaluating osmo-equivalence using 100 mM mannitol. The *red line* shows changes of leaf thickness and the *black line* changes of leaf growth rate. The *first vertical line* (*left to right*) shows the time of adding 100 mM mannitol to the medium. The immediate changes of leaf growth rate and thickness are obvious near to this vertical line. The *second black vertical line* shows the time of replacing mannitol with 50 mM NaCl. The changes in leaf thickness were consistent over a number of measurements and different from the changes of the blank (not shown). The increment of leaf growth rate is seen near to this *second vertical line*, which indicates the effective osmolality of 50 mM NaCl is less than 100 mM of mannitol. The *third vertical line* is for the time when the salt was removed from the medium. Each 50 su or units equals to 13 μm. (**b**) Evaluating osmo-equivalence using 85 mM mannitol. The *red line* again shows changes of leaf thickness and the *black line* changes of leaf growth rate. The *first vertical line* (*left to right*) shows the time of adding 50 mM salt to the medium. The *second vertical line* represents the time of removing NaCl and adding 85 mM mannitol to the root medium. After a few minutes from changing salt for mannitol the growth rate was approximately similar to that in the salt medium. Thus, 85 mM mannitol was considered an osmo-equivalent concentration to 50 mM of NaCl. Each 50 su or units equals to 13 μm

50 mM NaCl. At first, 100 mM mannitol in nutrient solution was used (as its osmolality is approximately equal to that of 50 mM of NaCl), by changing the root medium directly from nutrient + 100 mM mannitol to nutrient + 50 mM NaCl. Leaf growth rate increased after this treatment (Fig. 4a). So, a lower concentration of mannitol was tested to match the osmotic effect of the NaCl. 85 mM mannitol had virtually no effect on leaf growth rate or thickness when added directly after 50 mM NaCl (Fig. 4b) and was therefore assumed to be osmotically equivalent to 50 mM NaCl.

cDNA-AFLPs

The 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 utilized to identify differences between responses of the two genotypes exposed to ionic plus osmotic stresses (salt) and osmotic stress (mannitol). Three bands *(afb1–3)* that were differentially expressed between IR4630 and IR15324 were recognized (Fig. 5). These bands, which derived from the 0.5 and 192 h (after salinisation) time-points, 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.

After cloning, sub-cloning, PCR screening, DNA purification, and sequence determination (data not presented), BLAST analysis was carried out using 'Genbank' database for these three specific AFLP products, which appeared differentially in the profile of salt tolerant rice: present in the tolerant line, but absent from the sensitive line. Table 1 shows the result of the best homologues in the database for cloned AFLP bands, *afb1*, *afb2* and *afb3* were *Zea mays* PRP gene, a putative senescence-associated protein (*Pisum sativum*) and *Homo sapiens* HSPCO25-like protein (*Oryza sativa*).

Results obtained using RT-PCR (Fig. 6) confirmed those obtained using the cDNA-AFLP procedures. Figure 6 shows the PCR products using primers designed according to the nucleotide sequence of *Os PRP*, *PeaSAG and OsHSPCO25* genes for cDNA samples (from the salt stressed shoots of IR4630 at 0.5 h and IR4630 at 192 h, respectively) were clearly apparent on the 1% agarose gel.

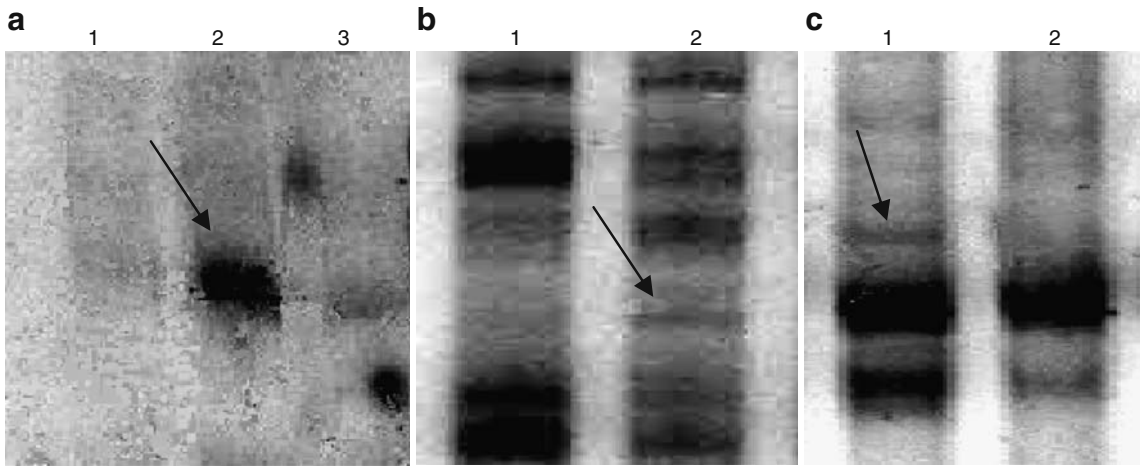

Fig. 5 Differentially expressed cDNA-AFLP bands in two genotypes of rice (IR4630 and IR15324) exposed to 50 mM NaCl for periods of time varying from 0.5 h to 192 h (**a**) Visualisation of *afb1*. Lanes *1*, *2* and *3* derive, in turn, from the control, salinised and mannitol stressed shoots of IR4630 at the first harvest (0.5 h after stress treatment). The *arrow* in lane *2* shows the band *afb1* from the cDNA-AFLP profile (prepared with E(AC) as a selective primer), which is differentially expressed under salt treatment. **b** Visualisation of *afb2*. Lanes *1* and *2* derive, in turn, 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 *afb2*, the second isolated band from cDNA-AFLP profile (prepared with E(AC) as a selective primer), which is expressed differentially in response to salinity; **c** Visualisation of *afb3*. Lanes *1* and *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 expressed differentially in response to salinity

Table 1 Determination of the identities of the genes represented by the cDNA-AFLP bands that were induced in a tolerant genotype of rice (IR4630) exposed to 50 mM NaCl and that were not present after exposure to an osmotically equivalent concentration of mannitol or in plants exposed to neither stress (controls) or in a sensitive line (IR15324)

Clone ID	Best homologue in the Genbank databases and accession numbers	Score (bits)	*E* value	Sequence identity (%)
afb1 from shoot of IR4630, Salt-specific at 0.5 h	Similar to *Zea mays* PRP gene (*Oryza sativa*); Accession number: NM_185672.1	194	4e-48	100
afb2 from shoot of IR4630, Salt-specific at 192 h	Putative senescence-associated protein (*Pisum sativum*); Accession number: AB0497231.1	356	9e-97	72
afb3 from shoot of IR4630 at 192 h, Salt-specific	*Homo sapiens* HSPCO25-like protein (*Oryza sativa*); Accession number: NM_188595	1090	0.0	96

BLAST searches were performed using the sequences of the fragments identified by cDNA-AFLP

afb1 = The first isolated AFLP band belonged to the salt stressed shoots of IR4630 at 0.5 h; *afb2* = The second isolated AFLP band belonged to the salt stressed shoots of IR4630 at 192 h; *afb3* = The third isolated AFLP band belonged to the salt stressed shoots of IR4630 at 192 h

Discussion

Growth and salt accumulation

The degree of sensitivity of the two lines was defined by differences in reduction in biomass after exposure to NaCl for 8 days. It was clear that IR15324 was much more sensitive than IR4630 in 50 mM NaCl. The salt reduced dry weight of both lines, but this reduction was much greater in IR15324 than IR4630. In contrast to IR4630, the sensitivity of IR15324 to 50 mM NaCl (reduction of dry weight in shoots and roots) was observed from the first harvest (24 h after salinisation) and increased with time. Growth inhibition was clearly correlated with increasing sodium concentration in the leaves: the sodium concentration in IR15324 was about twice that in IR4630 after only 24 h of salinisation. Furthermore, external sodium clearly had a greater effect on potassium uptake in IR15324 than in IR4630, suggesting an effect on plant nutrition.

In both lines, root sensitivity to NaCl (resulting in reduction of root dry weight) was greater than that of shoots. Furthermore, roots of the sensitive line were much more sensitive than those of IR4630 (the dry root weight reduction was three times greater). This conclusion is in agreement with Neumann's results (1997). He pointed out that salinity can rapidly inhibit root growth and hence capacity for uptake of water and essential mineral nutrient from the soil.

According to the Munns's two-phase growth hypothesis, the length of the phases 1 and 2 (short- and long-term effects of salt; osmotic and ionic effects), depends on the sensitivity of plants to salinity and is variable between species (Munns 2002); in more sensitive plants salt-specific effects appear in days after salinisation, because the sensitive plants accumulate salt in their tissues much faster than

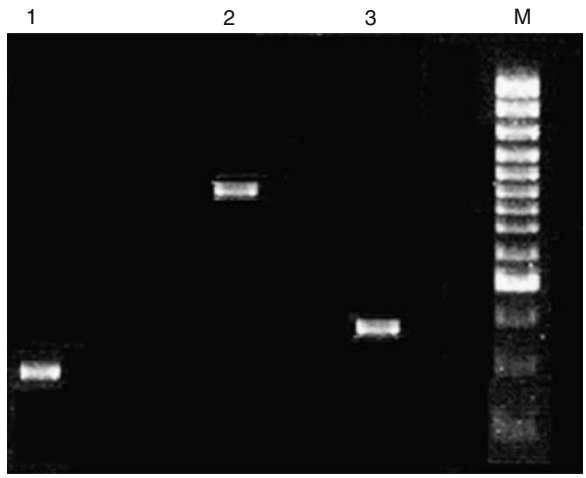

Fig. 6 The results of validation experiments using primers designed according to the nucleotide sequence of *OsPRP*, *PeaSAG and OsHSPCO25* genes for cDNA samples on the 1% agarose gel (*w*/*v*). Lanes *1–3* represent the PCR products using – in turn – *Os PRP, PeaSAG and OsHSPCO25* primers. *M*: DNA marker (100 bp)

tolerant plants. The duration of the short-term (phase 1) in rice is minutes to hours and describes only the transient changes in leaf expansion that occurs when turgor is suddenly changed (Yeo et al. 1991). There are also changes in metabolism and gene expression during the transient response to sudden change in salinity i.e. osmotic shock, which has been reported by Kawasaki et al. (2001) in rice.

Measurements of sodium in rice seedling of both lines revealed concentrations of: 1178 and 634 μmol g^{-1} dry weight in the shoots and roots, respectively, of IR15342 and 646 and 609 μmol g^{-1} dry weight in the shoots and roots of IR4630 after 24 h of exposure to NaCl (50 mM). These rapid increases suggest that ionic effects of salt in rice seedlings had already started by the end of the first day of salinisation. The start of phase 2 in rice is much sooner than in wheat or barley. The concentration of Na^+ in the shoots of a tolerant line of wheat (Khachia) after 13 days of 150 mM of NaCl only reached 0.18 mmol g^{-1} dry weight (Munns et al. 1995); in rice sodium concentration was 1.8 mmol g^{-1} dry weight in IR15324, ten times more than of this value in wheat, after only 48 h from adding 50 mM NaCl to the medium.

Although the K^+ content of shoots increased over time in both lines, particularly in IR4630, the K^+/Na^+ ratio in shoots showed a marked decrease during salinity. The K^+/Na^+ in IR4630 (the tolerant genotype), after eight days of salinisation, was about 1.6 times greater than that of IR15324 (the sensitive line). This result was similar to that reported by Khan and Hamid (1997), who found that salt-tolerant cultivars of rice accumulated less Na^+ and more K^+ in comparison to susceptible ones. Asch and Wopereis (2001) proposed a screening system for salinity resistance of rice, particularly in arid and semi-arid climates, based on the correlation between K^+/Na^+ in leaves under salinity and salinity-induced yield losses. They reported that the most susceptible cultivars had lowest K^+/Na^+ in leaves and the strongest yield reductions. Differences in Na^+/K^+ ratio appear to be an important determinant of salt tolerance in rice.

Changes in gene expression: *afb1* and proline-rich proteins (PRPs)

By establishing a mannitol treatment with an effective osmotic potential equivalent to 50 mM NaCl, we have been able to separate the ionic from the osmotic effects of salinisation. After 0.5 h we observed the expression of a gene encoding a *proline-rich protein (PRP)* in the tolerant but not in the sensitive genotype. The proline-rich domain of the protein encoded by *Zm*PRP is similar to that of soybean PRP protein (Jose-Estanyol et al. 1992). They established that a single gene encoded this protein and its mRNA accumulated during the developmental stages in the maize embryo. They assumed that the maize PRP protein is a wall protein and suggested that the presence of *Z. mays* PRP could modify the structure of the wall protecting the cell during later embryogenic development. Proline-rich proteins consist of a group of cell-wall proteins, which are distinguished by several repeated proline residues in their structures. It is known that the PRP genes are induced in developmental programs (Jose-Estanyol et al. 1992), biotic stresses such as pathogen attack (Sheng et al. 1991), wounding (Keller 1993) and also abiotic stresses, such as salinity and water deficit (He et al. 2002; Verdoy et al. 2004), cold (Goodwin et al. 1996) and heavy metals (Chai et al. 1998). There are some suggestions in the literature for the roles of PRPs; for example, some (e.g. Ristic and Ashworth 1994; Goodwin et al. 1996) have hypothesised that these proteins may help to maintain cellular integrity in stressed tissue by forming additional strong linkage between the cell wall and plasma membrane – because, stress-induced cellular dehydration (such as high salinity, water stress and freezing) causes a reduction in cell volume, contraction of the protoplasm and ultimately collapse of the cell. Others (Chai et al. 1998) suggested PRPs act through maintenance of cellular integrity by forming strong linkages within the cell walls. Pont-Lezica et al. (1993) expressed the importance of these proteins in conveying physical stress from the cell wall to the plasma membrane. Lamport et al. (2006) observed arabinogalactan proteins (AGPs), which are concerned in cell expansion, have had a massive (up to a six-fold increment) up-regulation under salt stress in tobacco cells, indicating the involvement of cell wall components in response to salinity.

afb2 and senescence-associated genes (*SAGs*)

A 72% identity of *afb2* to a senescence-associated gene of *Pisum sativum* (*PeaSAG*). is an interesting result as the primers used were designed according to a pea gene, which was not previously reported for rice

in 'Genbank' database. Molecular biological studies have shown a large range of genes that are expressed in senescing leaves and known as *SAGs* (senescence-associated genes; Dangel et al. 2000). Some of the *SAGs* are genes involving in regulatory processes such as phosphorylation and protein–protein interaction (Guterman et al. 2003); one example is a gene encoding a putative senescence-related receptor kinase (Hajouj and Michelis 2000). Receptor kinases are involved in cellular signal transduction and also protein trafficking. Another group mediates membrane vesiculation and transportation into vacuoles and also activation of vacuolar proteins. Membrane vesiculation facilitates the movement of ions directly from the apoplast into vacuoles. In addition, it is found that the expression of many types of *SAG* is induced by increments of reactive oxygen species (ROS) levels (Navabpour and Morris 2003). It is known that the production of reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radicals, occurs in all phases of plant growth and development, but is increased when plants are exposed to various abiotic stresses (Dat et al. 2000) such as salinity (Vaidyanathan et al. 2003; Menezes-Benavente et al. 2004). During leaf senescence, degradation of proteins, lipids, chlorophyll and nucleic acids, and production of ROS increase (Thompson and Froese 1998) and plants respond to this by production of certain antioxidant enzymes (Jimenez et al. 1995).

afb3 and Heat-shock proteins (HSPs)

HSPs are reported as molecular chaperones, which are involved in protein synthesis, maturation and degradation in the metabolic processes in non-stressed cells. In addition, molecular chaperones function in the stabilisation of proteins and membranes and are also able to bring about the refolding of proteins denatured under stress in an ATP-independent manner (Torok et al. 2001). Although HSPs are so-called because they provide basal tolerance to heat, their induction is observed under other abiotic stresses such as salinity, desiccation and chilling (Sun 2002). Among five conserved families of HSPs (HSP100, HSP90, HSP70, HSP60 and sHSP), it is believed small heat-shock proteins (sHSPs) play an important role in plant stress tolerance (Wang et al. 2003). sHSPs are shown to prevent aggregation of unfolding proteins and increase reactivation of denatured substrate proteins (Lee et al. 1995). Ehrnsperger et al. (1997) investigated the interaction of HSP25 with unfolding protein under heat shock conditions. They showed that HSP25 forms complexes with unfolding intermediates and prevents their aggregation and insolubilization. Although this result is presented for murine HSP25, the conserved nature of sHSPs suggests this model could be generalised to other organisms such as plants. Whether the protein encoded by *OsHSPCO25* functions as an HSP or in the detoxification processes due to oxidative stress caused by salt or dehydration remains to be evaluated.

Conclusions

This is the first report of changes in the expression of genes encoding proline rich proteins, senescence associated proteins and heat-shock proteins as being responsive to the ionic, rather than osmotic, effects of salt in rice: the expression of the PRP occurred soon after salinisation, while expression of the other two genes only occurred after more than a week of exposure to salinity. Our data are novel in that they differentially highlight changes induced by the ionic rather than osmotic effects of salt and in a tolerant rather than a sensitive genotype.

Acknowledgment The authors wish to thank Dr. Mike Malone for his help and encouragement. These experiments were conducted at the School of Life Science of the University of Sussex, Brighton, UK and supported by a scholarship from the Ministry of Sciences, Research and Biotechnology, Iran.

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