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Expression of the plasma membrane H$^+$-ATPase gene in response to salt stress in a rice salt-tolerant mutant and its original variety

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Abstract Plasma membrane (PM) H$^+$-ATPase plays an important role in the establishment and maintenance of ion homeostasis. To investigate its expression in the rice salt-tolerant mutant M-20 and the original variety 77–170 during salt stress, a cDNA fragment corresponding to the PM H$^+$-ATPase gene was obtained by PCR from rice *japonica* variety 77–170 and designated as *OSA3*. Sequence analysis of *OSA3* revealed its high homology with two other published PM H$^+$-ATPase genes, *OSA1* and *OSA2*, in rice. Southern-blot analysis detected a RFLP between M-20 and 77–170, and one copy of the *OSA3* gene was mapped to a position on rice chromosome 12 where a salt tolerance QTL was closely located. The expression of the PM H$^+$-ATPase gene, as revealed by the *OSA3* fragment, was compared between M-20 and 77–170. The results demonstrated that M-20 shoots accumulated less transcripts than 77–170 shoots at a later stage of salt treatment, and M-20 showed high expression at 300 mM NaCl while 77–170 reached its maximum at 200 mM NaCl. In roots, the difference in the level of the PM H$^+$-ATPase gene expression between stressed and non-stressed plants was substantially greater in M-20 than that in 77–170. The relative abundance of PM H$^+$-ATPase gene transcripts in M-20 roots may indicate the active role of this gene in the strict control of Na$^+$ and Cl$^+$ uptake into root symplast and apoplast, and further translocation into the shoot, hence leading to the reduced gene expression of M-20 shoots under salt-stress conditions.

Key words Plasma membrane H$^+$-ATPase gene · Salt stress · Salt-tolerant mutant · Rice

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Introduction

When plants are exposed to salinity, the ion homeostasis in cells is disturbed. To adapt to the osmotic and ionic imbalance, plants develop numberous responses. These include the induction of many genes whose products are classified into two groups. One group involves protein factors in the regulation of signal transduction and gene expression in stress responses. Another group involves proteins that possibly function in stress tolerance, e.g. transport proteins, key enzymes required for the biosynthesis of different osmoprotectants, and proteins protecting macromolecules and membrane structures (for a review see Shinozaki and Yamaguchi-Shinozaki 1997). Among the transport proteins, plasma membrane (PM) H$^+$-ATPase plays a key role in the establishment and maintenance of cellular ion homeostasis. The proton and electrical gradient produced by PM H$^+$-ATPase is the driving force for active secondary transport and the regulation of Na$^+$ and Cl$^-$ uptake (for a review see Niu et al. 1995). The H$^+$-pumping capacity of PM H$^+$-ATPase has been reported to increase in *Atriplex nummularia* roots upon salt treatment (Braun et al. 1986). Comparison of PM H$^+$-ATPase gene expression was also performed in a glycophyte and a halophyte under salt-stress conditions (Niu et al. 1993).

Rice is an important crop worldwide. Salinity causes a significant loss of rice yield. Using the anther-culture method, a salt-tolerant mutant (M-20) has been obtained from *japonica* variety 77–170 (Chen 1988) and extensively studied. Chen et al. (1991) reported the presence of allelic differences in M-20 at two linked loci, RG711 and RG4, on chromosome 7. Using an F_2 population from a cross between M-20 and its original variety 77–170, the major gene for salt tolerance has been mapped on chromosome 7 with a genetic distance of 7.0±2.9 cM in relation to the marker RG4 (Zhang et al. 1995a). A RAPD marker was also identified to link to the salt tolerance gene using the same population (Ding et al. 1998). In addition, several salt-inducible genes have been isolated, characterized and their expression

compared in M-20 and 77–170 (Zhang et al. 1995b, 1996; Zhang and Chen 1996). Biochemical and physiological studies were also carried out in the two materials (Chen et al. 1991; Zhang et al. 1997).

In the present study, a cDNA fragment representing a new isoform of the PM H^+-ATPase gene was isolated by a PCR approach. Its genomic organization and gene expression in response to salt stress were compared in the rice salt-tolerant mutant M-20 and its original variety 77–170.

Materials and methods

Plant material and treatments

Seeds of rice (*Oryza sativa* L. var. 77–170) and its salt-tolerant mutant (M-20) were imbibed in water at 37°C for 2 days and then germinated on wet cheesecloth at 26°C in a controlled environment with a photoperiod of 12 h. When seedlings grew to the three-leaf stage (around 17 days), Yoshida nutrient solution (Yoshida et al. 1976) was added to maintain the normal nutrition requirement. NaCl treatments were performed at this stage by including different concentrations of NaCl in the Yoshida solution. The solutions were changed every 1 or 2 days. The shoots and roots were harvested at the indicated time, frozen in liquid nitrogen and stored at –70°C for DNA or RNA extraction.

DNA and RNA analysis

Genomic DNA isolation followed the method of Chen et al. (1991). Genomic DNA was digested overnight with restriction enzymes, electrophoresed on an 0.8% agarose gel and transferred onto Hybond N^+ nylon membranes for Southern-blot analysis. Total RNA was isolated from frozen materials by the acid guanidinium thiocyanate-phenol-chloroform extraction procedure (Zhang et al. 1996). For Northern-blot analysis, 30 ug of total RNA was separated on a 1% agarose gel containing formaldehyde and transferred onto Hybond N^+ nylon membranes. Pre-hybridization, hybridization and washing procedures were done according to standard protocols (Sambrook et al. 1989). Probe labelling used the random-priming method. The membranes can then be stripped and re-probed with other probes. The 18S rRNA probe was used to detect the quality and quantity of RNA. Quantitation of the signals on autoradiograms was completed on an Imaging Densitometer (Bio-Rad). Northern blottings were repeated with two independent preparations of RNA and representative experiments are reported.

Reverse transcription and PCR

Four micrograms of total RNA from rice 77–170 was reverse-transcribed following the instructions of the GIBCO-BRL kit.

Two microliters from 20 µl of first-strand cDNAs were used as templates for amplification of the PM H^+-ATPase gene fragment. The sense primer 5′-CTTTGCAGTGATAAAACAGG-3′ and the antisense primer 5′-TCGTTTACACCATCACCAG-3′ were designed according to the published sequence from rice (Wada et al. 1992). The PCR reaction was performed at 94°C, 50 s; 56°C, 1 min 30 s; 72°C, 1 min 30 s for 35 cycles and kept at 94°C for 3 min. The amplified DNA fragment (0.8 kb) was cloned into pGEM-T easy vector (Promega) and analyzed further.

Mapping of the PM H^+-ATPase gene

The mapping of the PM H^+-ATPase gene *OSA3* was performed on the basis of the RFLP linkage map constructed using a rice DH (double-haploid) population of 127 plants (Lu et al. 1997). The DH population was derived from a cross between *indica* rice ZYQ8 and *japonica* rice JX17. The genomic DNAs from parents ZYQ8 and JX17 were digested with three enzymes and polymorphism was detected between the two parents when using *Dra*I for digestion. Genomic DNAs from 127 individual plants were then digested with *Dra*I and the segregation data analyzed using the MAPMAKER/QTL program. The distances between markers are presented in centiMorgans (cM).

Sequencing and data analysis

Sequencing of double-stranded DNA was completed on an automated DNA sequencer (Applied Biosystems, model 373 A) using a sequencing kit (Amersham). The nucleotide and deduced amino-acid sequences were compared with those in the GenBank database using the BLAST analysis program.

Results

Isolation and characterization of the rice PM H^+-ATPase gene fragment

Using the PCR method, a cDNA fragment corresponding to the PM H^+-ATPase gene was amplified and cloned from rice *japonica* variety 77–170. Sequencing analysis of three independent clones revealed that they all had an identical nucleotide sequence of 798 bp and were different from the published PM H^+-ATPase genes *OSA1* and *OSA2* in rice (Wada et al. 1992; Ookura et al. 1994). The gene for this sequence was therefore designated as *OSA3* (Oryza sativa, PM H^+-ATPase gene) following the nomenclature of Wada et al. (1992). A comparison of the three genes is presented in Fig. 1. It can be seen that, in the compared portion, *OSA3* showed 88% identity with *OSA1* and 80% identity with *OSA2*. At the amino-acid level, *OSA3* exhibited 92% identity (96% similarity) with *OSA1* and 88% identity (93% similarity) with *OSA2* (data not shown). The differences observed among the three genes may indicate that *OSA3* was a new isoform of the PM H^+-ATPase gene in rice.

To compare the genomic organization of the *OSA3* gene in the salt-tolerant mutant M-20 and its original variety 77–170, genomic DNAs from them were digested with three restriction enzymes and subjected to Southern-blot analysis. The results in Fig. 2 showed that, in *Eco*RI-digested DNAs, a polymorphism between 77–170 and M-20 was detected at around 10 kb, indicating that an altered arrangement of *OSA3* occurred in the M-20 genome. In addition, 3–4 major bands were visualized in each digestion. Because there were no *Eco*RI, *Hind*III or *Dra*I sites in the *OSA3* fragment, it is believed that 3–4 copies of *OSA3* exist in the rice genome. Minor bands may represent other homologous genes.

Using a rice DH population derived from a cross between ZYQ8 and JX17, and a RFLP linkage map constructed from it (Lu et al. 1997), one copy of the *OSA3* gene has been mapped to rice chromosome 12 between markers RG457 and Y12817R with a distance of 17.6 cM in relation to RG457 and a distance of 9.9 cM in relation to

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OSA3   TTTGCAGTGA TAAAACAGGA ACTTTGACCT TGAATAAGCT CACCGTGGAC AAGAACCTCA
OSA1   ---------- ---------- --C----T- -A-------- --A------ -----T----
OSA2   ---------- C--------- --AC-A---- ----C---T- G--T-----T ----GT--T-

OSA3   TTGATGTCTT TGAAAGAGGA ATCACTCAGG ACCAAGTGAT TCTCATGGCT GCTAGAGCAT
OSA1   -----GA-T-- ---------- G--------- --------A- ---G------ ----------
OSA2   -----G--T-- CC--------T G-TGAC---- -TACT--C-- A--A------ --C-----T-

OSA3   CACGAACAGA AAACCAGGAT GCTATTGATA CTGCAATAGT TGGGATGCTA GCTGATCCAA
OSA1   -C--------- ---T--A--- ---------- -----T----- C--------C ----------
OSA2   -C--T--C--- ---T------- --C--A---G -CA------- ---T-----T ----------

OSA3   AAGAGGCCCG TGCTGGTATT CAAGAAGTTC ATTTCCTGCC ATTCAATCCT ACTGACAAAA
OSA1   --------A- ---------- -----G---- -C--------- ---------G --------G-
OSA2   --------T- ---------- -----G---- ---------- ---------A ----------

OSA3   GAACAGCATT GACATACATT GATGGTGATG GCAAAATGTA TCGTGTTAGT AAGGGTGCAC
OSA1   ----T----- ---------- ---A------ -A--G----- C--------- --A--C----
OSA2   -G--T--T-- A--C------- -------G-- -G------C- C-----A--C -----A----

OSA3   CTGAGCAGAT TCTCCACCTT GCTCATAACA AACCGGAGAT AGAGCGGAGG GTCCATGCTG
OSA1   -A--------- -----A---G --A--C---- -GA-AC---- ---A--AC-A ----------
OSA2   -A--A------ C--TA-T--- --A--C---- --A-T----- T--AA-AA-A ---AGA----

OSA3   TGATTGACAA ATTTGCAGAA CGTGGACTTA GATCGCTTTC TGTAGCATAC CAGGAAGTAC
OSA1   -A-----T-- G--------- ---------C -----A--G- ---------T ---------T-
OSA2   -A--------- -------T--G -----CT-GC -----A---G- ---GCA---- --TC----G-

OSA3   CAGAGGGGAC GAAAGAAACC CCTGGTGGCC CATGGCATTT TGTTGGTCTC ATGCCACTTT
OSA1   -----T--A-G ------G-GT ---------G- -------GC- ------C---- C-T-----C-
OSA2   -----T--T-G ---------GT ------A--A- --------A- -------G--- T---------

OSA3   TTGATCCTCC AAGGCACGAC AGTGCTGAAA CAATTCGGCG GGCACTTAAT CTTGGTGTCA
OSA1   ---------- -------T--T ------A----- --------AA- ---------C ---------A-
OSA2   -----C----- TC-A-----T ------A----- ---CA------ -------C--C ----------

OSA3   ATGTCAAGAT GATCACAGGT GATCAGCTGG CAATTGGAAA AGAAACAGGG CGTCGCCTGG
OSA1   -----T----- ---------- --C--A--A- -G-G---G-- ---------- -----T--A-
OSA2   -----T--A-- ---T------- --C--A--T- -----A--C- G--------CA --C-------

OSA3   GAACTGGTAC AAACATGTAC CCTTCACTCC CTTTGCTGGG ACAGAACAAG GATGAGTCCA
OSA1   ---TG------ ---------- -------TCTG -----T---- ---------- --------TG
OSA2   ---TG------ T--T------T --C---TCTG -----A----- ---AG-T--- -----A--A-

OSA3   TTGCCGCTTT ACCAGTTGAT GATCTCATTG AGAAAGCTGA TGGCTTTGCT GGTGTATTCC
OSA1   -----T------ ---------C ------A--C- ---------- ---T------ ----------
OSA2   ---TG------ G--------- --AT-A----- ---------- ---------- --A--C--T-

OSA3   CAGAGCACAA GTATGAGATT GTGAAACGTC TGCAAGCAAG AAAGCACATA TGTGGAATGA
OSA1   ---------- ---------- --A------- ---------C- G--------C ----------
OSA2   -T--------- ---------- --------CT ---------- ---A--T--- -----T----

OSA3   CTGGTGATGG TGTAAACG
OSA1   ---------- --------
OSA2   ------A----- ---G--T-
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Fig. 1 Comparison of the plasma membrane H⁺-ATPase gene *OSA3* with its isogenes *OSA1* and *OSA2* in rice. The *OSA1* gene fragment represents positions from 1019 to 1816 (Wada et al. 1992). The *OSA2* gene fragment represents positions from 1119 to 1916 (Ookura et al. 1994). *Dashes* indicate identity of the nucleotide sequence. The nucleotide sequence of the *OSA3* gene has been deposited in Genbank under the accession number of AF 110268

Y12817R (Fig. 3). This position was very close to a QTL for salt tolerance, which was located between markers G1184C-1 and RG361 A in the same region of RG457–G1184C-4–G1184C-1–RG361A–Y12817R (Gong et al. 1999). The genetic distances between the adjacent markers listed above were 6.8, 5.6, 7.7 and 10.5 cM respectively.

Expression of the PM H⁺-ATPase gene in response to salt stress

The expression of the PM H⁺-ATPase gene was investigated in seedlings of the salt-tolerant mutant M-20 and its original variety 77–170 using an *OSA3* gene fragment

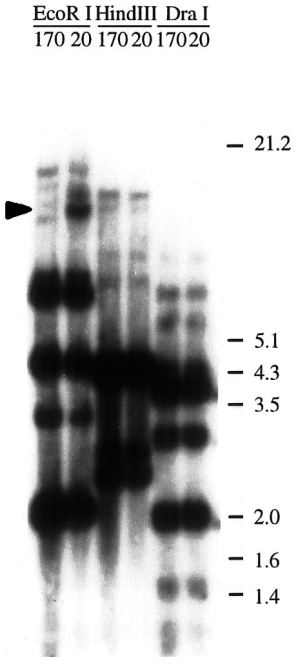

Fig. 2 Southern blot analysis of genomic DNAs from the salt-tolerant mutant M-20 (20) and its original variety 77–170(170). Eight micrograms of genomic DNA were digested with *Eco*RI, *Hind*III and *Dra*I. After electrophoresis on an 0.8% agarose gel, fragments were transferred onto a Hybond N⁺ nylon membrane and hybridized with a labelled *OSA3* probe. Washings were performed at 65°C with 2×, 1× and 0.5× SSC plus 0.1% SDS, each for 20 min. The *arrow* on the left indicates the position of the RFLP band detected in M-20 DNA. *Numbers* on the right are in kb

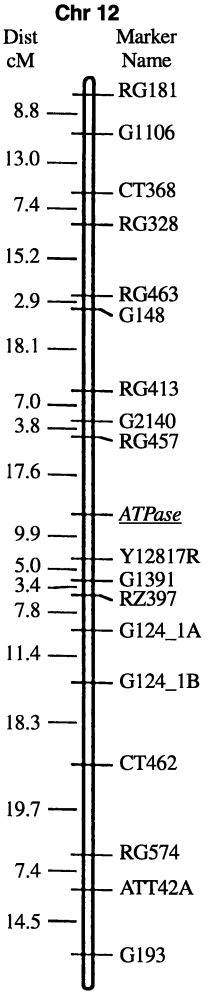

Fig. 3 Mapping of the plasma membrane H⁺-ATPase gene *OSA3* on rice chromosome 12. The RFLP linkage map used was based on a DH population of 127 plants which was derived from a cross between ZYQ8 and JX17. *Numbers* on the left are centiMorgan (cM) distances between adjacent markers. The names of the markers are listed at the right of the chromosome. The marker underlined (*ATPase*) represents the map location of the *OSA3* gene

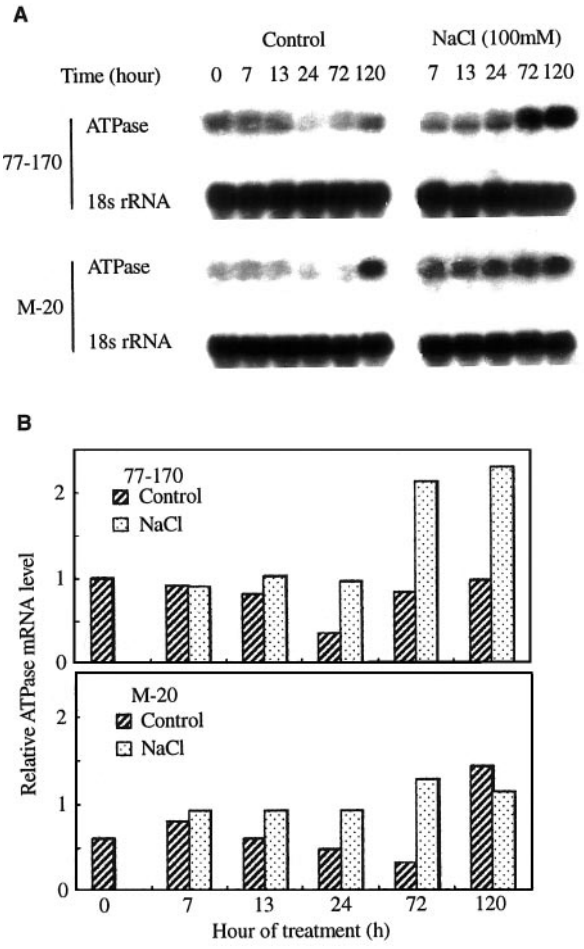

Fig. 4 **A** Transcript level of the plasma membrane H^+-ATPase gene in shoots of the salt-tolerant mutant M-20 and its original variety 77–170 during salt stress. Seedlings were treated with 100 mM NaCl for different times and total RNA was isolated. Thirty micrograms of total RNA from each sample were run on 1% agarose gels containing formaldehyde, transferred onto the same Hybond N^+ nylon membrane and then hybridized with the labelled *OSA3* probe. The membranes were stripped and hybridized with 18S rRNA probes to normalize the RNA loading. **B** Quantitation of the transcript level shown in **A**. The signals on RNA autoradiograms were quantitated using an imaging densitometer (Bio-Rad). The relative amount of mRNA was determined by the ratio of ATPase density to 18S rRNA density. The 0-h value in 77–170 was arbitrarily set to 1 and all other values were compared with it

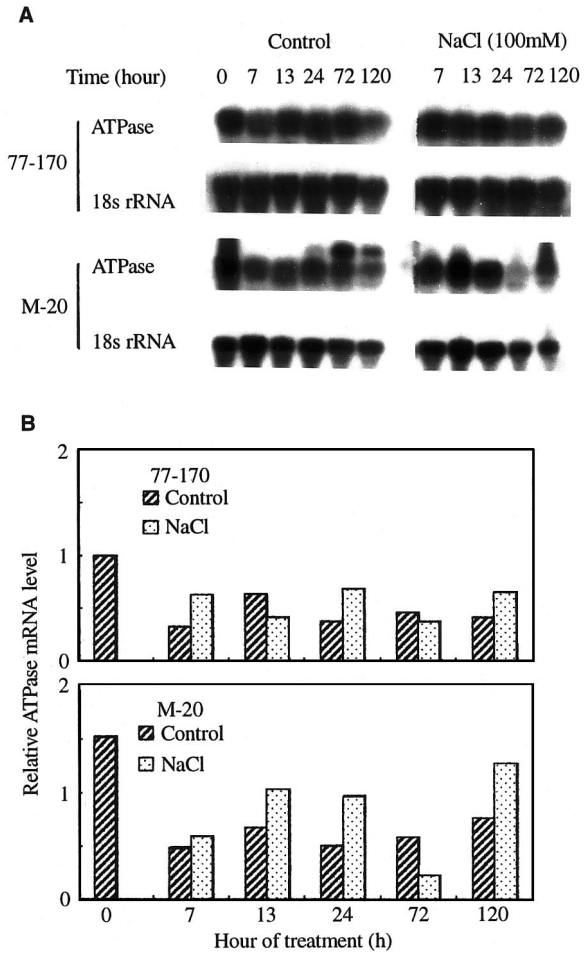

Fig. 5 **A** Transcript level of the plasma membrane H^+-ATPase gene in roots of the salt-tolerant mutant M-20 and it original variety 77–170 during salt stress. Other details are the same as in Fig. 4. **B** Quantitation of the transcript level shown in **A**. Details are as in Fig. 4

as probe. Due to the homology of *OSA3* with other isoforms of the PM H^+-ATPase gene, it is possible that transcripts of different isoforms were detected. The results in Fig. 4A,B showed that, in shoots of 77–170 seedlings, expression of the PM H^+-ATPase gene was relatively constant in controls without salt treatment. However, under salt stress its expression increased by more than one-fold 3 days after initiation of the treatment and remained similar after 5 days. In shoots of M-20 seedlings without treatment, expression of the PM H^+-ATPase gene had a low level in the first few days and increased 5 days after the experiment was started. In shoots of M-20 with salt treatment, its expression level went up slightly, but steadily, over the course of treatment.

The expression of the PM H^+-ATPase gene in roots was also studied. It is shown in Fig. 5A,B that its expression was high in 0-h 77–170 seedlings and declined when a nutrient solution (for control seedlings) or a nutrient solution plus NaCl (for stressed seedlings) was added. There was no significant difference in the expression between stressed and non-stressed 77–170 roots although some fluctuations existed. In roots of M-20 seedlings, expression of the PM H^+-ATPase gene showed a distinct pattern. A higher level of PM H^+-ATPase gene transcripts was observed in M-20 roots of 0-h seedlings. When a nutrient solution (for controls) or nutrient solution containing NaCl (for stressed seedlings) was added, the PM H^+-ATPase gene expression decreased and NaCl-stressed roots had more transcripts than non-stressed roots, especially in the first day of the treatment. The relatively high expression of the PM H^+-ATPase gene in both 77–170 and M-20 roots of 0- h seedlings may indicate the physiological requirement for nutrient ions from the surrounding environment before the addition of nutrient solution. It is interesting to note that a transcript of

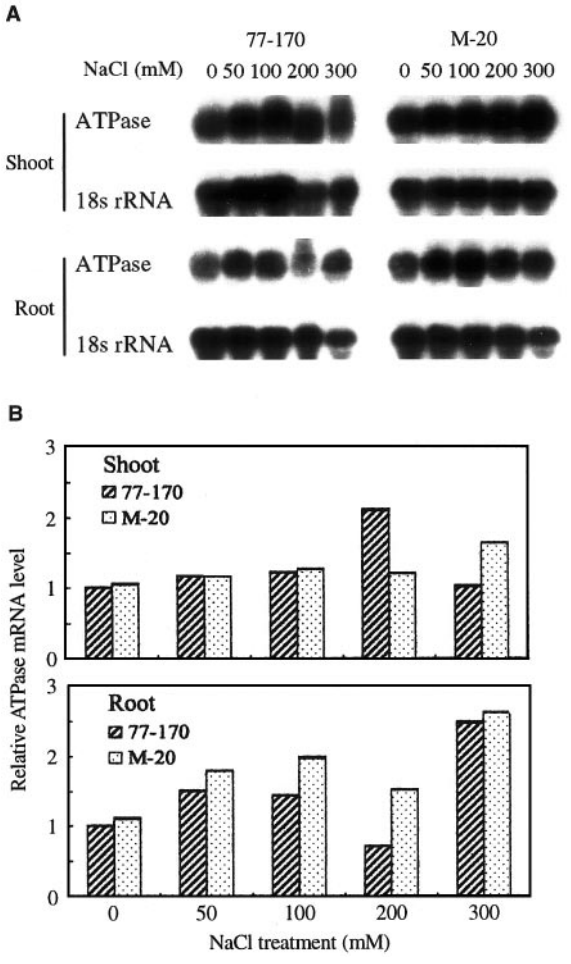

Fig. 6 **A** Transcript level the of plasma membrane H^+-ATPase gene in shoots and roots of the salt-tolerant mutant M-20 and its original variety 77–170 under treatments with different concentrations of NaCl. Seedlings were treated with 0, 50, 100,200 and 300 mM NaCl for 2 days. For other details, see Fig. 4. **B** Quantitation of the transcript level shown in **A**. The 0-mM values in shoots and roots of 77–170 were arbitrarily set to 1 and other values were compared with them. The details are as in Fig. 4

larger size (about 4.0 kb) occurred in non-stressed M-20 roots 3 days after the initiation of the experiment and disappeared in NaCl-treated roots. This phenomenon remains to be investigated.

The expression of the PM H^+-ATPase gene was analyzed in seedlings with treatments of different concentrations of NaCl for 2 days. The results (Fig. 6A, B) showed that 77–170 shoots had more transcripts at 200mM NaCl when corrected with 18S rRNA, whereas M-20 shoots had higher expressison at 300 mM NaCl. In roots, PM H^+-ATPase gene transcripts were more abundant in M-20 than those in 77–170 when treated with 50, 100 and 200 mM NaCl. The very high level of PM H^+-ATPase gene expression at 300 mM NaCl treatment (Fig. 6B, lower panel) was apparently due to the low amount of 18S rRNAs, which possibly resulted from the treatments with a high NaCl concentration.

Discussion

PM H^+-ATPase plays a critical role in the maintenance of ion homeostasis. Using the PCR technique, a cDNA fragment corresponding to the PM H^+-ATPase gene *OSA3* was isolated from rice *japonica* variety 77–170 and characterized. The *OSA3* gene showed high homology to two other PM H^+-ATPase genes *OSA1* and *OSA2*, of rice (Wada et al. 1992; Ookura et al. 1994); hence it may represent a new isogene. Multiple genes encoding PM H^+-ATPase have been cloned in other plants including tomato (Mito et al. 1996), *Arabidopsis thaliana* (Harper et al. 1994) and *Nicotiana plumbaginifolia* (Moriau et al. 1993).

The rice salt-tolerant mutant M-20 and its original variety 77–170 are useful materials for comparing of the genomic organization of the PM H^+-ATPase gene. Using an *OSA3* gene fragment as a probe, a RFLP was detected between M-20 and 77–170, indicating the occurrence of either a mutation or a rearrangement in the PM H^+-ATPase gene in M-20. Based on a DH population derived from a cross between ZYQ8 and JX17, one copy of the *OSA3* gene was mapped to a locus on rice chromosome 12. This locus was very near to a salt tolerance QTL which was obtained using the same DH population (Gong et al. 1999). The coincidence may imply that PM H^+-ATPase plays a role in the salt tolerance of rice.

Our previous studies have shown that shoots of the salt-tolerant mutant M-20 were less responsive to salt stress than its original variety 77–170 with respect to the expression of several genes (Zhang et al. 1996). This observation led us to assume that in roots of the salt-tolerant mutant M-20 some mechanism may be involved by means of which the rice plant could control the flux of Na^+ and Cl^- into roots, and hence make shoots at a relatively normal ion level. In the present study, expression of the PM H^+-ATPase gene was investigated in the salt-tolerant mutant M-20 and its original variety 77–170. In shoots, the PM H^+-ATPase gene was expressed at a higher level in 77–170 than in M-20, especially at a later stage of salt treatment. When stressed with various concentrations of NaCl, 77–170 shoots showed a relatively higher expression at 200 mM NaCl while M-20 shoots had abundant transcripts at 300 mM NaCl. The difference of PM H^+-ATPase gene expression in shoots was consistent with our previous observation that M-20 shoots were less responsive to salt stress than 77–170 shoots. This decrease in responsiveness may be due to the greater accumulation of PM H^+-ATPase messages in M-20 roots upon salt stress, as demonstrated in the present research.

As the primary barrier to the uptake of Na^+ and Cl^-, roots controlled their entry into symplasm. When treated with NaCl, the ion homeostasis was disturbed in root tissues. Increased pump activity was required at this time for the establishment and maintenance of the electrochemical potential gradient across the plasma membrane of epidermal, cortical and endodermal cells. This electrochemical gradient prevented further apoplast transport of

Na^+ and Cl^- into the xylem, through which the ion will be translocated into the shoots. In this way, the salt-tolerant mutant reduced the Na^+ and Cl^- accumulation in shoots and less H^+-pump activity was needed to maintain the ion homeostasis. In line with our current results, a low level of Na^+ content has been reported in the root and shoot of M-20 seedlings under salt treatment when compared with 77–170 seedlings (Zhang et al. 1997). Message accumulation of the PM H^+-ATPase gene in roots was also greater in the halophyte *A. nummularia* than that in glycophyte tobacco (Niu et al. 1993). In *A. nummularia*, the H^+-pump message was mainly localized in the epidermis and endodermis of the root, and the bundle-sheath cells in expanded leaves (Niu et al. 1996). These tissues were involved in the active symplast/apoplast transport of Na^+ and Cl^-. Elevated H^+-translocating activity was also observed in *A. nummularia* roots but not in the roots of glycophyte cotton after salt treatment (Braun et al. 1986; Hassidim et al. 1986).

Our results indicated a role for PM H^+-ATPase in roots of the salt-tolerant mutant M-20 during salt stress, and this role was probably achieved by transcriptional activation. The possible responsive elements and their related transcription factors remain to be investigated. Meanwhile, the PM H^+-ATPase may contribute only partly to salt tolerance due to the fact that some other major QTLs for salt tolerance have been identified in rice and tomato (Zhang et al. 1995a; Foolad et al. 1998; Gong et al. 1999). The cloning and characterization of these QTLs, together with a study of their cooperative interactions with the PM H^+-ATPase gene, should elucidate more details of the mechanisms of salt tolerance in plants.

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