2.5.3 DNA purification

2.5.3.1 Phenol Chloroform Extraction

An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the DNA solution, vortexed for one minute and centrifuged at 12,000 rpm for 5 min. The upper aqueous phase was removed into a fresh tube. DNA was precipitated by adding 0.1 volume of 3M sodium acetate, pH 5.2, and 2.5 volumes of 100% ethanol. The sample was kept on ice or at -20°C for 15 to 30 min, then centrifuged at 12,000 rpm for 10-30 min at 4°C. The supernatant was discarded and the pellet air-dried or dried in a vacuum desiccator. The DNA pellet was dissolved in sterile water or TE.

2.5.3.2 Bresa-Clean

Bresa-Clean™ kits (Bresatec) were used to clean DNA from TBE agarose gels and enzymatic reactions according to manufacturer's instructions

2.5.4 Total RNA Extraction from Rice Tissue

Total RNA was extracted from fresh mature leaf, young shoots inside the leaf sheath and from fresh root tissues using a modified version of the procedure of Logemann, *et al.,* (1987) and was quantified by UV spectrophotometry (Sambrook, *et al.,*; Fritsch, 1989). Rice tissue was collected and frozen immediately in liquid N_2 and stored at -80°C. They were ground in a sterile mortar and pestle with liquid nitrogen and transferred to a sterile 15 mL disposable plastic centrifuge tube. Before the powder thawed, 2 mL (2X volume) extraction buffer (8 M Guanidine hydrochloride, 20 mM MES (4-morpholineethan-sulfonic acid), 20 mM EDTA, 50 mM mercaptoethanol) and 3 mL (equal volume) phenol/chloroform/isoamyl alcohol (25:24:1) were added, and the mixture was shaken. The aqueous fraction containing the RNA was separated from the non-aqueous fraction by centrifugation for 10 minute at 3,000 g and 4°C. The aqueous fraction was removed and extracted once more with an equal volume of phenol/chloroform/isoamyl alcohol. RNA was precipitated overnight at -20°C by adding 0.2 volume of 1M acetic acid and 0.7 volume of cold ethanol. Precipitated RNA was collected by centrifugation at 9,500 g for 25 min at 4°C, then washed with 70% ethanol. The pellet was then dissolved in 1 mL of DEPC-saturated water. Soluble polysaccharides, genomic DNA and low molecular weight RNA material were

removed by precipitation with 2 M LiCl (final concentration) overnight at 4°C. LiCl was removed from RNA by resuspension of the pellet in 0.5 mL DEPC-saturated water followed by overnight ethanol/Na acetate precipitation at -20°C. The RNA was finally pelleted by centrifugation at 10,000 X g for 15 min at 4°C, washed with 70% ethanol, and stored at -80°C.

2.6 DNA Cloning Methods

2.6.1 cDNA Cloning

2.6.1.1 Synthesis and Adaptor Ligation

Poly (A)+ RNA was isolated from 0.5 - 1.0 mg of total RNA using oligo- (dT) cellulose spin columns (Pharmacia), following the manufacturers instructions. Using Poly (A)+ RNA (2 μg), cDNA was synthesized using a cDNA synthesis kit (TimeSaver cDNA synthesis kit from Pharmacia). First strand cDNA synthesis was performed by Moloney murine leukemia virus (MMLV) reverse transcriptase. Second strand cDNA was synthesized by nick translational replacement of the mRNA using *E. coli* DNA polymerase in combination with *E. coli* RNAse H. Following second -strand synthesis, Klenow polymerase (Large fragment) was added to blunt the ends of the cDNA. An *Eco*RI/*Not*1 adaptor was then ligated to each end. The adaptor was composed of two oligo-nucleotides, which form a duplex containing a phosphorylated blunt end and a non-phosphorylated *Eco*RI overhang, as well as an internal *Not*1 site:

AATTCGCGGCCGC

$GCGCCGGCG_P$

2.6.1.2 Ligation of cDNA into λExCell

The adaptor-ligated cDNAs were cloned into *Eco*RI digested, and CIAP (calf intestinal phosphatase) treated vector λExCell(Pharmacia), that contains an internal linearized phagemid vector. In an Eppendorf tube, 2 μL (1μg) of vector, 15 μL (1 μg) of cDNA and 15 μL of STE (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) were mixed and DNA was co-precipitated with 3 M sodium acetate and ethanol.

The pellet was resuspended in 8 µL of sterile distilled water and ligated by adding 1 µL ligation buffer, 0.5 µL of T4 DNA ligase (5 Weiss u/µL) and incubating overnight at 12°C.

2.6.1.3 Packaging of Ligated DNA

The entire ligation reaction was added to 50 µL ice thawed packaging extract, mixed gently by tapping, and incubated at 22°C for three h. After this period, 445 µL of phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM MSG) and 25 µL of chloroform were added and the mixture was vortexed briefly.

2.6.1.4 Titre Determination/Plating of Phage

Phage stock was diluted to 10, 10^2 and 10^3 fold and plated on LB medium. The number of resulting plaques was counted to calculate the viable number of recombinant phages per mL phage stock. A suitable aliquot of phage stock was added into 100 µL phage buffer followed by 50 µL of overnight cultured strain NM522 (Table 2.1). This was incubated at 37°C for 30 min with constant shaking before plating with molten LB soft top agar (0.5% bacto yeast extract, 1% bacto tryptone, 1% NaCl, 0.85% bacto-agar w/v). The top agar Phage mix was spread over pre-warmed 82 mm (for titring) or 150 mm (for library screening and clone purification) LB plates and then incubated overnight at 37°C.

2.6.1.5 Plaque Blotting and Hybridization

Plates were chilled at 4°C for at least one hour to allow the LB soft top agarose to harden. Nitrocellulose filters (Hybond^{TM}-N$^+$, Amersham) were numbered with a soft pencil and placed onto the soft top agar using sterile forceps, without trapping air bubbles. Filters were marked in three asymmetric locations by stabbing through the filter and into the agar with an 18 gauge needle attached to syringe containing radioactive ink. Filters were peeled off after one minute and laid (plaque side up) on Whatman 3MM chromatography paper moistened with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 7 min. The membranes were then transferred to another Whatman 3MM chromatography paper moistened with neutralizing solution (1.5 M NaCl, 0.5 m Tris-HCl pH 7.4, 0.001 M EDTA) for 5 min, rinsed briefly in 2X SSC and dried on a Whatman 3MM paper. The DNA was fixed to the membrane by

ultraviolet cross-linking using the auto cross-link setting of UV Stratalinker™ (Stratagene).

Filters were pre-hybridized, hybridized to ^{32}P-labelled *OsAMT1;1* and washed as described in section 2.7. Plaques that hybridized to the probe were visualized by autoradiography using phosphor screens (Molecular Dynamics).

2.6.1.6 Elution of Positive Plaques

The plaques showing positive signals were recovered using Pasteur pipettes and the phages were eluted into 250 µL of phage buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM $MgSO_4$) containing 50 µL of chloroform. After brief vortexing, tubes were kept at room temperature for an hour and then at 4°C for elution. In cases where a single isolated positive plaque could not be picked, agar containing several plaques was removed, plaques re-plated, and re-screened as described above.

2.6.1.7 Phagemid pExCell release

To release phagemid pExCell from the λ vector, 100 µL of prepared NP66 cells (section 2.1.1) were first incubated at 39°C for 20 min in order to express the proteins required for site specific recombination between the *att*L and *att*R sites. To these cells, 100 µL of phage solution was added, followed by an additional 20 minute incubation at 39°C, and the addition of 200 µL of 1M sodium citrate (room temperature 22°C) and 5 mL of 2x YT broth (pre-warmed 32°C) containing 50 µg/mL spectinomycin. The culture was then incubated at 32°C for 1.5 h with moderate shaking, before being used for DNA mini preps as described in section 2.5.2

2.6.2 Gene Constructions

2.6.2.1 Insert DNA Fragment Preparation.

DNA samples were digested with appropriate restriction endonucleases (RE) in specified buffers according to the manufacturer instructions. To create blunt ends of digested DNA, 5' or 3' overhangs were treated with Klenow polymerase (1 unit/µg of DNA) in the presence of 33 µM dNTPs at 25°C for 15 min. The reaction was stopped with the addition of EDTA (10 mM final concentration) and incubation at 75°C for 10 minute. Required restriction fragments were separated by electrophoresis on a 1% low

melt agarose gel in the 1x TBE buffer and then excised and purified using Bresa-Clean™ DNA purification kit (Bresatec), according to the manufacturer's instructions.

2.6.2.2 Vector Preparation

Following digestion with a single restriction enzyme, vector DNA was treated with Calf Intestinal Alkaline Phosphatase (CIAP) to remove the phosphate groups from the 5'-ends and prevent self-ligation of the vector molecules. CIAP was used at a concentration of 0.01 μ/pmol of ends (1 μg of 1 kb DNA fragment = 1.52 pmol DNA, or 3 pmol of ends) in 1X CIAP buffer. In the case of 5' -protruding ends, the reaction was incubated for 30 min at 37°C then another 0.01u CIAP/pmol of ends was added and incubated for an additional 30 min at 37°C. When the end is 5' -recessed or blunt, the reaction was incubated first at 37°C for 15 min then at 56°C for 15 min. Another 0.01u CIAP/pmol of ends was added and the incubation repeated at both temperatures. The reaction was stopped by incubating at 65°C for 5-10 min. The DNA was then purified using the Wizard™ DNA purification kit (Promega).

2.6.2.3 Ligation of Insert DNA and Vector

Concentrations of both insert and vector were estimated by agarose gel electrophoresis along with molecular weight standards of known concentration. Using an appropriate vector insert ratio (such as 1:3 molar ratio) ligations were set up using T4 DNA ligase, under conditions specified by the manufacturer, and incubated overnight at 16°C.

2.6.2.4 Transformation of Plasmids into Bacteria

Preparation of Competent Cells

Competent cells of *E. coli* DH5α and JM109 were prepared as previously described by Hanahan (1983). A single colony from an LB plate (LB solid medium contains 1% bacto-tryptone, 0.5% bacto-yeast extract, and 1% NaCl and 1.5% agar) was inoculated into 10 mL of liquid LB medium and incubated overnight at 37°C with shaking (225 rpm). The entire overnight culture was then transferred into 1L YENB medium (containing 7.5 g bacto yeast extract, 5g bacto beef extract, 3g bacto peptone) and incubated at 37°C for 2-3 h to an OD_{600} of 0.5 to 0.6. Cells were chilled on ice, collected by centrifugation at 5,000 g for 10 min at 4°C, then resuspended in 20 mL cold 10% glycerol, and transferred to a small tube. After centrifugation the cells were

finally resuspended in 10% glycerol to total volume of 3 mL. Aliquots (40µl) were frozen in liquid nitrogen and stored at -80°C.

Electro-transformation

For transformation, an aliquot of competent cells was thawed on ice and mixed with a small amount (10-50 ng) of ligated DNA, transferred into a chilled cuvette and electroporated at 2.5KV with a time constant of 3.7 sec. After electroporation, cells were mixed with 400 µL of cold LB, and the mixture then transferred into a microfuge tube. Cells were incubated at 37°C for 15-60 min (depending on the antibiotic selection to be used) before plating onto LB plates containing appropriate antibiotics.

Selection of Recombinant Plasmids

To identify vector clones containing inserts, blue/white selection was used (Sambrook, *et al.*, 1989). In this case, transformed cells were plated onto LB plates containing 30 µL of IPTG (20%) and X-GAL (20 mg/mL), with either ampicillin (100 µg/mL) or kanamycin (50 µg/mL). Isopropyl-thio-β-D-galactoside (IPTG) was used to induce expression of the α-fragment of GUS, while 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was the chromogenic substrate for the functional β-galactosidase to produces blue colour product. Thus α-complementation of DH5α cells in the presence of X-gal and IPTG results in blue colonies while cells containing vectors with an insert will produce white colonies. Plasmid DNA was isolated as described in section 2.5.2 and analyzed by restriction enzyme digestion or sequenced as described in sections 2.8.3.

2.7 Rice Genomic Library Screening

2.7.1 Genomic Library

A rice (*Oryza sativa* L. indica, var. IR36) genomic library (Clontec, Australia) made from 5-d-old etiolated seedlings was used for screening. For this library, total genomic DNA partially digested with *Sau*3A1 to produce segments ranging in size from 8 to 22 kb, was cloned into the *BamH*1 site of bacteriophage vector λEMBL-3 SP6/T7. Insert

DNA can be excised from the vector by *Xho*1, *Sac*1 or *Sfi*1. λ vectors and their recombinants were grown on the *E. coli* host strain K802.

2.7.2 Steps in Genomic Library Screening

The steps described in cDNA library screening (section 2.6.1) were also followed for the genomic library screening. Phage stocks isolated by hybridization as described in sections 2.6.1.5 and 2.6.1.6 were used for isolation of λ DNA containing the genomic DNA fragment of interest.

2.7.2.1 Isolation of DNA from Lambda Lysates

Preparation of *E. coli* K802 host cells

A single colony of K802 was inoculated to 15 mL of LB broth containing 10 mM $MgSO_4$ and 0.2% maltose and incubated at 37°C overnight. After harvesting of the cells by centrifugation, they were resuspended in 7.5 mL of 10 mM $MgSO_4$ and stored at 4°C prior to use.

Phage Stock Preparation

Phage stock, (isolated as described in section 2.6.1), was mixed with *E. coli* strain K802 and grown on a plate at 37°C overnight. A single plaque was then transferred to a microcentrifuge tube containing 200 µL of 1X lambda dilution buffer (0.1 M NaCl, 0.01 M $MgSO_4.H_20$, 0.35 M Tris-HCl, pH 7.5). A drop of chloroform was added followed by brief vortexing and incubation at 4°C overnight. Bacterial cell debris was removed by centrifugation at 8,000g for 2 min and the phage titre in the supernatant was determined as described in section 2.6.1.4. Based on the titre, enough phage was plated with *E. coli* strain K802 onto an LB agarose plate + 10 mM $MgSO_4$ and incubated overnight at 37°C so that confluency was obtained. High titre stock was increased by adding 10 mL of 1x lambda buffer to the plates and incubating overnight at 4°C. A few drops of chloroform was also added to the plates, swirled briefly, and the liquid collected into a sterile 50 mL polypropylene tube. To the plate lysate, 2 mL chloroform was added and vortexed for 2 min, and the supernatant was collected after centrifugation at 7,200 g for 10 min. This high-titre stock was used for liquid lysate preparation.

Liquid Lysate Preparation

Liquid lysate preparation commenced with the addition of 3 mL of high-titre stock to 1 L of K802 host cells grown in LB broth at 37°C overnight with shaking to an OD_{600} of 0.6. The cells, which were not lysed during overnight incubation, were lysed by the addition of 10 mL of chloroform with an additional 15 min of shaking at 37°C. Cell debris was removed by centrifugation (in a Beckman J2-21 at 8,500 rpm for 10 min). The lysate was incubated with DNAse I (1 μg/mL) and RNAse A (5 μg/mL) to remove cellular nucleic acids. Phage particles were extracted by adding 100% chloroform to the supernatant to a final concentration of 5% and centrifuged at 8,500 rpm for 10 min at 4°C. Phage particles in the extract were precipitated by adding an equal volume of 20% PEG/2.0 M NaCl on ice for 2 h, and followed by centrifugation at 8,500 rpm for 15 min. The resulted pellet was resuspended in 32 mL of 1X lambda dilution buffer. Phage DNA was released by the addition of 20 mM EDTA and 5% SDS, 50 μg/mL proteinase K, and incubation at 65°C for one hour and purifiation by phenol/chloroform extraction and ethanol precipitation at -20°C. The DNA was precipitated by centrifugation, and the pellet was washed with 70% ethanol, dried, and resuspended in 500 μL of water.

2.7.2.2 Small Scale Plasmid DNA Preparation

To isolate individual clones, 200 μL of release culture was streaked out onto a plate with LB medium containing ampicillin and incubated overnight at 37°C. Individual colonies were then further streaked to prepare plasmid mini-preparations (section 2.5.2) for restriction digestion (section 2.6.2.1), and subsequent sequencing (section 2.8.3).

2.8 DNA/RNA Analyses

2.8.1 Southern Blot Hybridization

2.8.1.1 Preparation of DNA Probes

Labelled DNA probe were produced from specific DNA fragments that were first separated on a 1% (w/v) low melt agarose gel in 1X TBE buffer, then extracted and purified using a Bresa-Clean™ DNA purification kit (Bresatec). The probes were radioactively labeled with ^{32}PdCTP by random-priming using *E. coli* DNA polymerase

1 (Klenow fragment) from the Megaprime™ DNA labelling system (Amersham™). Five µL DNA (5-25 ng) was denatured by heating for 5 min in a boiling water bath then cooled to room temperature. Denatured DNA was mixed with 5 µL primer and kept at room temperature for 1-2 min. Four µL of dATP, dGTP and dTTP (2.5 mM each), 5 µL of reaction buffer (10X concentrated buffer containing Tris-HCl pH 7.5, 2-mercaptoethanol and $MgCl_2$), 16 µL of sterile distilled water, 5 µL of ^{32}PdCTP and 2 µL Klenow enzyme were then added and mixed. The reaction was carried out at 37°C for 30 min. The synthesized probe was cleaned by separating the unincorporated nucleotides from the labeled DNA by spermine precipitation. To the labelled reaction, 1.2 µL of a 0.25 M solution of spermine was mixed gently by tapping and then kept on ice for 15 min or longer. The supernatant was removed after centrifugation for 15 min, and the pellet was resuspended by adding 100 µL of buffer comprised of 10 mM EDTA and 0.5% SDS.

2.8.1.2 Southern Blot Preparation and Hybridization

Rice genomic DNA from wild type and transgenic plants was isolated by a method adopted from a nucleic acid isolation kit (Puregene, Gentra systems Inc, Minneapolis). DNA (2 µg) was digested overnight with *Bgl*II, *Eco*RI, *Hind*III or *Sac*I and separated on a 0.8 % agarose gel in 1X TBE (Sambrook *et al.*, 1989). The gel was stained with ethidium bromide, visualized under UV light using a trans-illuminator, and photographed. The gel was then denatured in 0.25 M HCl (until the blue dyes turned yellow) and washed in distilled water for 5 min with gentle shaking. DNA fragments were capillary blotted onto Hybond-N+ membrane (Amersham™), using alkali transfer buffer (0.4M NaOH). The blot was pre-hybridized and hybridized according to Khandjian (1987) with ^{32}P-labeled probes prepared by random priming (Feinberg and Vogelstein, 1983). Membranes were then washed twice for 15 min in 2X SSC containing 1% SDS at ambient temperature, twice at 60°C for 30 min in 0.1X SSC containing 1%(w/v) SDS and twice at ambient temperature in 0.1X SSC, 0.1% (w/v) SDS. Radiolabeling was visualized by autoradiography using phosphor screens (Molecular Dynamics).

2.8.2 PCR Analysis

2.8.2.1 Transgenic Plants

Hygromycin resistance gene (*hph*) primers (Table 2.4) were used to detect the presence of the transgene in putative transgenic plants by the polymerase chain reaction (PCR). Primers specific to the endogenous rice sucrose synthase gene (*RSc1*) were used as controls (Wang, Boulter, and Gatehouse, 1992). A typical PCR reaction solution of 20 µL contained the following:-

Template DNA	~100 ng of Plant DNA or 1 ng of Plasmid DNA
Primers	1 µL each of a 10 pmol/µL stock
dNTPs	0.2 µL of a 10 mM stock
Taq polymerase	0.2 µL of a 5 units/µL stock
10X PCR buffer	2 µL (500 mM KCl, 100 mM Tris-HCl pH 9.0, 1.0% Triton® X_100)

Typical PCR cycle used was as follows:

No. of Cycles	Step	Temperature	Time
1	1	95°C	3 min
20	1	95°C	30 seconds
	2	56°C	30 seconds
	3	70°C	1 minute
10	1	95°C	30 seconds
	2	70°C	1 minute
1	1	70°C	5 min
1	1	20°C	3 minute

2.8.2.2 Expand^{Tm} Long Range PCR

A modified Expand^{Tm} Long Template PCR System (Boehringer Mannheim) was used to amplify the full length *OsAMT1;1* coding region (section 5.2.3.1) and promoter

region from clone pPIMP770 (Section 5.2.5). The following 33cycle program was used for cycle sequencing.

No. of Cycles	Step	Temperature	Time
1	1	94°C	2 min
30	1	94°C	10 seconds
	2	58°C	30 seconds
	3	68°C	4 minute
1	1	68°C	7 minute
1	1	4°C	3 minute

2.8.3 Sequencing

Forward and reverse internal primers (Table 2.4) or the two M13 universal primers, 17-mer forward primer (5' GTAAAACGACGGCCAGT 3') and the 24-mer reverse primer (5' AGCGGATAACAATTTCACAGGA 3'), were used for Big Dye Terminator DNA sequencing. Sequencing reactions were set up by mixing 2 μL (300-600 ng) of plasmid DNA, 1 μL (5 pmole) of primers 5 μL of Big Dye mix and 12 μL of sterile distilled water in a 250 μL PCR tube (Astral). The reaction mix was then loaded in the wells of PC- 960C Cooled thermal Cycle sequencer (Corbett, Australia). The following 27-cycle program was used for cycle sequencing:-

No. of Cycles	Step	Temperature	Time
1	1	96°C	1 min
25	1	96°C	20 seconds
	2	50°C	15 seconds
	3	60°C	4 minute
1	1	80°C	1 minute

At the end of the cycle, reactions were cleaned by ethanol/sodium acetate precipitation and processed by the PI CSIRO sequencing facilities. Sequence data were analyzed using the programs of the University of Wisconsin Genetics Computer Group (GCG) sequence analysis software (Devereux *et al.*, 1984).

2.8.4 Northern Blot Analysis

Twenty five micrograms of total RNA was separated on 1.5% w/v formaldehyde agarose gels and transferred to nylon membrane (Hybond N; Amersham) with 20x SSC, overnight using a standard capillary transfer protocol (Sambrook *et al.,* 1989). The membrane was washed in 2x SSC for 5 min and dried with filter paper blotting. The RNA was fixed to the membrane by ultraviolet cross-linking using the auto cross-link setting of UV Stratalinker™ (Stratagene). Pre-hybridization was performed (Promega protocol) for one hour at 65°C in 50% deionized formamide 0.25M $NaPO_4$ pH 7.2, 0.25M NaCl, 1mM EDTA and 7% SDS. The filter was then hybridized in the same buffer, overnight at 65°C, with α^{-32}PrUTP labeled *OsAMT1;1* Riboprobe prepared using Promega's Riboprobe™ *in vitro* transcription system. Filters were then washed twice in 2x SSC at 22°C for 5 min, twice in PSE buffer (0.25M sodium phosphate, pH 7.2, 2% SDS, 1mM EDTA) at 65°C for 20 min, followed by another two washes in PES buffer (0.04M sodium phosphate, pH 7.2, 1% SDS, 1mM EDTA) at 65°C for 20 min each. After a further two washes in 2x SSC at 22°C for 5 min each, membranes were treated with RNAse for 15 min in 2xSSC containing 2 µg/mL RNAse at 22°C. Finally, membranes were washed in 0.1% SDS and 0.1x SSC for 15 min at 22°C and then visualized by autoradiography using phosphor screens (Molecular Dynamics).

2.9 Physiological Analyses

2.9.1 Measurement of Cell Membrane Potential (Em)

Depolarization of the electrical potential difference across the plasma membrane of rice root cells was measured as described by Glass *et al.,* (1992). In short, the roots of rice plants were mounted in a Plexiglass chamber and secured in a horizontal position with silicon grease and small plastic blocks. Before measuring the electrical potential difference, plants were equilibrated for 30- 40 min in an ammonium free nutrient

solution that flowed through the chamber. Impalements with borosilicate glass microelectrodes (Clark Electromedical Instruments, Reading UK) were made in a region about one to two centimetre behind the root tip using a manually operated micromanipulator (Narishige, Japan). The electrodes were filled with 2M KCl and connected to an electrometer (FD 223, World Precision Instruments, Sarasota, FL USA) via an Ag-AgCl half-cell. The membrane potential was monitored continuously on a chart recorder (Fig. 2.1). To minimise the difficulties associated with variable unstirred layers the solution flow rate across the root surface was maintained at a constant rate during an experiment at approximately 0.5 cm s^{-1} (Walker *et al.,* 1979). Once a stable measurement of membrane potential was obtained in the NH_4^+ free solution (-90 to -200 mV), a similar solution containing one of four NH_4^+ concentration (5, 10, 20 and 100 μM of NH_4Cl) was flowed into the chamber. When the membrane potential had stabilized to a new value (usually 1-3 min) the NH_4^+-free solution was again flowed into the chamber. This was repeated until all four NH_4^+ concentrations were used at least twice. Data were collected from several plants and the Michaelis-Menten equation fitted using excel 5.0 software.

2.9.2 Ammonium Determination in Rice Roots

The rate of ammonium uptake was estimated by measuring the depletion of ammonium from the culture solution. Three-week-old single seedlings from different entries were transferred at time 0 in a small tube with 25 mL MJN solution containing 50 μM of NH_4^+ as the sole source of nitrogen. Tubes were provided with gentle air bubble to ensure uniform distribution of NH_4^+. After one hour the seedlings were removed from the solution and immediately root and shoot weight were recorded. Finally, ammonium was measured in the crude extract of roots and from nutrient solution using a colorimetric assay modified from Solorzano (1969). For ammonium determination in the roots of rice seedlings, total roots from individual seedlings were homogenized with a mortar and pestle using 2 mL 0.3 mM sulphuric acid (pH 3.5). The homogenate was centrifuged for 10 min at 5000 rpm. For the colour reaction 0.5 mL of clear supernatant was used. The assay is based on an indophenol reaction and required four stock solutions.

Solution A: 55 w/v phenol in water

Solution B: 0.5% w/v sodium nitroprusside.

Solution C: 5% w/v (= 1.5 M) sodium hypochlorite

Solution D: 20% w/v trisodium citrate with 1% w/v sodium hydroxide

Before use, a volume of solution A was added to B in the ratio of 2:1, and C was added to D in the ratio 1:4. To a 1 mL volume of unknown sample, 75 µL of A+B solution was added and mixed thoroughly and then 75 µL of C+D solution was added and mixed. The tubes were incubated at 37°C for 15-20 min and then absorbancy was read at 640 nm using the Labsystems Multiskan plus (Pathtech Diagnostics pt. Ltd.) vertical light path filter photometer.

Table 2.1 Bacterial strains used in this study

Strain	Characteristics	References
	E. coli	
NM522	*SupE*, Δ(*hsd*MS-*mcr*B)5, Δ(*lac-pro*AB), F'[*pro*AB, *lacI*^{q}, *lacZ*ΔM15]	Pharmacia Biotech
NP66	thr, leu, pro, thi, bio, cI^{857}int^{+}xis^{+}, ΔH1, *lacZ*ΔM15, Chloramphenicol^{R},/pJN13 [ϕ80 represor, pACYC184 replicon]/pXis [xis+, Streptomycin^{R}, spectinomycin^{R}, pSC101 replicon]	Pharmacia Biotech
JM109	F' *tra*Δ36 *laqlq* Δ(*LacZ*)M15 *proAB*/*recA*1 *endA*1 *gyrA*96 (Nalr) *thihsdR*17 (rK-mK+) *supE*44 *relA*1 Δ(lac-proAB)	(Yanisch-Perron *et al.*, 1985)
DH5α	*SupE*44 Δ*lacU*169(ϕ80*lacZ*ΔM15) *hsdR*17 *reca1 gyra* 196*thi*-1*rel*A1	(Hanahan, 1983)
	Agrobacterium	
AGL1	*A tumefaciens* AGL0 rec::*bla* pTiBo542ΔT Mop^{+} Cb^{R}	(Lazo *et al.*., 1991)

Table 2.2 Plasmid vectors used in this study

Plasmids	Features	Source/Reference
pUbi1cas	Kan^r, *Ubi1(I)* promoter, nopaline synthase terminator, a polylinker having sites for *Sac*I/*Sst*I, *Kpn*I, *Sma*I, *BamH*I.	(Li *et al.*, 1997)
pWBVec8	Spec^r, CaMV35S promoter, coding region of the hygromycin phosphotransferase gene (*hph*), nopaline synthase terminator, Left Border, Right Border, a polylinker having sites for *Xba*I, *Sph*I, *Aat*II, *Apa*I, *Xba*I, *Not*I, *Nsi*I. *Hind*III, *Eco*RI.	(Wang *et al.*, 1998)
pGEM®-7Zf$^+$(+/-)	Amp^r, *lacZ*, a I23 bp polylinker having sites for *Apa*I, *Aat*II, *Sph*I, *Xba*I, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, Csp451, *Cla*I, *Hind*III, *Bam*HI, *Sac*I, *Bst*XI, *Nsi*I.	Promega
pGEM®-T	Amp^r, *lacZ*, a 126 bp polylinker having sites for *Apa*I, *Aat*II, *Sph*I, *Bst*ZI, *Nco*I, *Sac*II, TT, *Spe*I, *Not*I, *Bst*ZI, *Pst*I, *Sal*I, *Nde*I, *Sac*I, *Bst*XI, *Nsi*I.	Promega
pK^{r}Bluescript SK(-)	Kan^r, *lacZ*, a 109 bp polylinker having sites for *Kpn*I, *Apa*I, *Dra*II, *Xho*I, *Sal*I, *Acc*I, *Hinc*II, *Cla*I, *Hind*III, *Eco*RV, *Eco*RI, *Pst*I, *Sma*I, *Bam*HI, *Spe*I, *Xba*I, *Not*I, *Eag*I, *Bst*XI, *Sac*II, *Sac*I,	Stratagene
pABCD	Amp^r, RBTV Promoter, *E. coli uidA* gene encoding β-glucuronidase, nopaline synthase gene terminator.	Claude Fauquet ILTAB

Table 2.3 Gene constructs used in this study

Plasmids	Coding region/Features	Bacterial Selection	Plant Selection	References
pPIMP56	*OsAMT1;1* sense in Plzubi1cas	Kan		This study
pPIMP58	*OsAMT1;1* antisense in pLZUbi1cas	Kan		This study
pPIMP145	*Ubi1(I)* P-*OsAMT1;1* antisense-*nos* in pWBVec8	Kan, Spec	hyg	This study
pPIMP161	*Ubi1(I)* P-*OsAMT1;1* sense-*nos* in pWBVec8	Kan, Spec	hyg	This study
pPIMP321	A3 genomic clone digested with *Sfi*1&*Kpn*1, 7.3 kb band eluded and digested with *Sac*1 cloned into *Sac*1 digested pGEM-7Zf(+). Contains 4.8 kb *OsAMT1;1* genomic clone.	Amp		This study
pPIMP567	PCR product of E2 λ clone with the AMT45^{+} forward primer and AMT2058^{-} reverse primer, cloned into pGEM-T vector. Contains 2.46 kb genomic *OsAMT1;1* ORF.	Amp		This study
pPIMP770	E2 genomic clone digested with *Sac*1, 12 kb fragment eluted and cloned into *Sac*1 digested K^{r} Bluescript SK(-). Contains 12 kb insert that included 2.46 kb genomic *OsAMT1;1* ORF.	Kan		This study
pPIMP827	Genomic clone J digested with *Sac*1 and eluded and cloned into *Sac*1 digested pK^{r}Bluescript SK(-). Contains 5 kb insert that is identical to clone pPIMP321 except it has an additional 150 bp 5' end sequence.	Kan		This study
pPIMP834	Genomic clone K digested with *Sac*1 and eluted and cloned into *Sac*1 digested pK^{r} Bluescript SK(-). Contains 6 kb insert that included 1.7 kb genomic *OsAMT1;3* ORF.	Kan		This study
pPIMP841	A promoter region of 2.38 kb from pPIMP321 in pABCD pABCD vector replacing the *Ubi1(I)* promoter	Amp		This study
pPIMP920	A PCR product from pPIMP770 (promoter region) amplified with SH74-668^{+} forward primer and AMT-90-*Nco*1 reverse primer replacing and cloned into pABCD vector replacing *Ubi1(I)* promoter	Amp		This study
pPIMP925	1.4 kb promoter region from pPIMP834 was cloned into pK^{r} Bluescript SK(-).	Kan		This study
pPIMP934	1.4 kb promoter region from pPIMP925 in pABCD vector replacing the *Ubi1(I)* promoter	Amp		This study

Table 2.4 List of Primers Used in This Study

Primer Name	Oligo-Nucleotides	Reference
AMT-44+	5' AACGAGGGATCGTAGAGAG 3'	This study
AMT-90-(*Nco*1)	5' CTTCCCATGGCTTCCTCCCCTCCCTCAC 3'	This study
AMT-275-	5' TGATGTTCATCGTGTTCTTGG 3'	This study
AMT-864+	5' CTGTCACCACCATCCTCAAGAC 3'	This study
AMT-1143-	5' TTGAGGCCGATGAGCACCCAC 3'	This study
AMT-1562+	5' TGCCTCCAACAGCAACAAC 3'	This study
AMT-1698+	5' ATGCTTTTGCCGCTCTCTC 3'	This study
AMT2058+	5' CCAGGAAATAAGCCAAAG 3'	This study
UBIPRO-106-	5' TTTTTTAGCCCTGCCTTCATAC 3'	This study
SH7-696+	5' ACGTTCCAGTGCCCCGTC 3'	This study
SH7-237-	5' ACGTGGACGAGGACGAAG 3'	This study
SH17-658+	5' CACGTTGGTGAGCATGATG 3'	This study
SH20-649+	5' AAGAACGAGCAGTTCACCTC 3'	This study
SH8-559+	5' AAAGCATTACAAAGCTTCAG 3'	This study
SH23-423+	5' ATGAGCGACTCAACTAGCC 3'	This study
SH79-597+	5' CGCATCCTGCAAGCTCATAGAC 3'	This study
SH77-651+	5' GAGCAACAGCAACCAGACAAC 3'	This study
SH71-414+	5' CGCCTTCATTTTTCGTGCTTGTG 3'	This study
SH74-668+	5' TCGAGCATTGACTAGTACTTTG 3'	This study
SH69-557+	5' TTTCCCCATATTTAATATCC 3'	This study
SH70-554+	5' TGGGCAACTATTCAAACTTC 3'	This study
SH47-538+	5' AACAGCGCCGTGAAGATGAC 3'	This study
SH43-274+	5' ATAGAAGATAACCGTTGAG 3'	This study
SH47-116-	5' ATGCTCAAGTCGGCGCAC 3'	This study
SH57-528+	5' AAGTCGATGACGCCGGAC 3'	This study
SH62-516+	5' TATCCGCGCACGTTGCCATC 3'	This study
Universal F	5' GTAAAACGACGGCCAGT 3'	(Sambrook *et al.*, 1989)
Universal R	5' AGCGGATAACAATTTCACAGGA 3'	(Sambrook *et al.*, 1989)
Hph5-5	5' AAAAGCCTGAACTCACCGC 3'	(Upadhyaya *et al.*, 1998)
Hph3-515	5' TCG TCC ATG ACA GTT TCG C 3'	(Upadhyaya *et al.*, 1998)
SSIC-R	5' AAGCAAGTGGATGGTGTCAAG 3'	(Wang *et al.*, 1992)
SSI- F	5' TGCCTTGATCGAAGCTGAC 3'	(Wang *et al.*, 1992)

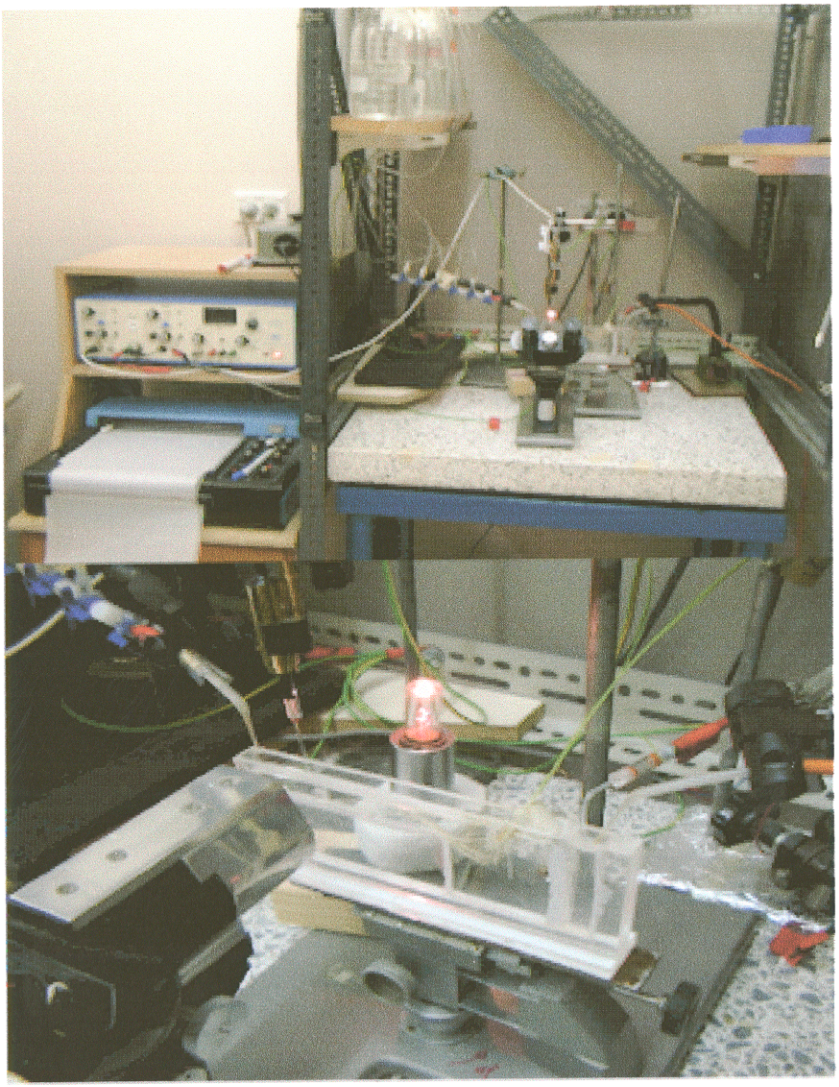

Figure 2.1 Experimental setup to measure rice root E_m.

CHAPTER 3

MOLECULAR BIOLOGY OF *OsAMT1;1* IN RICE

3.1 Introduction

Plants obtain their nitrogen primarily as nitrate and ammonium from the soil but can also utilize gaseous ammonia. Nitrate and ammonium are derived mainly by mineralisation of soil organic matter. The supply of ammonium and nitrate ions varies with environment, soil type, fertilizer practices and cropping practices. Well-aerated soils are rich in NO_3^- and poor in NH_4^+ (Kronzucker *et al.*, 1995) whereas in anaerobic soil, such as irrigated or rain-fed low land rice systems, NH_4^+ is the prevalent source of nitrogen (Sasakawa and Yamamoto, 1978; Smith and Waker, 1978). Ammonium is not only the predominant and most available N form in rice soil (Yu, 1985) but also the preferred nitrogen source for rice (Dijkshoorn and Ismunadji, 1972; Fried *et al.*, 1965; Sasakawa and Yamamoto, 1978; Yoneyama and Kumazawa, 1974). Nearly 70% of the world's rice is produced in rainfed or irrigated lowland systems in Asia (IRRI, 1997), where nitrogen fertilizer is the only limiting factor for yield. Most of this nitrogen is applied in the form of urea, which is converted to ammonium before being taken up by plants.

In plants ammonium influx into roots is mediated by two discrete transport systems (Fried *et al.*, 1965; IRRI, 1997; Ullrich *et al.*, 1984; Wang *et al.*, 1993). The first is an energy dependent system that operates at low external $[NH_4^+]_O$, exhibits saturation kinetics, and leads to depolarization of the plasma membrane electrical potential (Ayling, 1993; Ullrich *et al.*, 1984; Walker *et al.*, 1979; Wang *et al.*, 1993). The second system operates at high external $[NH_4^+]_O$ and exhibits non-saturation kinetics (Fried *et al.*, 1965; Ullrich *et al.*, 1984; Wang *et al.*, 1993). The first high affinity ammonium transporter identified in plants was *AtAMT1;1* from *Arabidopsis* (Ninnemann *et al.*, 1994) which was isolated by complementation of a yeast mutant deficient in ammonium uptake (Dubois and Grenson, 1979; Marini *et al.*, 1994). Since then ammonium transporters from different crop plants have been isolated and characterized.

This project was initiated in late 1996. A rice EST (GeneBank Accession D39189) with 63% identity with *AtAMT1* (Ninnemann *et al.*, 1994) was available in the GenBank database (Nov 11 1994). This putative rice ammonium transporter EST clone was obtained from MAFF DNA Bank, NIAR, Japan. At the time of commencement of this project little was known about the number, regulation, and

physiological role of *AMT*'s in plants. In this chapter results from Southern blot hybridization of rice genomic DNA and Northern blot hybridization of RNA transcripts from rice plants grown under different nitrogen regime with the above mentioned rice EST (putative *OsAMT1*) probe are presented. These experiments were aimed at understanding the genetic complexity of *AMT* genes, their expression and regulation in rice. Attempts on cDNA cloning of *AMT1* genes are also described.

3.2 Results

3.1.1 Genetic Complexity of Ammonium Transporters in Rice

To gain an insight into the genetic complexity of ammonium transporters in rice with respect to gene family and copy number, Southern blot analysis was performed. Total genomic DNA from rice cultivars Taipei 309 and Jarrah, digested with selected restriction enzymes, were blotted and probed with a 604 bp 3' end fragment of the EST clone (Fig. 3.1) and the full-length EST clone (Fig 3.2). Hybridization with the 604 bp probe revealed one major band with each of the RE digests (~10 kb, 3kb and 2.8kb for *Bgl*II, *Sma*I and *Sac*II, respectively) and at least two other faint bands. The size of the bands is similar for both cultivars. Hybridization of *Eco*RI, *Hin*dIII and *Sac*I digested Taipei 309 genomic DNA with the full-length EST probe showed several bands.

3.1.2 Expression of Ammonium Transporter/s in Rice

Northern blot analysis was performed using the above mentioned putative rice ammonium transporter to study the expression patterns in different plant tissues. This putative ammonium transporter was found to express in mature leaves and in roots but not in young and newly emerging leaves (Fig. 3.3). The expression appeared to be higher in roots than in mature leaves.

3.1.3 Regulation of Ammonium Transporter/s in Rice Roots

Expression of the putative ammonium transporter/s was also studied under different nitrogen regime by Northern blot analysis. No substantial changes in expression were observed in nitrogen deprived (0 to 142 h) seedlings over a 7 d period (0 to 142 h) (Fig. 3.4). No substantial changes in expression patterns were observed in rice roots of 6 d and 11 d old seedlings grown under different nitrogen conditions (Fig.3.5).

3.1.4 Isolation Putative *OsAMTs* from cv. Taipei 309

In order to isolate possible homologues/paralogues or members of related ammonium transporters a cDNA library was constructed using poly-A RNA isolated from roots of ammonium starved rice seedlings (see section 2.6). The estimated number of plaque forming units (pfu) in the un-amplified library was 2.5 x 10^5 plaque/ml. Approximately 1.25 x 10^5 plaques were screened using the full length EST (isolated previously) as the probe under low stringent conditions. Three plaques with positive signals were obtained and named F1, G1 and J1. The positive plaques containing cDNA inserts (*Eco*RI fragments) in pExCell were released from the λpExCell vector following manufacturer's instructions and designated pPIMIN1, pPIMIN2 and pPIMIN3. Initial *Eco*RI restriction digestion revealed that the two clones pPIMP1 and pPIMP2 were similar with 760 bp inserts and that pPIMP3 was smaller with a 500 bp insert. Sequences of these clones revealed 99% identity with the published rice *OsAMT1;1* (Von Wiren *et al.*, 1997). All three clones were shorter than the original EST. The 5' end of clones pPIMP1 and pPIMP2 coincided with position 1198 of the full-length EST clone. The smaller clone pPIMP3 extended from nt 1637 and stretched beyond 3' end of the original EST clone by 138 nt (Fig 3.6).

3.3 Discussions

A multiple banding pattern observed with a low stringency hybridization of appropriately digested Taipei 309 genomic DNA with the full-length EST cDNA suggests that this gene belong to a multi-gene family. The variation in the intensities of different hybridizing bands suggest that there are different classes of ammonium transporters in rice or that other genes share considerable homology with the putative ammonium transporter used as probe. When the 3' region of the putative *OsAMT1* was used as probe against different digests of Taipei 309 and Jarrah genomic DNA, one major hybridizing band and 2-3 faint bands were observed. The strong hybridising band is most probably the *OsAMT1;1* paralogue. The faint hybridizing bands probably correspond to other members of the *OsAMT1;1*.

Northern blot analyses indicated that this gene is expressed in both roots and matures leaves under different nitrogen treatments with a higher expression level in roots than that in other parts of the plant. Similar expression patterns have been reported in *Arabidopsis* (Gazzarrini *et al.*, 1999; Ninnemann *et al.*, 1994) and tomato

(Lauter *et al.*, 1996). The expression of *AMT;1* in roots suggests its role in root ammonium uptake. The evidence for this comes from the fact that the *AMT1;1* gene from tomato and *Arabidopsis* show high affinity uptake in yeast mutants defective in NH_4^+ transport (Lauter *et al.*, 1996; Ninnemann *et al.*, 1994). Moreover, the *AtAMT1;1* mRNA in roots increase rapidly during nitrogen starvation, and transcript levels decrease rapidly in response to high nitrogen supply (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999). Unlike *AtAMT1;1*, *OsAMT1;1* did not show higher expression with the ammonium starvation treatments as its expression was constitutive under all nitrogen conditions and growth stages tested. The constitutive expression of the ammonium transporter was also reported in tomato (Lauter *et al.*, 1996), and with *AtAMT1;2* in *Arabidopsis* (Gazzarrini *et al.*, 1999) suggesting that this class of ammonium transporters may have a general role in uptake of NH_4^+. Although cereal plants grown in absence of nitrogen displayed increased ammonium uptake rate upon re-exposure to ammonium (Gazzarrini *et al.*, 1999; Lee and Rudge, 1986; Morgan and Jackson, 1988). This did not reflect in the activity of this particular class of ammonium transporters. One explanation could be alteration of ammonium influx and efflux (Morgan and Jackson, 1988). Other possibility is that there are several classes of ammonium transporters in operation. Some of which could be responsive to external ammonium concentrations as in the case of *Chlamydomonas* (green alga) where one ammonium transporter is constitutively expressed while the other repressed by NH_4^+ (Franco *et al.*, 1988). The data presented in this study indicates that the EST used in this study is constitutively expressed.

The physiological role of ammonium transporters in leaves could be for NH_4^+ accumulation from the vascular system across the mesophyll plasma membrane. Ammonium concentration in the xylem quite often can rise to mM levels (Cramer and Lewis, 1993) which need to be transported away from the xylem. Ammonium transporters in mesophyll cells could also be involved in the recovery of photorespiratory NH_3/NH_4^+. Photorespiratory NH_3 is likely to be re-protonated during passage to the cytosol or when released to the leaf apoplast and hence may require re-import by an ammonium transporter. It will be of particular interest to determine the cellular localization and regulation under different photorespiratory conditions.

Screening of a rice cDNA library of the cultivar Taipei 309 with the same EST probe yielded three positive clones. Sequencing of these clones showed near 100%

identity with the putative *OsAMT1;1*(Accession AF 001505, dated 29/04/1997) indicating that they are paralogues. Instead of re-isolation of *OsAMT1;1* from Taipei 309 cDNA library, the published *OsAMT1;1* was therefore used in this study. A further attempt to isolate cDNA corresponding to other possible ammonium transporters (faintly hybridizing bands in the Southern blot) was not successful. However, success in isolation of genomic clones of other ammonium transporters is described in chapter 5.

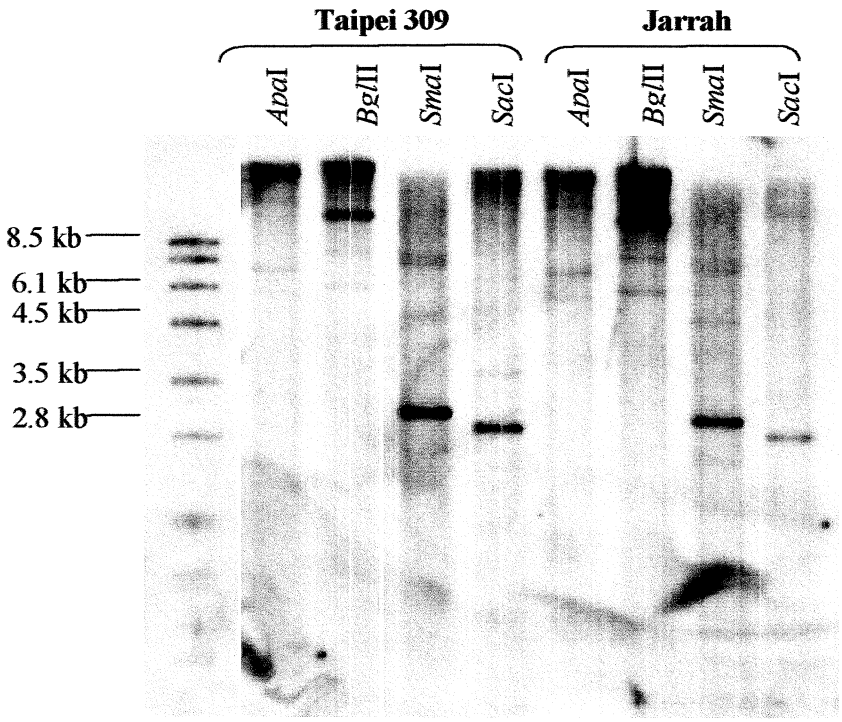

Figure 3.1 Southern blot showing *OsAMT1* copy number in rice cvv. Taipei 309 (lane 2 to 5) and Jarrah (lane 6 to 9). Genomic DNA (2 µg) from each cultivar was digested with *Apa*I, *Bgl*II, *Sma*I and *Sac*II and probed with 604 bp 3' end fragment of EST (Accession D39189). Lane 1 is the molecular marker.

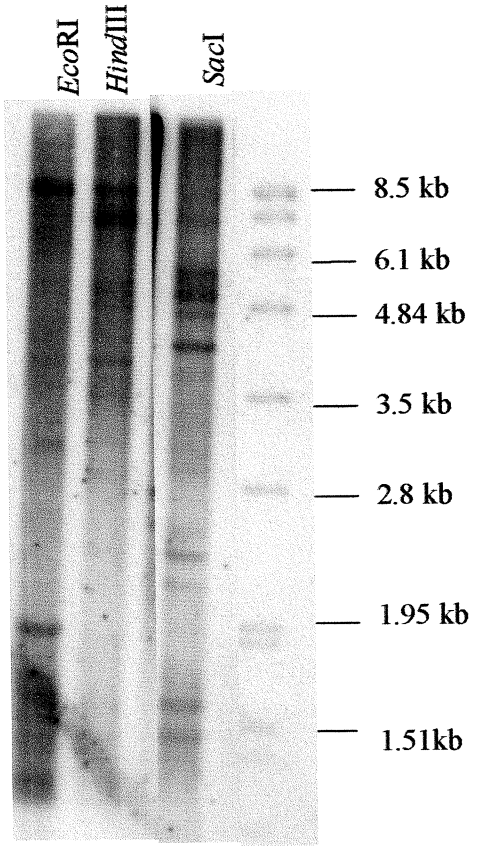

Figure 3.2 Southern blot showing *OsAMT*1 copy number in rice cv. Taipei 309. Genomic DNA (2 µg) was digested with *EcoR*I (lane 1), *Hind*III (lane 2) and *Sac*I (lane 3) and probed with full length of EST (Accession D39189). Lane 4 is the molecular marker.

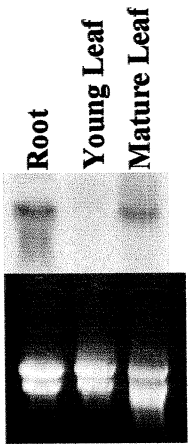

Figure 3.3 Northern blot showing expression of *OsAMT1;1* in different organs of rice. **(A)** Northern blot (upper panel) probed with rice EST (GeneBank Accession No. D39189) and ethidium bromide staining (lower panel) of electrophoretically separated total RNA from three week rice roots (lane 1), young leaves from inside leaf sheath (lane 2), mature leaves (lane 3) of plants grown in 2 mM NO_3^- and 1 mM NH_4^+. The blot was autoradiographed for 16 h using phosphor screens (Molecular Dynamics).

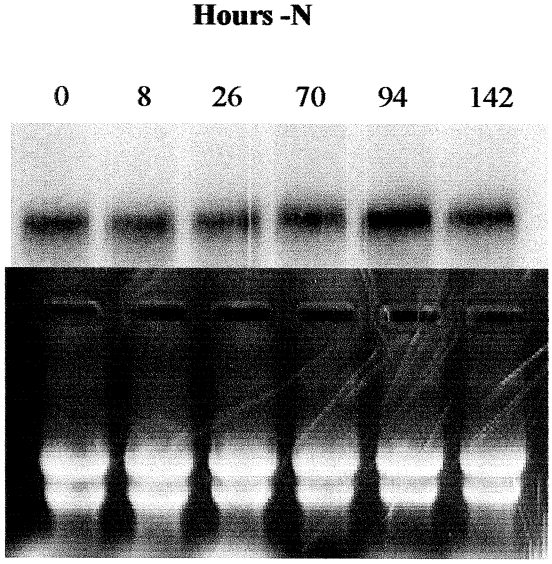

Figure 3.4 Northern blot showing the effect of nitrogen starvation on *OsAMT1;1* expression in rice. Northern blot (upper panel) probed with rice EST (Accession D39189) and ethidium bromide staining (lower panel) of electrophoretically separated total RNA from three week rice roots from plants grown in 2 mM NO_3^- and 1 mM NH_4^+ (lane 1), similarly treated plants but deprived of N for 8 h (lane 2), 26 h (lane 3), 70 h (lane 4), 94 h (lane 5), and 142 h (6); The blot was autoradiographed for 16 h using phosphor screens (Molecular Dynamics).

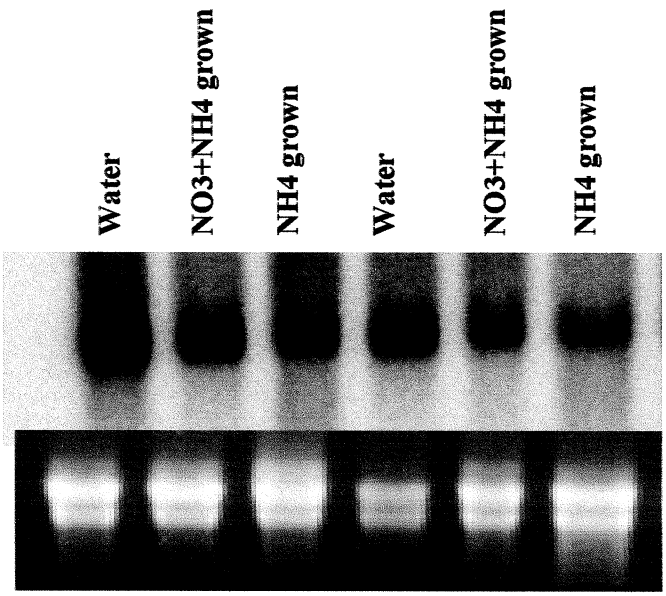

Figure 3.5 Northern blot showing the effect of different nitrogen sources and age of seedlings on *OsAMT1;1* expression in rice. Northern blot (upper panel) probed with rice EST (Accession D39189) and ethidium bromide staining (lower panel) of electrophoretically separated total RNA from from roots of 6 d old seedlings geminated in water (lanel), 1 mM NO_3^- + 0.5 mM NH_4^+ (lane 2) or 0.5 mM NH_4^+ (lane 3), and of 11 d old seedlings geminated in water (lane 4), 1 mMNO_3^- + 0.5 mM NH_4^+ (lane 5) or 0.5 mM NH_4^+ (lane 6). The blot was autoradiographed for 16 hr using phosphor screens (Molecular Dynamics).

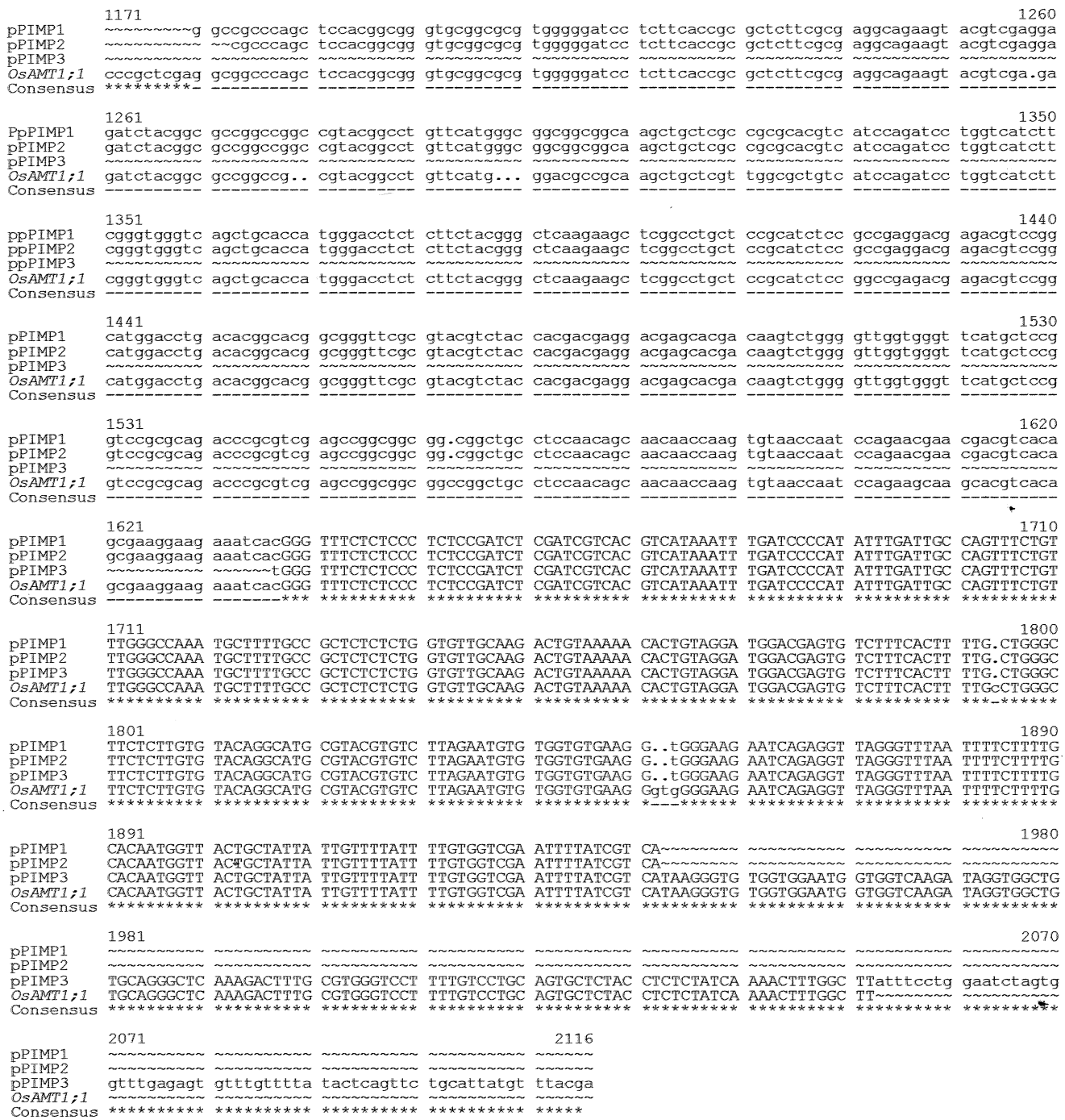

Figure 3.6 Sequence alignment of *OsAMT1;1* clones from Taipei 309 and the original *OsAMT1;1* clone from Nipponbare.

CHAPTER 4

MOLECULAR AND PHYSIOLOGICAL CHARACTERIZATION OF RICE LINES THAT OVER-EXPRESS *OsAMT1;1*

4.1 Introduction

Around 70% of the worlds rice is cultivated in irrigated lowland system in Asia (IRRI, 1997) where nitrogen is the main limiting factor for yield. Large amounts of fertilizer nitrogen are therefore, used in this system, mainly in the form of urea which is converted to ammonium before uptake by the plant. Less than 50% of applied fertilizer is utilized by rice plants the rest being lost into the environment (Hauck *et al.*, 1997). Effective use of N is required not only to increase the rice yield but also to protect the environment. Following fertilization with urea, waterlogged soils rapidly hydrolyse urea to NH_4^+, consequently ammonium concentrations in the root rhizosphere rise transiently before falling again. In one laboratory experiment it has been found that 460 mg urea placed in 100 cm^3 of waterlogged soil was completely hydrolyzed within 3 days (Craswell and Vlek, 1979). This period represents a window of opportunity for rice plants to absorb NH_4^+. This window can be widened by the use of slow release fertilizers such as sulphur-coated urea (Oertli, 1980) and shellac-coated urea (Chauhan and Mishra, 1989), or by repeated fertilizer applications. However, plants have to compete with other physical, chemical and biological factors for this transient ammonium. Soil microbes are effective scavengers of mineral N, including ammonium. Physicochemical factors such as conversion of NH_4^+ to gaseous NH_3 and subsequent loss to the atmosphere together with movement of dissolved ammonium away from the root zone probably result in more significant ammonium losses. Losses of ammonium through ammonia volatilization from fertilized flooded soils could be up to 8% to 19% (Chauhan and Mishra, 1989; Ventura and Yoshida, 1977). In intermittently flooded soils loss of added ammonium due to nitrification and denitrification processes was up to a maximum of 63% (Reddy and Patrick Jr, 1975). Losses of applied N through leaching was from 3.4 to 25.5% (Koshino, 1975). The share of soil ammonium going to plants, or the N-use efficiency, could, in principal be increased by increasing the rate at which ammonium is taken up by roots. Depending on whether ammonium transport or ammonium metabolism is rate limiting, uptake could be enhanced by increasing one or the other of these processes.

The hypothesis that *OsAMT1;1* is involved in high affinity ammonium uptake into roots is based on its expression pattern (see chapter 3) and on the biochemistry of the possible orthologue *AtAMT1;1* from *Arabidopsis* (Ninnemann *et al.*, 1994).

AtAMT1;1 expressed in yeast had a K_m for ammonium around 1 μM (Ninnemann *et al.*, 1994). This estimate has been reduced since then, and affinities in the μM range have also been demonstrated for other *AMT1* family members (Gazzarrini *et al.*, 1999). To test the hypothesis that ammonium transport rather than metabolism limits ammonium assimilation under different physiological conditions, the alteration of ammonium transport in rice was attempted by using a transgenic approach. Antisense inhibition of *OsAMT1;1* gene expression would provide information about the normal physiological role of *OsAMT1;1*, as well as an indication of whether the transporter is rate-limiting for ammonium assimilation under certain conditions. Over-expression of the same gene in rice also could indicate whether the transporter is rate limiting for ammonium assimilation and whether it is possible to increase net ammonium flux into plants simply by increasing transport into roots. If these were true, the over-expression of ammonium transporters in rice and other crops could be a rational way to improve N-use efficiency, and reduce fertilizer use and loss to the environment.

This chapter describes the results of experiments to alter *OsAMT1;1* gene expression in rice cultivars Taipei 309 and Jarrah. The molecular and physiological characterization of several *OsAMT1;1* over-expressing lines are described.

4.2 Results

4.2.1 Gene Construction

A *Mlu*I-*Not*I fragment from the clone R2952 containing the *OsAMT1;1* cDNA was end filled using DNA polymerase (Klenow fragment) and inserted at the *Sma*I site of the plant expression vector pLZUbi1cas, downstream of the maize *ubiquitin* (*Ubi1(I)*) promoter (Christensen and Quail, 1996). The orientation of insertion was determined by the size of the fragments liberated following *Bam*HI digestion. Recognition sites for this restriction enzyme are adjacent to the *Sma*I site and 1214 nt from the original *Mlu*I site in *OsAMT1;1* (Fig. 4.1, A & B). In the case of the sense construct (pPIMP56) *Bam*HI digestion produced a 1214 bp fragment, and in case of antisense construct (pPIMP58) it produced an 830 bp fragment (Fig 4.1, C). The sense and the antisense cassettes from pPIMP56 and pPIMP58 were recovered as *Eco*RI(end filled)/*Hind*III fragments and then cloned into a *Xba*I(end filled)/*Hind*III digested binary vector pWBVec8 to produce binary vector constructs pPIMP161 and

pPIMP145, respectively (Fig. 4.1, D & E). The binary vector pWBVec8 contains a cauliflower mosaic virus 35S (CaMV35S) promoter driven, intron-interrupted (catalase intron from castor bean) hygromycin resistance gene (*hph*) as the selectable marker (Wang *et al.*, 1998).

4.2.2 Rice Transformation

Calli derived from mature embryos of rice cultivars Taipei 309 and Jarrah (detailed in section 2.2.1) served as the target material for *Agrobacterium*-mediated transformation. Co-cultivation of *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991) containing pPIMP161 or pPIMP145 (Fig. 4.2, A) with this target material for two days, followed by thorough washing and culture in the first selection medium (NB medium containing 30 mg/l hygromycin and 150 mg/l Timentin) for 3-4 weeks produced hygromycin resistant outgrowths. Suppression of bacterial overgrowth was adequate with the level of Timentin used. Resistant outgrowths were excised and cultured on a second selection medium (NB medium with 50 mg/l hygromycin and 150 mg/l Timentin) for two weeks, healthy looking hygromycin resistant calli were obtained with variable frequencies (Fig. 4.2, B). Healthy calli were taken through pre-regeneration (Fig. 4.2, C) and regeneration (Fig 4.2, D & E) steps resulting in the production of putative transgenic lines. A number of resistant callus lines, regenerated lines and fertile plants were produced with sense and antisense constructs and are summarised in Tables 4.1 and 4.2. T2 transgenic lines containing *OsAMT1;1* transgenes are summarised in Table 4.3. Transgenic lines containing the *OsAMT1;1* antisense gene showed a higher degree of sterility than those containing the *OsAMT1;1* sense transgene. Transgenic lines containing sense *OsAMT1;1* transgene only were obtained for cv. Jarrah.

4.2.3 Transgene Analyses

4.2.3.1 Preliminary PCR analyses

Genomic DNA isolated from each T_0 plant was analyzed using the polymerase chain reaction (PCR) with *hph* gene specific primers and rice sucrose synthase *SSc1* specific primers (as internal control) (Table 2.4 in chapter 2). The results are presented in Figure 4.3 showing a 720 bp *hph* gene specific product and a 1016 bp *SSc1* specific product.

4.2.3.2 Northern Blot Analysis

T_1 seeds from 12 Taipei 309 transgenic lines (Line 27, 28, 31, 35, 37, 38, 40, 41, 42 46, 47 and 51; Table 4.2) and 5 Jarrah transgenic lines (Line 71, 74, 75, 76 and 77; Table 4.2) containing *OsAMT1;1* in the sense orientation and from Taipei 309 transgenic lines (Line 1, 3, 4, 5 and 11; Table 4.2) with antisense *OsAMT1;1* construct were germinated in 1/2 strength MS medium with 75 mg/l hygromycin. Hygromycin resistant seedlings (Fig. 4.4) were grown hydroponically in MJN solution (Epstein, 1972) with 4.0 mM NO_3^- and 1.0 mM of NH_4^+ for three weeks. The seedlings were then transferred to fresh nutrient solution devoid of nitrogen and grown for three days. Total RNA was extracted from the roots for Northern blot analysis, and was probed using *OsAMT1;1* cDNA as a template. Ten out of 12 Taipei 309 transgenic lines and all 5 Jarrah transgenic lines showed higher expression of *OsAMT1;1* compared to wild type plants (Figure 4.5, A & C). The transcript levels in lines 46, 38, 41, 47, 74 & 75 were at least 5 to 6 times higher than that in the wild type plants. Transgenic plants containing antisense constructs had *OsAMT1;1* expression levels similar to that of wild type plants (Fig. 4. 5, B). Three over-expressing lines (46, 38, 40), one line (line 35) with an *AMT1;1* expression level similar to that of the control plant Taipei 309, and all five lines from Jarrah were used for further analyses.

4.2.3.3 Southern Blot Analyses

Total genomic DNA isolated from Taipei 309 transgenic lines 35, 40, 38 and 46 and Jarrah transgenic lines 71, 74, 75, 76, and 77 were digested with *Bgl*II (for which there is only one recognition site in the gene construct), blotted onto Hybond N^+ nylon filter (Amersham^{TM}), and hybridized with a radioactively-labelled *hph* gene probe (Fig. 4. 6, C & D). The highest expressing line, 46 had 6 copies while line 38 had 3 copies. Lines 40 and 35 had a single copy of the transgene. A similar pattern was also observed in case of T_0 over-expressing lines of Jarrah where expression levels positively correlated with copy number (Fig. 4.7 C & D).

4.2.4 Physiological Analyses

The physiological impact of altering *OsAMT1;1* expression in transgenic rice was assessed in several ways. Firstly, ammonium dependent depolarization of plasma membrane electrical potential difference (E_m) in root cells was measured. Secondly, net ammonium influxes into roots of transgenic and wild type plants were measured.

Thirdly, ammonium concentrations in plant roots grown under different conditions were measured. Finally, growth analyses of plants were performed.

4.2.4.1 Depolarization of Cell Membrane Electrical Potential of Rice (E_m):

Depolarization of the plasma membrane electrical potential (E_m) of root cells was measured in two experiments. In the first experiment, 21-27 day old T_1 plants of transgenic line 46 (highest over-expresser) and the wild-type Taipei 309 plants (as control) were grown in MJN solution containing 2mM NO_3^- + 1mM NH_4^+. In the second experiment, homozygous T_2 plants from transgenic line 40 (40-1) were grown in MJN solution containing 1mM NH_4^+, along with control plants. Ammonium fed plants always had a greater (negative) membrane potential (E_m) compared to ammonium starved wild type plants as well as transgenic line 46 when roots were assayed in –N solution (Table 4.4). In contrast, E_m values were more or less similar in ammonium fed and 7-day nitrogen starved plants as in T2 line 40-1 (Table 4.4).

Ammonium transport into transgenic and wild type rice roots grown under different nitrogen conditions were measured indirectly by monitoring ΔE_m (or membrane depolarization) of root cells following ammonium addition. Results of a typical electrophysiology experiment are shown in Figure 4.8 and Figure 4.9. It is interesting to note that increasing concentrations of NH_4^+ led to greater depolarization. Estimated half-saturation values (K_m) for net depolarization were more or less similar in wild type and transgenic plants (Table 4.5), except in the ammonium fed plants in experiment 1. In contrast, the maximum depolarization (V_{max}) values were higher in transgenic plants in comparison to wild type plants in all growth conditions, except in the case of ammonium fed plants of T2 line 40-1 (Table 4.4).

4.2.4.2 Effect of High NH_4^+ Accumulations in Transgenic Rice Plants.

Net ammonium uptake rates were determined in three separate experiments. The ammonium uptake rate was determined as the amount of ammonium removed from the nutrient solution per unit time, expressed as μmol g^{-1} fresh root wt. h^{-1}. Associated phenotypes of wild type and transgenic plants from these experiments were observed.

Experiment 1

In the first experiment, four T_1 transgenic lines 35, 40, 38 and 46 (lowest to highest *OsAMT1;1* expression) and Taipei 309 control plants were used. Seedlings were 16 d,

17 d and 20 d old for ammonium fed, one day N starved and two day N starved, respectively. For ammonium uptake assays, five seedlings from each line were transferred at zero time to a tube containing 75 mL MJN solution with 75 μM of NH_4^+ as the sole nitrogen source. A gentle stream of air was bubbled through the solution to ensure uniform distribution of NH_4^+ and sufficient oxygen during the experiment. Sample solutions (1 ml) were taken after 10 min, 30 min, 1 h and 3 h. After three hours, plants were harvested and whole plant weight, root and shoot weights were recorded. Total roots from individual seedlings were homogenized in 0.3 mM sulphuric acid prior to measuring the ammonium content.

Plant phenotypes from lines 46 and 38 (containing multi-copy transgene) were highly variable in comparison to wild type plants. Plants from line 46 segregated into several plant types, such as normal height compared to wild type (Fig. 4.10, D, E & F), dwarf (Figure 4.2.10, C), and dwarf-lethal (Figure 4.10, B). Plants from line 40 (containing a single copy transgene) were all similar to wild type (Fig. 4.10, G-I). Due to low survival rate and high variability, no plant from line 46 was subjected to the two-day nitrogen starvation treatment. For the same reason, plants (of line 46) used in nitrogen fed and one-day nitrogen starvation treatments were not of uniform size. Fresh weights (whole plant, shoot and root), and root ammonium contents from ammonium fed 16 d old seedlings (A & D), one day N starved 17 d old seedlings (B & E), and two day N starved 20 d old seedlings (C&F) are presented in Figure 4.11. The biomass of transgenic lines (except line 35) was lower than that of wild type plants in all treatments. The reduction in biomass in transgenic plants (except line 35) was correlated with the copy number of the *OsAMT1;1* transgene. Root ammonium content on a root fresh weight basis, measured after three hours of uptake experiments in 75 μM of ammonium, were higher in transgenic lines 38 & 46 compared to wild type plants in all treatments (Fig. 4.11, D, E & F). Root ammonium content in wild type as well as in transgenic plants was higher in one day starved plants compared to that in nitrogen fed and two-day nitrogen starved plants.

For the ammonium fed plants, only the biomass and root ammonium content was recorded. Measurement of ammonium uptake was not possible because ammonium content of the nutrient solution at the early stage of uptake (10 and 30 min.) was much higher than the initial ammonium content (presumably because of ammonium efflux from these plants). No ammonium efflux was evident in 1 d and 2

d starved plants. Figure (4.12, A) shows the NH_4^+ uptake rates of plants deprived of N for 1 day. Uptake rates were significantly higher in two transgenic lines (40 and 38) than in control plants. Uptake of ammonium by line 35 was not significantly different to that of the wild type at any time point. Initial ammonium uptake rate in transgenic lines (40 & 38) was approximately twice that in wild type plants during the first 10 minutes. The rate of ammonium uptake decreased with time, although line 40 & 38 still took up more ammonium, on a fresh weight basis, than wild-type plants during the first 30 minutes. A similar trend was observed in 2 d N starved plants (Fig. 4.12, B). This higher NH_4^+ uptake rate correlated with the *OsAMT1;1* mRNA expression level (Fig 4. 12, C).

Experiment 2

Transgenic (T_1) plants that are homozygous for the hygromycin resistant gene would be expected to show no segregation for hygromycin resistance in the T_2 generation. In the second experiment T_2 plants from two such homozygous lines, 40-1, 77-1 and corresponding wild type Taipei 309 and Jarrah, respectively were used. Seedling age was 21 d for all three treatments. The first set of plants were grown in MJS containing $NH_4^+ + NO_3^-$ as the nitrogen sources. The second set was the same as the first, except that for last seven days plants were grown in MJS solution with NO_3^- as only nitrogen source. The third set of plants was the same as the first, except during the last seven days plants were grown without any nitrogen. Plant tissues were harvested from each treatment for Northern blot analysis and other measurements, as described in the last section for experiment 1.

Biomass (Fig. 4.13 A-C) and root ammonium contents (Fig 4.13 D-F) of all plants in each treatment were measured after the three hours exposure to NH_4^+. When plants were grown in adequate nitrogen conditions (2.0 mM NO_3^- and 1.0 mM NH_4^+) shoot and root weights were less in T_2 transgenic plants (Fig. 4.14, A) than in wild type controls. Fresh weights were 55%, 52% and 60% for whole plant, shoot and root fresh weights, respectively in line 40-1 compared to the wild type Taipei 309. In line 77-1 the reductions were 21% and 25% for shoot and root fresh weight, respectively compared to Jarrah. A similar result was observed in nitrate-fed plants (Fig 4.13 C).

The seven days of nitrogen starvation resulted in reduced biomass (mainly due to reduction in shoot weight) in the wild type as well as in the T_2 transgenic plants compared to nitrogen-fed plants. Wild-type plants deprived of N for 7 d exhibited reduction of 43% and 14% in shoot and root weight respectively, compared to N fed plants. For plants from line 40-1, shoot weights were reduced by 21% and root fresh weight increased by 3% compared to that of N-fed plants (Fig. 4.13, A & B). The same trend was observed in wild-type Jarrah plants, but with transgenic Jarrah (line 77-1), shoot weight as well as root weight decreased compared to N-fed plants (Fig. 4.13, A & B).

Transgenic plants from line 40-1 had significantly higher root NH^+_4 content than wild type Taipei 309 in all treatments. The same trend was observed (although not significantly different) with Jarrah transgenic line 77-1. Ammonium uptake rate was measured only in 7 d N starved plants during a 3 h uptake experiment. Uptakes were 35% and 27% higher in line 40-1 and line 77-1 compared to their respective wild type controls (Fig. 4. 14).

Experiment 3

A third experiment was conducted to assess the impact of high and low ammonium treatments in transgenic plants over-expressing *OsAMT1;1*. Three T_2 homozygous transgenic lines 40-1 (Taipei 309 background), 71-1 (Jarrah background), 77-1 (Jarrah background) and respective wild type plants were used in this experiment. Seeds were germinated in water and then grown for two weeks without nitrogen. After two weeks, one set of plants was supplied with MJS solution containing 10 μM of NH_4^+ (low ammonium) and the other set was provided with the same solution containing 2 mM of NH_4^+ (high ammonium). When the seedling age was 6 weeks observations were made on fresh weights, leaf numbers, leaf area and root ammonium content from 10 representative plants of each line and from both treatments.

Results of the two previous experiments indicated that reduction in root and shoot weights of transgenic plants could be due to increased ammonium accumulation. This experiment was designed to confirm these observations. When plants were grown in a low concentration of ammonium (10μM) for four weeks, root and shoot fresh weights remained similar in both transgenic and wild type plants (Fig. 4.15, A). On the other