

hand, when plants were grown in high ammonium (2 mM), the biomass production differed greatly between wild type and transgenic plants. A similar result was also found in the case of leaf area (Fig. 4.15, C). However, leaf numbers did not vary substantially with high ammonium levels (Fig. 4.15,B).

Root ammonium content was similar in wild type and transgenic plants grown in 10  $\mu$ M of ammonium (Fig. 4.15, D). At 2 mM ammonium, however, root ammonium content was greater in transgenic plants than in wild type plants (Fig. 4.15, E).

### 4.3 Discussion

The role of the ammonium transporter *OsAMT1;1* was studied in two rice cultivars Taipei 309 and Jarrah using a transgenic approach. The introduction of *OsAMT1;1* in these cultivars as transgenes led to an increase in the accumulation of *OsAMT1;1* transcripts and transcript level positively correlated with transgene copy number. Physiological studies with the over-expressing lines showed that, depolarization of the plasmamembrane electrical potential ( $E_m$ ) was substantially greater in transgenic rice roots (line 46) compared to wild type plants Taipei 309. Transgenic plants had increased ammonium uptake, root ammonium content, and decreased biomass when grown with high concentrations of  $\text{NH}_4^+$ .

#### 4.3.1 Rice Transformation

Two rice cultivars Taipei 309 and Jarrah were used in this study. The cultivar Taipei 309 was developed in Taipei, Taiwan 30 years ago (Harold E. Bockelman, National Small Grains Collection, USDA - Agricultural Research Service 1691 S. 2700 W. Aberdeen, Idaho 83210 USA, personal communication). According to the IRRI database, it is described as intermediate between japonica and indica. This variety has been widely used in tissue culture and rice transformation studies (Hamid *et al.*, 1996; Hiei *et al.*, 1994; Li *et al.*, 1993). Jarrah is a modern high yielding, nitrogen responsive and medium-grain japonica variety bred and released from Yanco Agricultural Institute, Australia in 1993. Jarrah has also been successfully used in rice transformation studies (Abedinia *et al.*, 1997; Upadhyaya *et al.*, 2000).

A well established *Agrobacterium*-mediated transformation system (Upadhyaya *et al.*, 2000; Wang *et al.*, 1997) was used to produce transgenic lines that

over-expressed *OsAMT1;1*. Attempts were also made to suppress *OsAMT1;1* expression using an antisense approach. Overall, transformation efficiency was higher in Taipei 309 compared to Jarrah (Table 4.1). Such differences in transformation efficiency between rice cultivars has been reported previously (Upadhyaya *et al.*, 2000). Interestingly, only a small number of Taipei 309 lines regenerated from calli transformed with the antisense *OsAMT1;1* construct. No antisense transgenic lines could be recovered from Jarrah. In addition, the proportion of sterile plants was higher with antisense transgenic lines than for sense transgenic lines. The reason for the low regeneration and high sterility of antisense lines could be due to suppression of ammonium transporters during callus induction or regeneration. The small number of antisense transgenic lines were probably the ones with less efficient co-suppression of ammonium transporters as indicated by the expression studies discussed later. The majority of the tested lines showed Mendelian segregation with a few exceptions, which was consistent with segregation reported previously (Upadhyaya *et al.*, 1998; Upadhyaya *et al.*, 2000; Wan and Lemaux, 1994).

#### **4.3.2 Expression of *OsAMT1;1* in Rice**

Over-expression of *OsAMT1;1* mRNA transcript was achieved with the introduction of *Ubi1(I)* promoter-driven *OsAMT1;1* cDNA in rice cultivars Taipei 309 and Jarrah. *OsAMT1;1* expression levels positively correlated with the transgene copy number. Differences in expression levels were also observed in lines with single transgene copy, which presumably resulted from transgene position.

The expression of transgenes can be influenced by the regulatory sequences of nearby host genes, especially if they are inserted into the transcriptionally active euchromatin (Herman *et al.*, 1990; Kerbundit *et al.*, 1991; Koncz *et al.*, 1989). On the other hand, transgene expression can be inactivated if insertion is on or near repetitive DNA or heterochromatin (Prols and Meyer, 1992). The T-DNA transfer system can insert two or more T-DNA copies at the same chromosomal site, and those T-DNAs can be arranged 'head-to-head' or 'tail-to-tail' as inverted repeat (IR) or 'head-to tail' as a direct repeat (DR) (Jorgensen *et al.*, 1987). Transgenes on T-DNAs often show low expression when they are organized as IRs, perhaps because of gene silencing (Jorgensen *et al.*, 1987). Transgene silencing can also occur when a transgene

integrates into a heavily methylated and/or repetitive regions of a chromosome (Prols and Meyer, 1992).

Antisense RNA is normally expected to interact with the endogenous sense message via base pairing, which promotes the degradation of that message. Thus, expression of antisense RNA is usually accompanied by a decrease in level of target message. Only 5 Taipei 309 transgenic lines could be produced with the *OsAMT1;1* antisense construct and none of these showed any down-regulation of the endogenous *OsAMT1;1* RNA. Thus, it is possible that efficient suppression of *OsAMT1;1* has a lethal effect on callus proliferation and/or regeneration.

### 4.3.3 Physiological Analyses

Plant root cells have an excess of negative charges on the inside of the cell or plasma – membrane and an excess of positive charges on the out side. This separation of charge is maintained due to the impermeability of ions through the lipid bilayer of the membrane and results in a negative potential difference inside the cell relative to outside. Ion transporters in the lipid bilayer provide a pathway for inorganic ions to carry electric charges across the membrane. The bilayer of lipid acts as a capacitor and the transporters act as conductors. The membrane capacitance and conductance accounts for the electrical behaviour of cell membrane. The conductance of a membrane is a measure of its permeability to ions. The greater the conductance, the more ionic charges will cross the membrane via transporters under a given electrical force, ie. membrane potential difference. Electrophysiological experiments have shown that the membrane potential,  $E_m$ , is the driving force for ammonium uptake and that ammonium uptake depolarizes  $E_m$  (Smith and Waker, 1978; Ullrich *et al.*, 1984; Wang *et al.*, 1994). The greater negative  $E_m$  values observed in this study for  $\text{NH}_4^+$  fed plants than  $\text{NH}_4^+$  starved plants (both in wild type and transgenic line 46) were probably due to the contributions to the membrane depolarization from the various ions present in MJN solutions. In rice this depolarization of  $E_m$  in response to  $[\text{NH}_4^+]_o$  ( $< 1\text{mM}$ ) is due to carrier-mediated  $\text{NH}_4^+$  uptake that exhibits Michaelis Menten kinetics (Wang *et al.*, 1994; Wang *et al.*, 1993). In the present study, addition of ammonium chloride into -N solutions induced a rapid depolarization of membrane potential in both wild type and transgenic rice root cells. The extent of the depolarization of  $E_m$  by  $\text{NH}_4^+$  was consistently higher in transgenic rice roots (line 46)

lines was lower than that of wild type plants in all treatments. The reduction in biomass in over-expressing plants was correlated with the copy number of transgene and the *OsAMT1;1* expression level. Root ammonium contents were much higher in transgenic plants compared to wild type plants in all treatments. At an elevated level of added ammonium, wild type plants grew normally but the *OsAMT1;1* over-expressing transgenic lines had increased root ammonium content as well as decreased biomass, possibly because of ammonium toxicity (Mehrer and Mohr, 1989). Accumulation of  $\text{NH}_4^+$  is energy dependent which may explain in part the reduction in root growth (Bowman and Paul, 1988). The transgenic lines may also have higher efflux rate and, therefore, higher ammonium cycling across the plasma membrane. This would result in energy loss to the plants. Others have showed that high internal ammonium concentration can also affect both morphology and physiology of plants. At the physiological level, excessive  $\text{NH}_4^+$  blocks ATP production and reducing  $\text{CO}_2$  fixation in the chloroplast (Ikeda and Yamada, 1981; Puritch and Baker, 1967), and affects starch synthesis (Marwaham and Juliano, 1976). High ammonium uptake may prevent water movement from root to shoot (Anderson *et al.*, 1991) and, as a result, some plants may die. This appears to be the case of  $T_2$  plants from line 46 (Fig. 4.10, B). It is seems clear from these results that increasing ammonium uptake is not necessarily beneficial for plants.

The two cultivars, Taipei 309 and Jarrah, possibly have differences in ammonium assimilation and utilization as evidenced by their differential response to N nutrition in (a) wild type and (b) in transgenic lines over-expressing ammonium transporters. Two homozygous  $T_2$  transgenic lines, one derived from Taipei 309 (40-1) and the other derived from Jarrah (77-1), had differences in biomass production and root ammonium concentrations compared to respective wild type plants when grown under high concentration of  $\text{NH}_4^+$  (2 mM). The reduction in biomass when plants were grown at high (2 mM)  $\text{NH}_4^+$  was less drastic in the Jarrah transgenic line compared to that of Taipei 309. This is consistent with the fact that cultivar Taipei 309 (which is closer to indica type) has a better ammonium absorption system and a poorer ammonium utilization system than Jarrah (pure japonica type). This is the reason why japonica varieties respond better to fertilizer nitrogen than the indica varieties (Augladette, 1965).

Flowering time appears to be affected by  $\text{NH}_4^+$  nutrition in the cultivars, Jarrah and Taipei 309. The wild type cultivar Jarrah flowered 14-16 d earlier than Taipei 309 when grown in 2 mM  $\text{NH}_4^+$ . There were also differences in flowering time within each cultivar under different  $\text{NH}_4^+$  nutrition. Flowering in Jarrah was delayed at least 4-5 days when grown in 2 mM  $\text{NH}_4^+$  compared to 10  $\mu\text{M}$  of  $\text{NH}_4^+$ , whereas for Taipei 309, this delay in flowering was at least 15-20 days. Transgenic line 77-1 took only two weeks more time to flower than the wild type Jarrah as compared to 40-1 which took 8 weeks more time to flower under adequate supply of  $\text{NH}_4^+$ . This reflects the differences in ammonium assimilation and utilization among these two cultivars. Hyper ammonium accumulation in the over-expressing transgenic rice plants might have reduced the activity of GS and GOGT which could delay physiological processes leading to flowering and senescence. Flowering time appears to require the balanced activity of  $\text{NH}_4^+$  uptake rate and assimilation. For example, high ammonium accumulation reduced the activity of ammonium assimilation pathways in *Neurospora crassa* by repressing NADH-GOGAT activity (Hummelt and Mora, 1980). Transgenic *Lotus* over-expressing GS and GOGAT showed lower rate of ammonium uptake under high  $[\text{NH}_4^+]_0$  nutrition and premature flowering presumably due to activation of certain physiological processes (Vincent *et al.*, 1997).

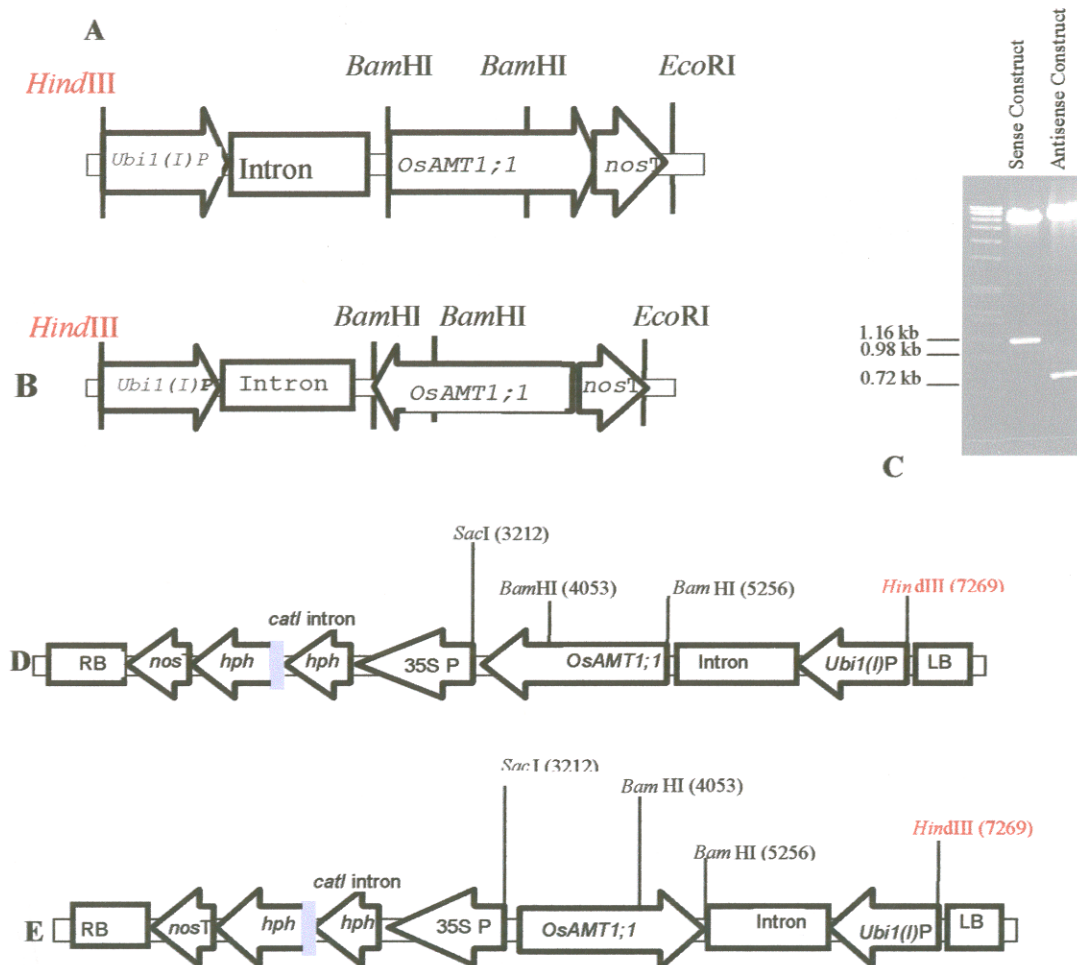
The results from the electrophysiological studies and from experiment 1 & 2 suggest that reductions in root and shoot weights of over-expressing plants are due to increased accumulation of ammonium. This was further confirmed in experiment 3 where low nitrogen treatment showed no differences among transgenic and wild type plants with respect to leaf numbers, biomass production and root ammonium content. The ammonium uptake rate, as measured by the amounts depleted from the nutrient solution per unit time, was higher in transgenic plants compared to wild type plants which was consistent with electrophysiological results and expression level of *OsAMT1;1*. The difference in the net ammonium uptake after 10 min in 1 d -N treated plants (17 d old seedlings) and 2 d -N treated plants (20 d old seedlings) is attributed to the rapid rate at which ammonium enters the plant (Fried *et al.*, 1965). Most of the ammonium was taken up during the first 10 minutes both in the 1 d -N treated plants and 2 d -N treated plants. A maximum amount of uptake could even have occurred in less than 10 minutes as revealed in Fig. 4.2.12. This is because 2 d -N treated plants (20 d old seedlings) are older and bigger than 1 d -N treated plants (17 d old

than in wild type plants at particular  $[\text{NH}_4^+]_o$ . For example, addition of  $100 \mu\text{M NH}_4^+$  into -N solution induced an average depolarization of  $53.4 \pm 10.4$  (mV) in the case of wild type plants while in transgenic plants (line 46) this value was  $108.6 \pm 12.1$  (-mV). This probably indicates the presence of a greater number of ammonium transporters in transgenic plants over-expressing *OsAMT1;1*.

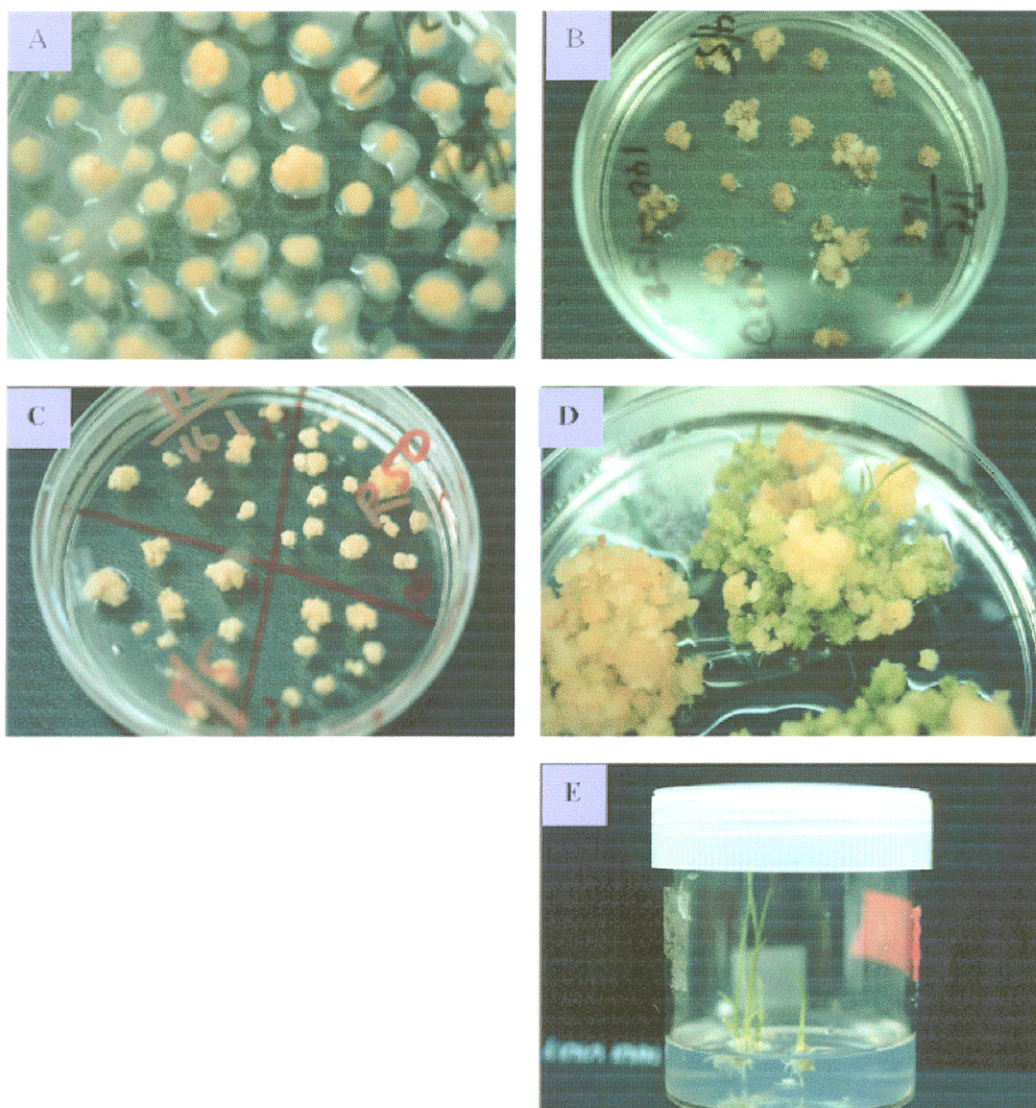
Kinetic parameters were obtained by fitting the data to the Michaelis-Menten equation. The estimated half-saturation values for net depolarization (equivalent to  $K_m$  values) were similar in each growth conditions in both wild type and transgenic plants except in the case of ammonium fed plants in experiment 1. The estimated  $K_m$  values were in the range reported for the high affinity system (HATS) in several species including rice (Kronzucker *et al.*, 1996; Ullrich *et al.*, 1984; Wang *et al.*, 1994; Wang *et al.*, 1993), indicating that both wild type and transgenic plants had similar affinity for ammonium. On the other hand, maximum depolarization values (similar to  $V_{\text{max}}$  values) were higher in transgenic plants compared to wild type plants in all growth conditions, except in case of ammonium fed plants in experiment 2. This again indicates that more transporters were involved in ammonium uptake by transgenic plants than in control plants. The  $V_{\text{max}}$  values in wild type plants grown under different nitrogen conditions differed. For example, the  $V_{\text{max}}$  value ( $35 \pm 8$  mV) in experiment 1 was higher than the values of 1 d -N treated and ammonium fed plants in experiment 2. This apparent inconsistency was probably due to the growth conditions and ages of seedlings used in the experiment. The net uptake of ammonium by plants is the result of two unidirectional flux components, influx and efflux (Morgan and Jackson, 1988). In the case of N fed plants, adequately supplied ammonium may result in higher accumulation of cytosolic  $\text{NH}_4^+$  and an increase in the rate of efflux compared to plants deprived of ammonium (Wang *et al.*, 1993). The efflux rate may also depend on the growth stage of plants, which can further complicate the estimation and interpretation of  $V_{\text{max}}$  values. If the data from the ammonium fed plants is considered, both the wild type and transgenic plants had a similar affinity ( $K_m$  values) for ammonium. However, transgenic plants had a higher ammonium uptake rate compared to wild type plants presumably due to over-expression of *OsAMT1;1*.

The physiological consequences of *OsAMT1;1* over-expression were studied in two rice cultivars under various nitrogen regimes. Total biomass of over-expressing

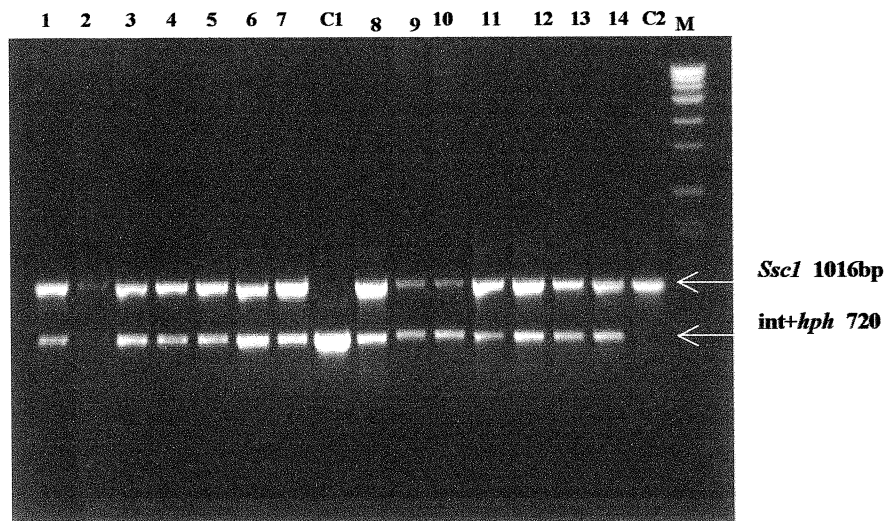
seedlings) and as a result, uptake capacities were higher in bigger plants. Uptake measurements were recorded at 10 min time, if measurement were to be made earlier the uptake rate could have been much higher.



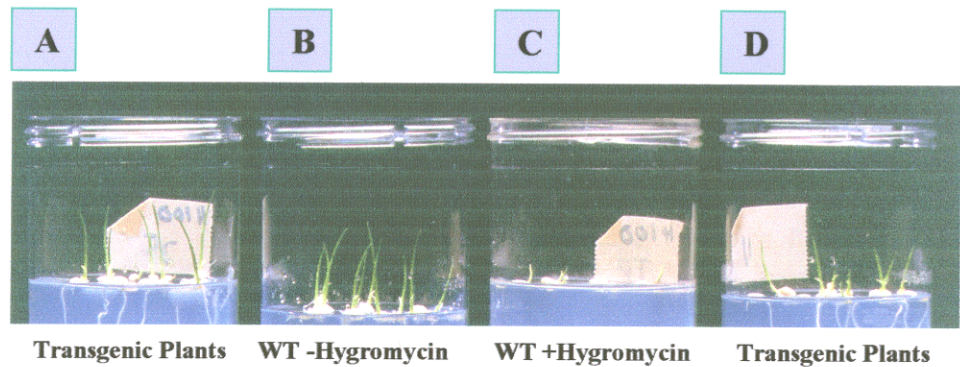
**Figure 4.1.** *OsAMT1;1* gene construction. (A) Construct pPIMP56 containing the 2.04 kb *OsAMT1;1* cDNA inserted in sense orientation between the maize ubiquitin promoter (*Ubi1(I)*) and the nopaline synthase gene terminator (*nosT*); (B) construct pPIMP58 containing the 2.04 kb *OsAMT1;1* cDNA inserted in antisense orientation between *Ubi1(I)* promoter and *nos* terminator; (C) Constructs pPIMP56 and PIMP58 digested with *Bam*HI showing sense (lane 2) and antisense (lane 3) orientations, with a molecular marker (lane 1); (D) binary vector construct pPIMP161 containing the expression cassette from pPIMP56 (*Eco*RI-endfilled/*Hind*III fragment) inserted into the binary vector pWBvec8 (*Xba*I-endfilled/*Hind*III digest) which contains CaMV35S promoter driven, intron-interrupted hygromycin resistance gene (*hph*) as selectable marker; (E) binary vector construct pPIMP145 containing the antisense expression cassette from pPIMP58 inserted into pWBvec8.



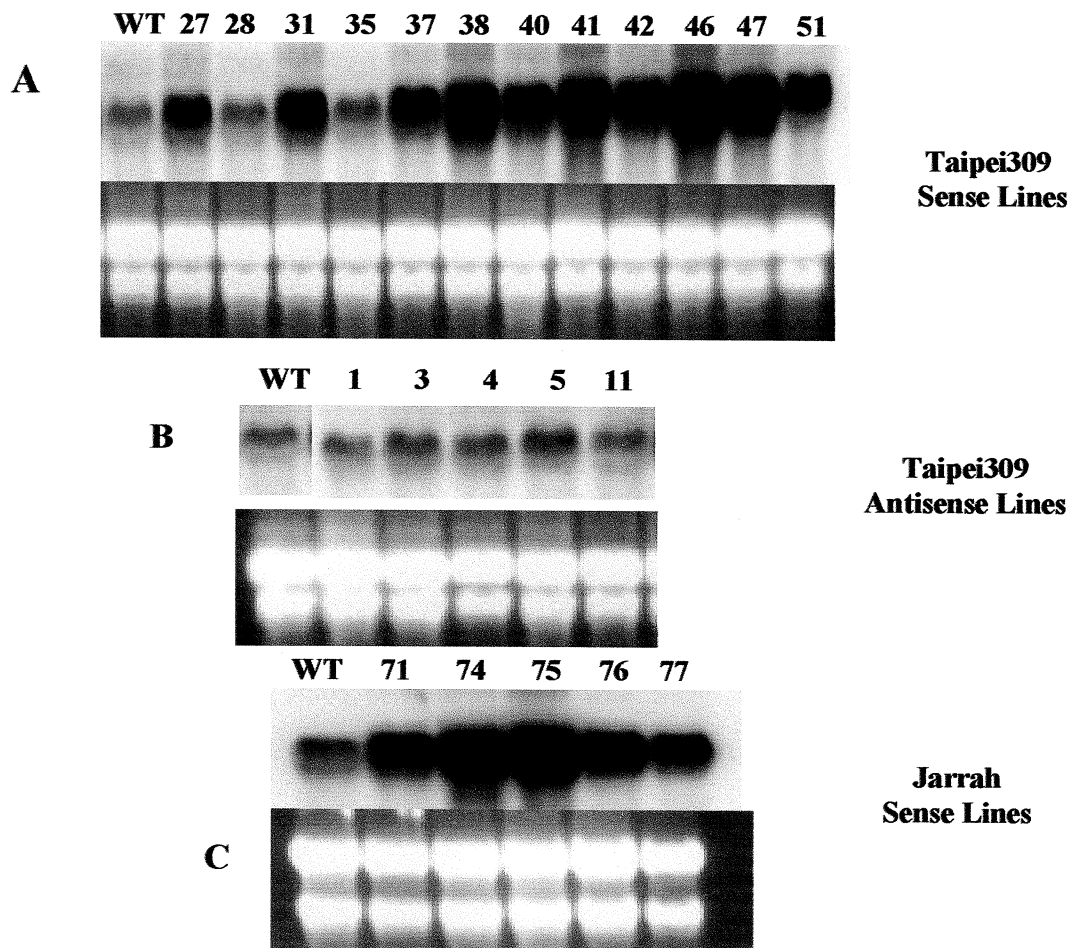
**Figure 4.2.** Stages in the production of fertile transgenic plants from rice cvv. Taipei 309 and Jarrah. **(A)** *Agrobacterium* (containing binary vector construct with *Ubi1(I)P-OsAMT1;1* and *CaMV35SP-hph* gene expression cassettes) and rice embryogenic calli co-cultivation; **(B)** *Agrobacterium* infected rice calli producing new growth in selection medium; **(C)** callus line formed from *hph* resistant cell cluster on pre-regeneration medium. **(D)** Plantlets regenerated from *hph* resistant callus line on regeneration medium; **(E)** Transgenic rice plants in half MS medium.



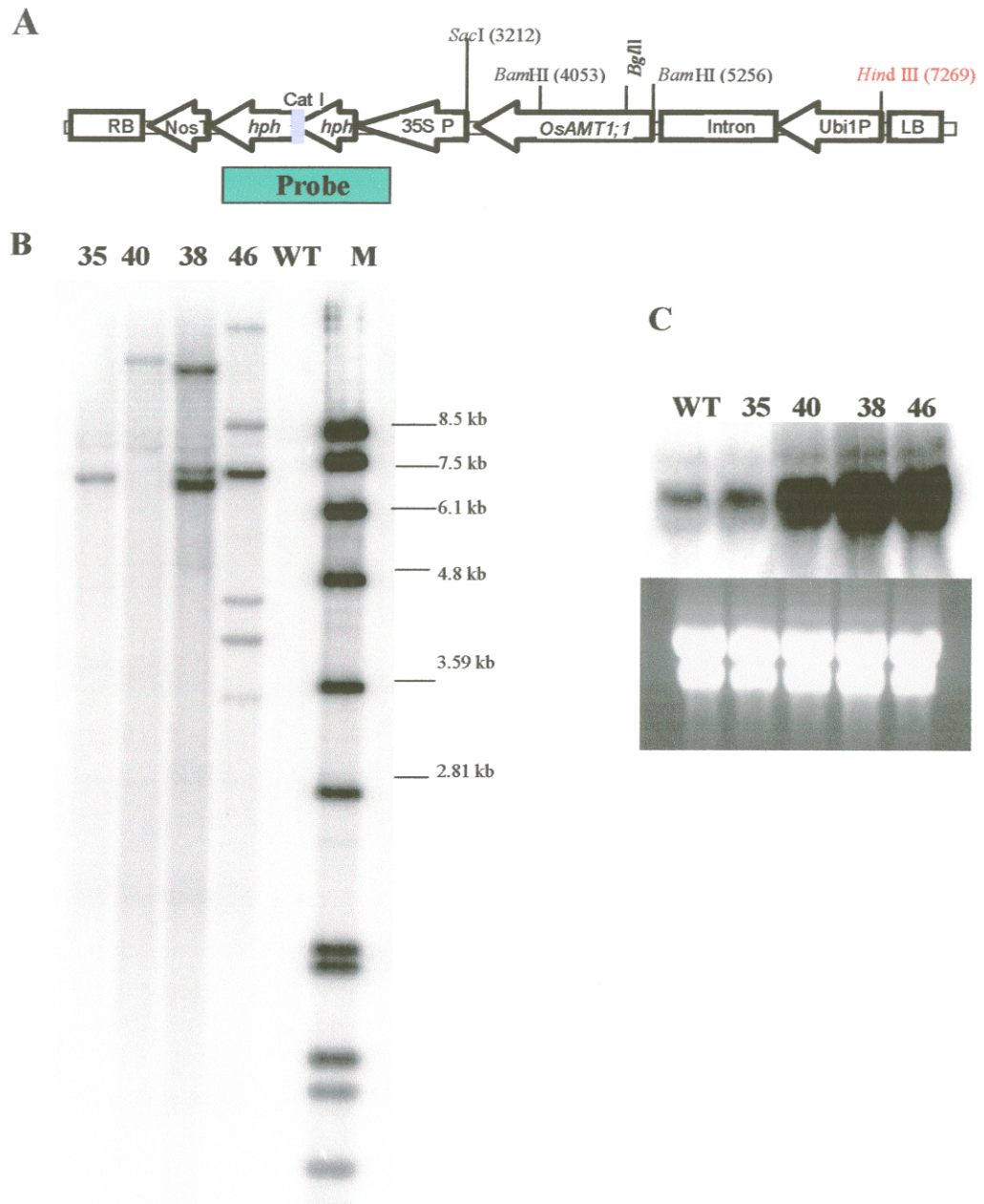
**Figure 4.3** Preliminary analysis of transgenic lines by polymerase chain reaction (PCR). Genomic DNA was isolated from primary transgenic plants ( $T_0$ ) and used in co-amplification of *hph* and *SSs1* (internal control) DNA fragments by PCR. PCR products from DNA of various transgenic lines having *hph* (1-14), +ve control (C1, plasmids containing *hph* gene), -ve controls (C2, wild type plants) and DNA size standard (M) are shown.



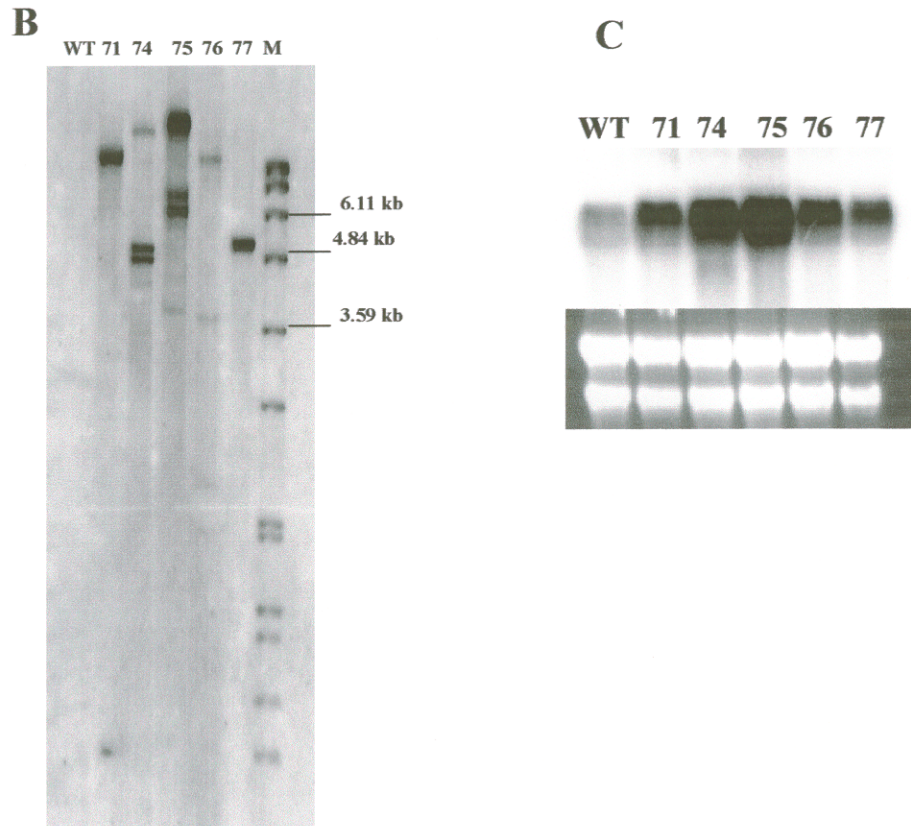
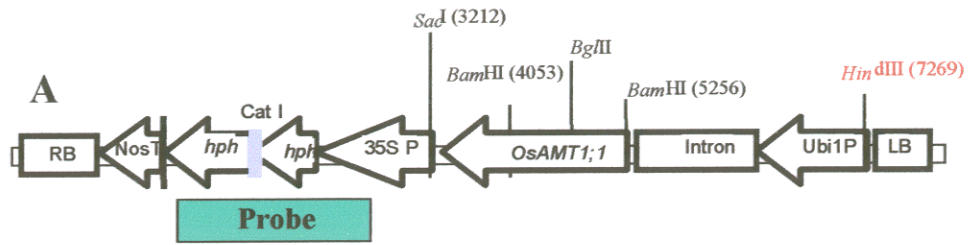
**Figure 4.4** Segregation analysis with progeny of transgenic lines. Sterile seeds of wild type (WT) and T<sub>1</sub> transgenic plants were germinated on half MS medium containing 100 mg/L hygromycin. Hygromycin-resistant transgenic plants (A&D) grew normally while wild type plants did not (C). Normal germination and growth of wild type plants in the absence of hygromycin is also shown (B).



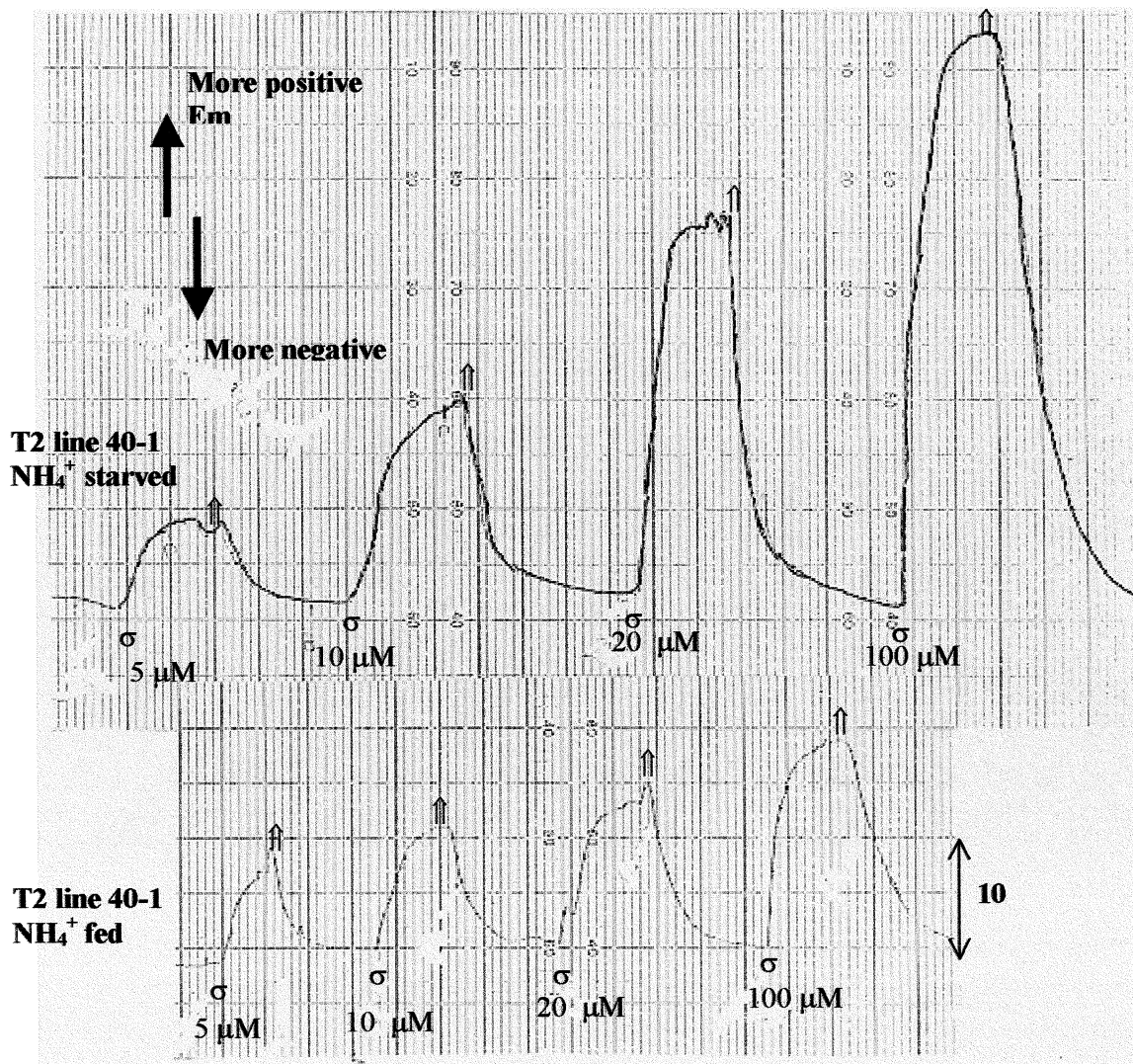
**Figure 4.5.** Northern blot analysis of *OsAMT1;1* expression in rice roots. Total RNA was isolated from the roots of hydroponically grown plants and 25  $\mu$ g was loaded in each lane. **(A)** Northern blot hybridization (upper panel) and ethidium bromide stained gel (lower panel) of total RNA from Taipei 309 (lane 1) and various T<sub>1</sub> Taipei 309 *OsAMT1;1* over-expressing lines (27, 28, 31, 35, 37, 38, 40, 41, 42, 46, 47 and 51); **(B)** Northern blot hybridization (upper panel) and ethidium bromide stained gel (lower panel) of total RNA from Taipei 309 (lane1), and various T<sub>1</sub> Taipei 309 *OsAMT1;1* anti-sense lines (1, 3, 4, 5 & 11); **(C)** Northern blot hybridization (upper panel) and ethidium bromide stained gel (lower panel) of total RNA from Jarrah (lane1) and various T<sub>1</sub> Jarrah *OsAMT1;1* over-expressing lines (71, 74, 75, 76 & 77). The blots were exposed to autoradiography using phosphor screens (Molecular dynamics) overnight.



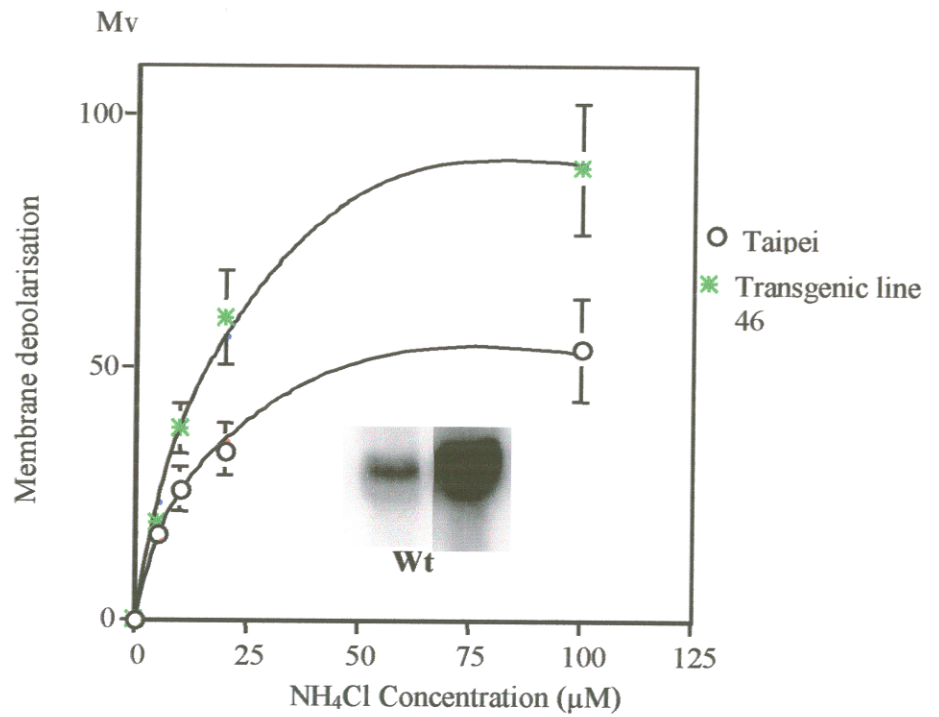
**Figure 4.6.** Estimation of *OsAMT1;1* transgene copy number in Taipei 309 T<sub>1</sub> transgenic lines by Southern blot analysis and corresponding transgene expression levels. **(A)** Construct showing *hph* gene used as a probe; **(B)** genomic DNA from Taipei T<sub>1</sub> transgenic lines (indicated by code number) and Taipei 309 (lane 5) digested with *Bgl*II, separated on a 0.8% agarose gel, blotted onto nylon membrane and hybridized with <sup>32</sup>P-labelled *hph* gene along with molecular wt marker (lane M); **(C)** Northern blot showing *OsAMT1;1* transgene expression in the same transgenic lines and wild type plants.



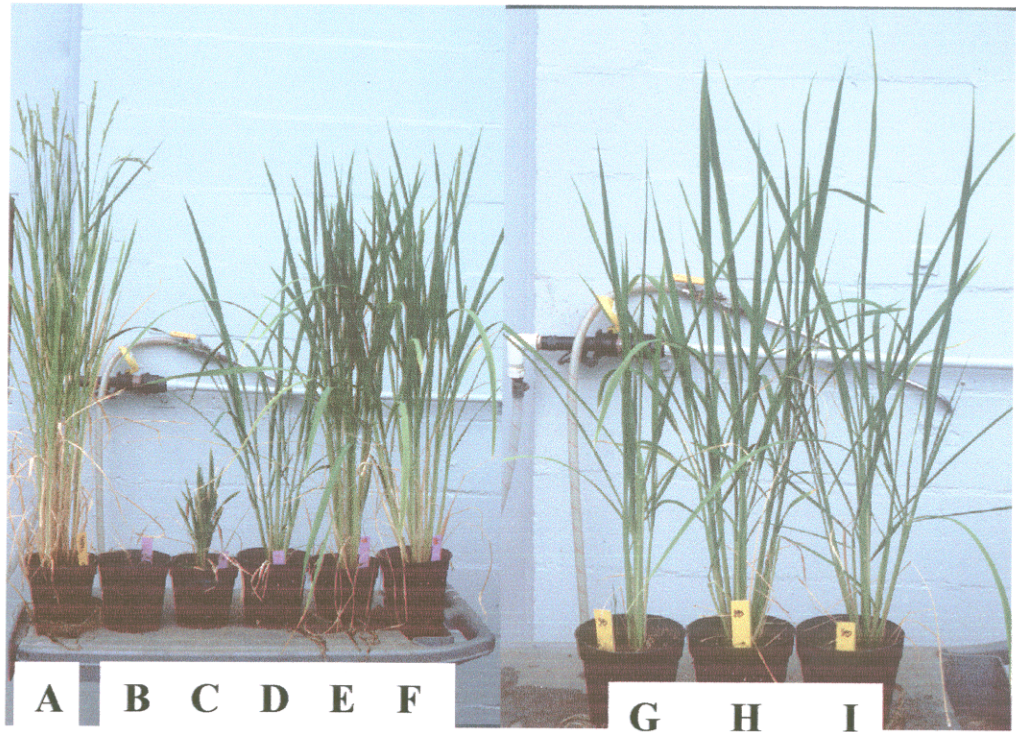
**Figure 4.7.** Transgene copy number and expression in Jarrah T<sub>1</sub> transgenic lines. **(A)** Construct showing *hph* gene used as a probe; **(B)** genomic DNA from Jarrah T<sub>1</sub> transgenic lines (indicated by code number) and Jarrah (lane 1), digested with *Bgl*II, separated on a 0.8% agarose gel, blotted onto nylon membrane and hybridized with <sup>32</sup>P-labelled *hph* gene along with molecular wt. marker (lane M); **(C)** Northern blot showing *OsAMT1;1* transgene expression in the same transgenic lines and wild type plants.



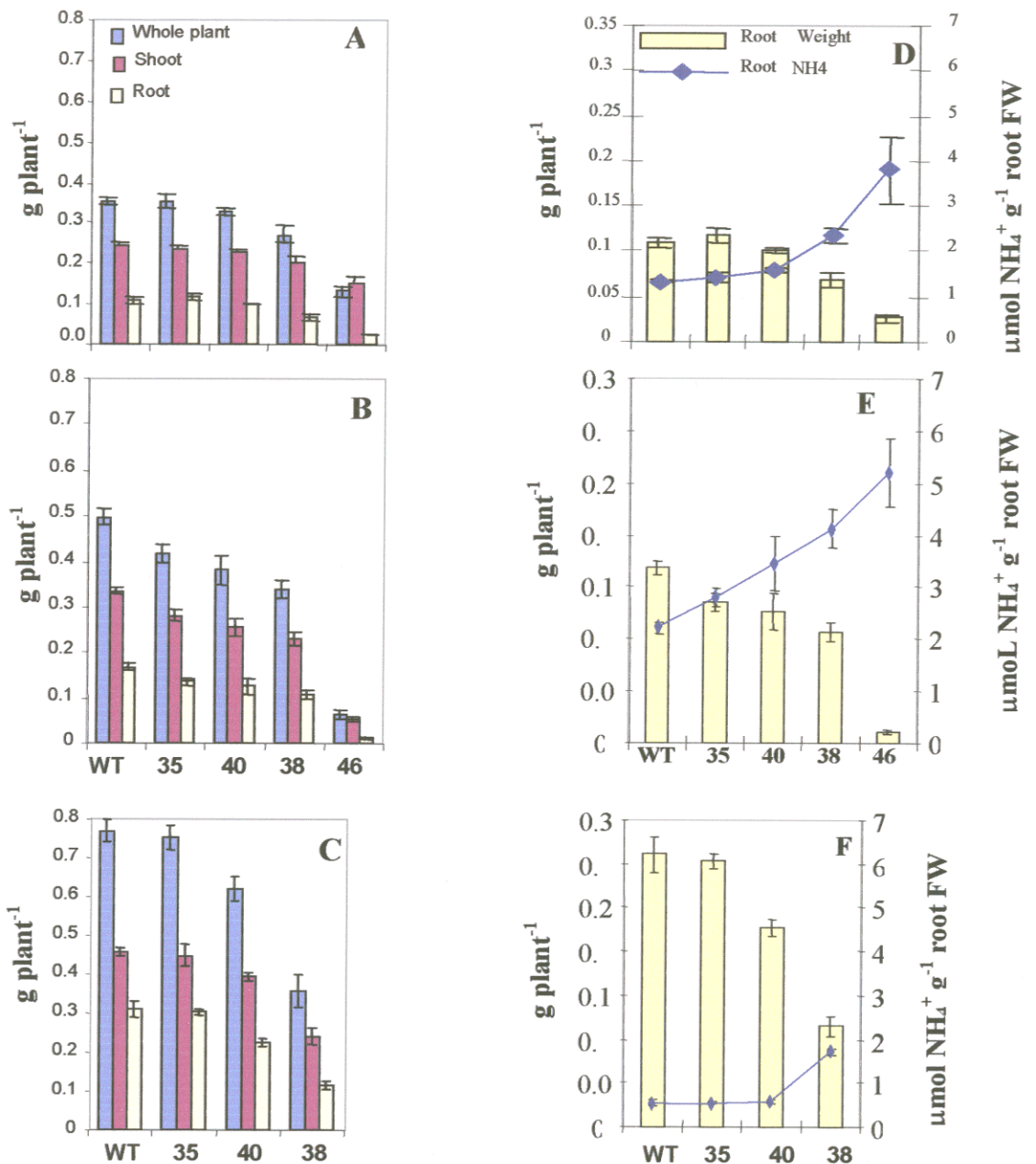
**Figure 4.8.** Representative traces from T<sub>2</sub> line 40-1 showing the depolarization of root cell E<sub>m</sub> induced by adding various concentrations of NH<sub>4</sub>Cl. (σ), various concentrations of NH<sub>4</sub>Cl was added into the nutrient solution. NH<sub>4</sub>Cl was withdrawn from the nutrient solution where indicated (↑).



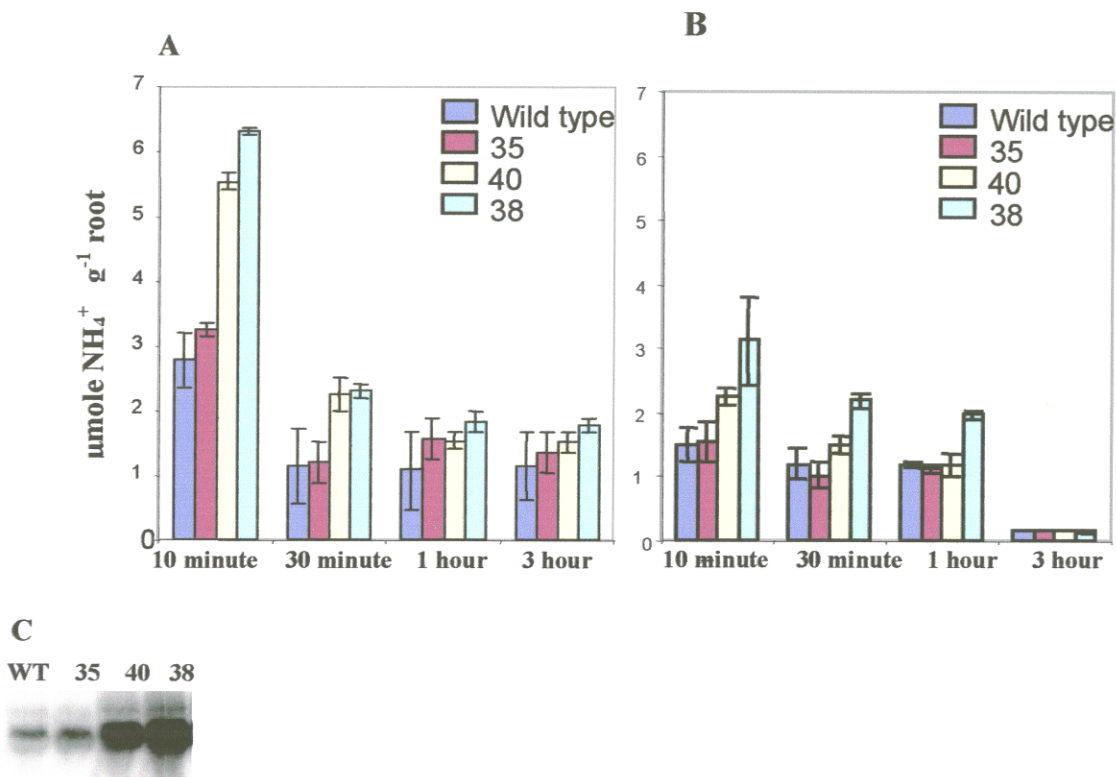
**Figure 4.9.** Membrane depolarization of transgenic and wild type rice root cells in response to increasing concentrations of  $\text{NH}_4\text{Cl}$ . Plants were grown in modified Johnson's nutrient solution (Epstein, 1972) with 2.0 mM  $\text{NO}_3^-$  and 1.0 mM of  $\text{NH}_4^+$  for three weeks. For the nitrogen starvation treatment the seedlings were transferred to nitrogen free nutrient solution and grown for 2 d. The -N nutrient solution was used as resting solution to measure the resting  $E_m$ . Results are the average of experiments with at least 4 plants and the bars indicate the standard error of the mean. The lines were obtained by fitting the Michaelis-Menten equation to the data.



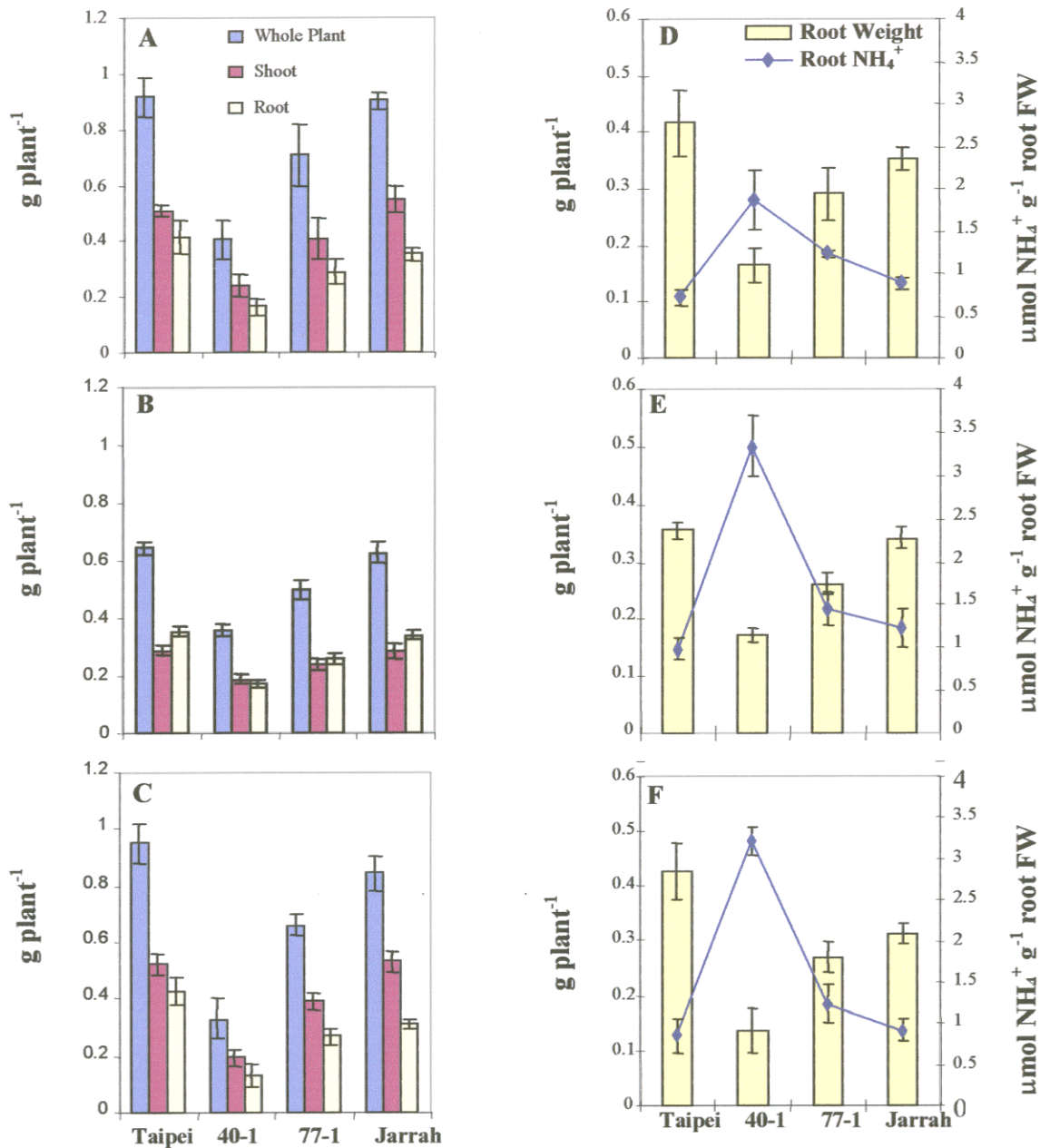
**Figure 4.10**  $T_1$  plants from transgenic lines 46 (B to F) and 40 (G to I) and Taipei 309 (A).



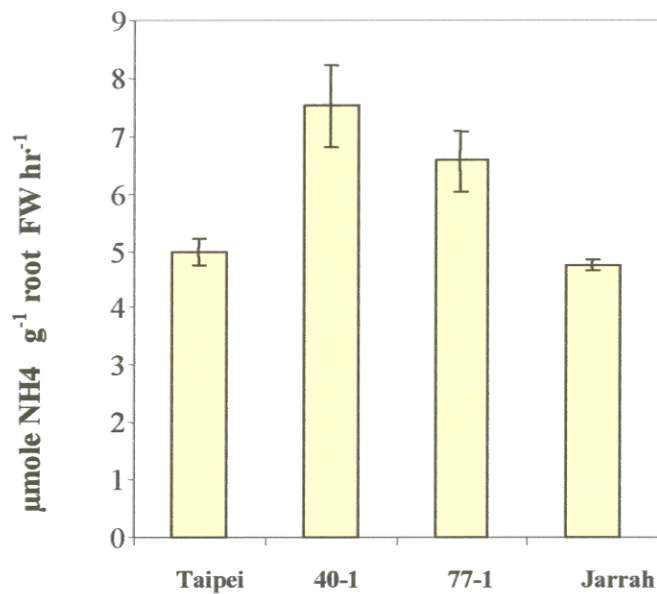
**Figure 4.11** Whole plant, shoot and root weights and root ammonium content of wild type (Taipai-309) and transgenic plants. Rice seedlings (wild type and transgenic) were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM  $NO_3^-$  and 1.0 mM  $NH_4^+$  for 16 d. First set, 16 d old, grown in the same solution (A & D), second set, 17 d old seedlings, last 1 d grown in -N solution (B & E), Third set, 20 d old seedlings, last two day was in nitrogen free solution (C & F). After three hours of  $NH_4^+$  uptake in 75  $\mu M\ NH_4^+$ , seedling were removed from the tubes and whole plant weight, root weight and shoot were recorded (A, B & C). Root fresh weight and ammonium content of the roots were measured immediately. Error bars indicate the standard error of the mean.



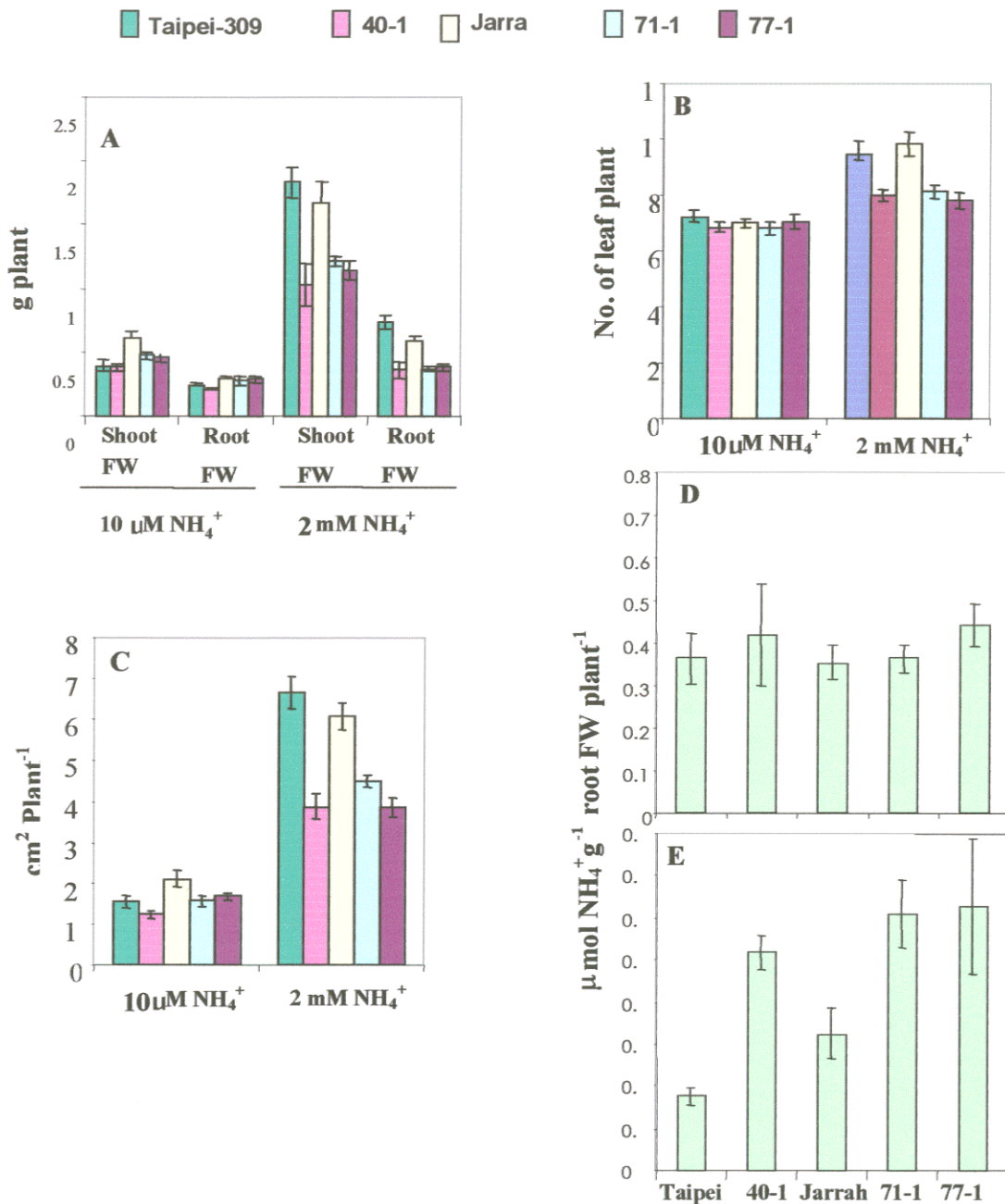
**Figure 4.12.** Ammonium uptake rate of transgenic and wild type (Taipei-309) rice plants. Rice seedlings were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM  $\text{NO}_3^-$  and 1.0 mM of  $\text{NH}_4^+$  for 16 d. **(A)** 17 d old seedlings, last 1 d grown in -N solution; **(B)** 20 d old seedlings, last two 2 d grown in -N solution. Five seedlings from each treatment were placed in a tube containing 80 mL of nutrient solution with 75  $\mu\text{M}$  of  $\text{NH}_4^+$  as the only nitrogen source at zero time. Samples of the growth solution were taken at 10 min, 30 min, 1 h and 3 h. After three hours, seedlings were removed from the tubes and the depletion of ammonium was measured and converted to  $\mu\text{mol}$  ( $\text{g}$  fresh root wt.  $\text{h}^{-1}$ ). Error bars indicate the standard error of the mean; **(C)** Northern blot showing *Os AMT1;1* transgene expression in wild type and transgenic lines.



**Figure 4.13** Whole plant, shoot and root fresh weights, and root ammonium content of wild type and transgenic plants. Rice seedlings (wild type and transgenic) were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM NO<sub>3</sub><sup>-</sup> and 1.0 mM NH<sub>4</sub><sup>+</sup> for two week. The 1st set was grown in the same solution for another one week (A & D), 2nd set was transferred to nitrogen free nutrient solution and grown for last seven days (B & E) and 3rd set was grown last seven days in NO<sub>3</sub><sup>-</sup> containing solution (C & F). After three hours of NH<sub>4</sub><sup>+</sup> uptake in 75 μM NH<sub>4</sub><sup>+</sup>, seedling were removed from the tubes and the whole plant weight, root and shoot weight were recorded (A, B & C). Root fresh weight and ammonium content of the roots were measured immediately after the NH<sub>4</sub><sup>+</sup> uptake experiments (D, E, & F). Error bars indicated the standard error of the mean.



**Figure 4.14.** Ammonium uptake rate of wild type and transgenic rice plants from nutrient solution during the 3 h period of growth. Rice seedlings (wild type and transgenic) were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM NO<sub>3</sub><sup>-</sup> and 1.0 mM of NH<sub>4</sub><sup>+</sup> for two weeks. Then transferred to nitrogen free same nutrient solution and was grown for one more week. Five seedlings from each entry were placed in a tube containing 75 mL of nutrient solution with 75 µM of NH<sub>4</sub><sup>+</sup> as the only nitrogen source at zero time. Tubes were gently aerated to ensure the uniform distribution of NH<sub>4</sub><sup>+</sup> throughout the solution. After three hours, depletion of ammonium in the culture solution was measured to determine the uptake rate in µmol (g fresh root wt.h<sup>-1</sup>). Error bars indicate the standard error of the mean.



**Figure 4.15** Shoot and root fresh weights of 6 week old wild type and transgenic plants grown in 10 μM and 2 mM NH<sub>4</sub><sup>+</sup> (A). Number of leaves plant<sup>-1</sup> (B) and leaf area (C) of 6 week old wild type and transgenic plants grown in 10 μM and 2 mM NH<sub>4</sub><sup>+</sup>. Root NH<sub>4</sub><sup>+</sup> content of 6 week old wild type and transgenic plants grown in 10 μM (D) and 2 mM NH<sub>4</sub><sup>+</sup> (E).

**Table 4.1** List of transgenic lines of different generations containing *OsAMT1;1* transgenes.

Cultivar	Construct	Number of hygromycin resistant callus lines	Number of transgenic (T <sub>0</sub> ) plant lines	Number of transgenic (T <sub>1</sub> ) plant lines	
				Fertile	Sterile
Taipei 309	Sense	48	17 (53)*	40	15
	Antisense	25	7 (23)*	5	8
Jarrah	Sense	9	4 (9)*	6	1
	Antisense	0	0	0	0

\* Number of Plants

**Table 4.2 List of Transgenic Lines**

Serial No.	Line No.	Pedigree	PCR	Southern	Northern	Fertility
<b>Antisense lines (Taipei 309)</b>						
1	16	Tp-145-0-1	Positive	Not tested		Sterile
2	17	Tp-145-0-2	Positive	Not tested		Sterile
3	18	Tp-145-0-3	Positive	Not tested		Sterile
4	19	Tp-145-0-4	Positive	Not tested		Sterile
5	20	Tp-145-0-5	Positive	Not tested		Sterile
6	21	Tp-145-0-6	Positive	Not tested		Sterile
7	22	Tp-145-0-7	Positive	Not tested		Sterile
8	23	Tp-145-0-8	Positive	Not tested		Sterile
9	13	Tp-145-2-1	Positive	Not tested		Sterile
10	14	Tp-145-2-2	Positive	Not tested		Sterile
11	6	Tp-145-3-1	Positive	Not tested		Sterile
12	7	Tp-145-3-2	Positive	Not tested		Sterile
13	8	Tp-145-3-3	Positive	Not tested		Sterile
14	24	Tp-145-4-1	Positive	Not tested		Fertile
15	1	Tp-145-10 -1	Positive	Not tested	No under expression	Fertile
16	2	Tp-145-10 -2	Positive	Not tested		Sterile
17	3	Tp-145-10 -3	Positive	Not tested	No under expression	Fertile
18	4	Tp-145-10 -4	Positive	Not tested	No under expression	Fertile
19	5	Tp-145-10 -5	Positive	Not tested	No under expression	Fertile
20	11	Tp-145-9-1	Positive	Not tested	No under expression	Fertile
21	12	Tp-145-9-2	Positive	Not tested		Sterile
22	9	Tp-145-13-1	Positive	Not tested		Sterile
23	10	Tp-145-13-2	Positive	Not tested		Sterile
<b>Sense lines (Taipei 309)</b>						
24	27	Tp-161-0-1	Positive	Not tested	over expressed	Fertile
25	80	Tp-161-0 -2	Positive	Not tested	Not tested	Sterile
26	44	Tp-161-8-1	Positive	Not tested	Not tested	Sterile
27	56	Tp-161-11d -1	Positive	Not tested	Not tested	Sterile
28	57	Tp-161-11d -2	Positive	Not tested	Not tested	Sterile
29	58	Tp-161-11d -3	Positive	Not tested	Not tested	Sterile
30	59	Tp-161-11d -4	Positive	Not tested	Not tested	Sterile
31	49	Tp-161-11d -5	Positive	Not tested	Not tested	Sterile
32	50	Tp-161-11d -6	Positive	Not tested	Not tested	Sterile
33	26	TP-161-13-1	Positive	Not tested	over expressed	Fertile
34	83	Tp-161-13-3	Positive	Not tested	Not tested	Fertile
35	64	Tp-161-14-1	Positive	Not tested	Not tested	Fertile
36	65	Tp-161-14-2	Positive	Not tested	Not tested	Fertile
37	66	Tp-161-14-3	Positive	Not tested	Not tested	Fertile
38	67	Tp-161-14-4	Positive	Not tested	Not tested	Fertile
39	33	Tp-161-14d-1	Positive	Not tested	over expressed	Fertile
40	34	Tp-161-14d-2	Positive	Not tested	Not tested	Fertile

Continued..

Continued...

Serial No.	Line No.	Pedigree	PCR	Southern	Northern	Fertility
41	35	Tp-161-14d-3	Positive	single copy	no over expression	Fertile
42	36	Tp-161-14d-4	Positive	Not tested	Not tested	Fertile
44	51	Tp-161-15 -1	Positive	Not tested	over expressed	Fertile
45	52	Tp-161-17-1	Positive	Not tested	Not tested	Sterile
46	53	Tp-161-17-2	Positive	Not tested	Not tested	Sterile
47	29	Tp-161-17 -3	Positive	Not tested	Not tested	Fertile
48	30	Tp-161-17 -4	Positive	Not tested	Not tested	Fertile
49	41	Tp-161-18-1	Positive	Not tested	over expressed	Fertile
50	42	Tp-161-18-2	Positive	Not tested	over expressed	Fertile
51	82	Tp-161-18-3	Positive	Not tested	Not tested	Sterile
52	37	Tp-161-19d-1	Positive	Not tested	over expressed	Fertile
53	38	Tp-161-19d-2	Positive	Three copy	over expressed	Fertile
54	39	Tp-161-19d-3	Positive	Not tested	Not tested	Fertile
55	40	Tp-161-19d-4	Positive	single copy	over expressed	Fertile
56	54	Tp-161-19d -5	Positive	Not tested	Not tested	Sterile
57	55	Tp-161-19d -6	Positive	Not tested	Not tested	Fertile
58	85	Tp-161-19d-7	Positive	Not tested	Not tested	Sterile
59	45	Tp-161-23-1	Positive	Not tested	Not tested	Fertile
60	87	Tp-161-25-1	Positive	Not tested	Not tested	Fertile
61	60	Tp-161-26 -1	Positive	Not tested	Not tested	Fertile
62	61	Tp-161-26 -2	Positive	Not tested	Not tested	Fertile
63	62	Tp-161-26 -3	Positive	Not tested	Not tested	Fertile
64	63	Tp-161-26 -4	Positive	Not tested	Not tested	Fertile
65	31	Tp-161-26-5	Positive	Not tested	over expressed	Fertile
66	32	Tp-161-26-6	Positive	Not tested	Not tested	Fertile
67	84	Tp-161-26-7	Positive	Not tested	Not tested	Fertile
68	68	Tp-161-27 -1	Positive	Not tested	Not tested	Fertile
69	69	Tp-161-27 -2	Positive	Not tested	Not tested	Fertile
70	46	Tp-161-30-1	Positive	Six copy	over expressed	Fertile
71	47	Tp-161-30-2	Positive	Not tested	over expressed	Fertile
72	48	Tp-161-30-3	Positive	Not tested	over expressed	Fertile
73	81	Tp-161-30-4	Positive	Not tested	Not tested	Sterile
74	86	Tp-161-30-5	Positive	Not tested	Not tested	Sterile
75	25	TP-161-32-1	Positive	Not tested	over expressed	Fertile

**Sense lines (Jarrah)**

76	43	Tp-161-33-2	Positive	Not tested	Not tested	Sterile
77	76	JA-161-10 -1	Positive	Two copy	over expressed	Fertile
78	75	JA-16 -13-1	Positive	Four copy	over expressed	Fertile
79	70	JA-161-14-1	Positive	Not tested	Not tested	Fertile
80	71	JA-161-14-2	Positive	Single Copy	over expressed	Fertile
81	72	JA-161-14-3	Positive	Not tested	Not tested	Sterile
82	73	JA-161-14-4	Positive	Not tested	Not tested	Fertile
83	74	JA-161-14-5	Positive	Three copy	over expressed	Fertile
84	77	JA-161-17 -1	Positive	Single copy	over expressed	Fertile
85	78	JA-161-17 -2	Positive	Not tested	Not tested	Fertile

**Table 4.3** Number of selected T<sub>2</sub> populations.

Pedigree	Cultivar Used	Transgene Copy Number In T <sub>0</sub> plants	Homozygous/Heterozygous
38-1	Taipei 309	3	Not Tested
-2	Taipei 309	3	Not Tested
-3	Taipei 309	3	Not Tested
-4	Taipei 309	3	Not Tested
-5	Taipei 309	3	Not Tested
-6	Taipei 309	3	Not Tested
-7	Taipei 309	3	Not Tested
40-1	"	1	Homozygous
-2	"	1	Heterozygous
-3	"	1	Not Tested
-4	"	1	Not Tested
-5	"	1	Not Tested
-6	"	1	Not Tested
-7	"	1	Not Tested
46-1	"	6	Heterozygous
-2	"	6	Heterozygous
-3	"	6	Heterozygous
-4	"	6	Heterozygous
-5	"	6	Heterozygous
-6	"	6	Heterozygous
-7	"	6	Heterozygous
-8	"	6	Not tested
-9	"	6	Not Tested
-10	"	6	Not Tested
-11	"	6	Not Tested
71-1	Jarrah	1	Not Tested
-2	"	1	Not Tested
74-1	"	3	Not Tested
-2	"	3	Not Tested
-3	"	3	Not Tested
-4	"	3	Not Tested
75-1	"	4-6	Not Tested
-2	"	4-6	Not Tested
-3	"	4-6	Not Tested
-4	"	4-6	Not Tested
76-1	"	2	Not Tested
-2	"	2	Heterozygous
-3	"	2	Heterozygous
-4	"	2	Not Tested
-5	"	2	Not Tested
77-1	"	1	Not tested
77-2	"	1	Heterozygous

**Table 4.4** Membrane potentials of root cells (mV) of wild type Taipei 309 and two transgenic lines.

	Taipei 309	T <sub>1</sub> line 46	T <sub>2</sub> line 40-1
Ammonium fed plants	-151±8	-154±9	-120±2
One day nitrogen starved	-125±5	-130±4	-
Two day nitrogen starved	-131±7	-135±4	-
Seven day nitrogen starved	-121±2	-	-123±3

**Table 4.5** Estimated half saturation ( $K_m$ ) and maximum depolarization ( $V_{max}$ ) values of wild type and transgenic rice roots exposed to varying ammonium concentrations.

		$K_m$ ( $\mu M$ )	$V_{max}$ (mV)
<b>Experiment 1</b>			
Ammonium fed plants	Taipei 309	$4.1 \pm 1.1$	$35 \pm 8$
	T <sub>1</sub> line 46	$16.2 \pm 1.4$	$71 \pm 14$
One day nitrogen starved	Taipei 309	$10 \pm 5$	$26 \pm 3$
	T <sub>1</sub> line 46	$9 \pm 3$	$50 \pm 3$
Two day nitrogen starved	Taipei 309	$13 \pm 4$	$52 \pm 4$
	T <sub>1</sub> line 46	$20 \pm 1$	$86 \pm 10$
<b>Experiment 2</b>			
Ammonium fed plants	Taipei 309	$4 \pm 12$	$4 \pm 1$
	T <sub>2</sub> line 40	$8 \pm 11$	$5 \pm 1$
Seven day nitrogen starved	Taipei 309	$22 \pm 7$	$43 \pm 6$
	T <sub>2</sub> line 40	$25 \pm 5$	$62 \pm 6$

**Table 4.6** Segregation analysis in the T<sub>1</sub> progeny of selected transgenic lines

Lines	Genotype Used in transformation	Hygromycin Resistant	Hygromycin Susceptible	Ratio
35	Taipei 309	64	28	2.28:1
40	"	75	21	3.57:1
38	"	85	51	1.66:1
46	"	37	46	0.80:1
71	Jarrah	36	8	4.5:1
74	"	79	14	5.6:1
75	"	15	3	4.6:1
76	"	66	27	2.4:1
77	"	32	9	3.5:1

# **CHAPTER 5**

## **ISOLATION AND CHARACTERIZATION OF THREE *OsAMT1* GENE FAMILY MEMBERS**

## 5.1 Introduction

Sequence similarity between ammonium transporter genes has allowed the isolation of several *AMT1* orthologues, not only from *Arabidopsis* (Gazzarrini *et al.*, 1999) but also from tomato (Lauter *et al.*, 1996; Von Wiren *et al.*, 2000) and rice (Von Wiren *et al.*, 1997). There are at least two ammonium transporter gene families in plants, *AMT1* and *AMT2* (Sohlenkamp *et al.*, 2000). Near completion of the genome sequence by the international *Arabidopsis* genome project has revealed the presence of at least five *AMT1* family members and one *AMT2* member. Three *AMT1* family members have been described in tomato (Von Wiren *et al.*, 2000).

As mentioned at the beginning of this study, no rice orthologues of *AtAMT* have been identified or characterized to date. Three cDNA clones corresponding to *OsAMT1;1* were isolated within the first year of this study, although the full-length sequence of *OsAMT1;1* was published by another group (Von Wiren *et al.*, 1997). The main aim of the research outlined in this chapter was to isolate and characterize different members of the *OsAMT1* gene family. It was expected that rice, like other plant species, would possess several *AMT1* family members. The sequence of *OsAMT1* genomic clones was expected to provide not only insight into the coding region and possible intron-exon structure of these genes, but also to identify promoter regions of these genes. In particular, research interest was focused on the possibility of identifying putative N-regulatory domains for *OsAMT1* promoters, given the observation that some *AMT1* genes are N-regulated.

This chapter describes the isolation of three *OsAMT1* genomic clones from rice, *OsAMT1;1*, *OsAMT1;2*, and *OsAMT1;3*. Construction of promoter-GUS fusions and transformation of the rice cultivar Taipei 309 with these constructs are also described.

## 5.2 Results

### 5.2.1 Isolation of Genomic Clones of *OsAMT1* Gene Family

A genomic library of *Oryza sativa* L. indica, var. IR36 (CloneTech Laboratories Inc, USA), constructed in the vector  $\lambda$ EMBL-3 SP6/T7 (Fig. 5.1, A) was used to isolate members of *OsAMT1* family. This library was made from 8-20 kb fragments

generated from partial digestion of rice genomic DNA with the restriction enzyme *Sau3A1* and resulting fragment were cloned into the *Bam*HI site in the vector  $\lambda$ EMBL-3 SP6/T7. The titre of the library was  $\sim 1.8 \times 10^6$  pfu ml<sup>-1</sup> ( $\sim 12$  genome equivalents).

A full-length *OsAMT1;1* cDNA was used as a probe to screen the library. Seven positive plaques namely, A3, A4, A6, E1, E2, J1 and K1 were isolated and studied further. Because *OsAMT1;1* cDNA has one *Bam*HI, two *Xho*I sites and no internal *Sac*I site (the three available restriction sites on either side of the insert in the recombinant plasmid (Fig. 5.1, A)), *Sac*I was selected to release the inserts in the hope that a single fragment would be released from the vector. Digested fragments were separated by electrophoresis, transferred onto nylon membranes and hybridized with radioactively labelled full-length *OsAMT1;1* cDNA. A high stringency wash (final two washes with 1XSSC, 0.2%SDS at 65°C for 15 min.) was performed. The *OsAMT1;1* probe hybridized to one band of the same size ( $\sim 5$  kb) in A3, A6, and E1, one different ( $\sim 12$  kb) band in E2, and two bands in both J1 ( $\sim 5$  kb and 4.5 kb) and K1 ( $\sim 5$  kb, 6 kb). The restriction pattern and hybridization data suggested that A3, A6 and E1 contained one member of the *OsAMT1* gene family, J1 and K1 contained a second member, and E2 encompassed a third member of the gene family (Fig. 5.1). Clones A3, E2, J1 and K1 were selected for further subcloning, sequencing and analysis.

Clone A3 was double digested with *Sfi*I and *Kpn*I producing 6 fragments of 15 kb, 14 kb, 8.5 kb, 7.5 kb, 5 kb and 1.5 kb (Fig. 5.2). A Southern blot analysis indicated the 7.5 kb fragment contained an *OsAMT1* gene (Fig. 5.2, B & C). This fragment was eluted from an agarose gel and digested with *Sac*I to produce a 4.8-kb fragment and three smaller fragments of different sizes. The 4.8 kb fragment (based on previous Southern data) was eluted and cloned into the vector pGEM<sup>®</sup>-7Zf<sup>+</sup> (Promega Corporation, USA) to obtain the recombinant plasmid pPIMP321 (Fig 5.2, D).

*Sac*I digestion of the clone E2 produced three bands (Fig. 5.3, A) of which the middle band of  $\sim 12$  kb contained the insert (based on Southern data, Fig 5.1, C). This 12 kb fragment was eluted from gel and cloned into pK<sup>+</sup>Bluescript SK(-) vector (Stratagene, USA) to produce pPIMP770 (Fig. 5.3, B).

Fragments generated by *SacI* digestion of clones J1 and K1 were subcloned into pK<sup>+</sup>Bluescript SK(-) using a shotgun approach due to difficulties in ascertaining the correct fragments. From J1 one clone named pPIMP827 was isolated with a 4.8 kb insert and from K1 several clones with different sized inserts were isolated (Fig. 5.4, A & B). Southern blot analysis showed that clones pPIMP827 and pPIMP834 (and presumably pPIMP936) contained *OsAMT1;1* homologues (Fig. 5.4, C & D).

## 5.2.2 Sequencing and Analysis of Genomic Clones

Clone pPIMP770 (~12 kb) was sequenced from both the 5' and 3' ends using M13 universal forward and reverse primers. Initial BLAST searches of the GenBank non-redundant nucleotide database revealed no homology to known ammonium transporter genes. Sequence obtained using M13 forward primer revealed *SacI/XhoI/BamHI* restriction recognition sequences from the cloning vector  $\lambda$ EMBL-3 SP6/T7. To make sure that the *OsAMT1* gene was within this large clone (~12 kb), two primers were designed to amplify *OsAMT1* gene sequences. These primers were designed to anneal to the 5' end of the *OsAMT1;1* cDNA sequence (*OsAMT1;1*-44<sup>+</sup>) and the 3' end (*OsAMT1;1*-2058<sup>-</sup>) (Refer to Table 3.1 and section 3.2). Using a modified Expand<sup>TM</sup> Long template PCR system (Boehringer Mannheim, Australia), a 2.04 kb PCR product was obtained and cloned into a pGEM<sup>®</sup>-T vector (Promega Corporation, USA Fig. 5.6). One of the resulting clones (pPIMP577) was sequenced from both ends using universal forward and reverse primers and the sequences were almost identical (99%) to *OsAMT1;1* cDNA (Figure 5.7, A & B). The data show that the coding region of *OsAMT1;1* gene does not contain any introns. Further sequencing of the pPIMP770 using primers designed from the sequence of pPIMP577 yielded sequence of the entire coding region as well as upstream promoter and downstream terminator regions. The sequencing strategy is shown in Figure 5.5. A 4123 nt sequence along with salient features is presented in Figure (5.14, A). Upstream of ATG there are several 5'-GAT(A/T)A-3' core sequences and putative TATA box (TATAAAT) and CAAT (CAT) sequences (found in almost all eukaryotes) were located at positions -111 and -166 from the start codon. A possible inverted repeat structure capable of forming a stem-loop structure was also located in the 3' region which might serve as a transcription terminator.

The sequence PISE7 obtained from pPIMP321 (4.8 kb insert) using the M13 universal forward primer revealed 86% identity with sequences complementary to *OsAMT1;1* cDNA and also revealed the *SacI/XhoI/BamHI* sequence from the cloning site of vector  $\lambda$ EMBL-3 SP6/T7. This possible second member of the *OsAMT1* gene family identified, was designated *OsAMT1;2* (Fig. 5.8 & Fig. 5.9). As expected (because of the size of the clone) the PISE8 sequence obtained using the M13 universal reverse primer did not show any similarity to *OsAMT1;1*. Upstream sequences were obtained by progressively using internal primers (Fig. 5.8). An additional 145 bp 3' end sequence of *OsAMT1;2* was obtained from the overlapping clone pPIMP827 (5 kb insert) using the M13 universal reverse primer. A total of 4654 bp sequences were obtained (Fig. 5.14, B). Several 5'-GAT(A/T)A-3' core sequences were also present in this clone in its putative promoter region. A putative TATA box (TATAAAT) and CAAT (CAT) are located at positions -111 and -166 upstream from the start codon, respectively. A possible stem-loop structure after the coding region is also present in this gene.

The sequence PISE 47 from clone pPIMP834 (6 kb insert) obtained with M13 universal reverse primer, showed 88% identity with sequence from *OsAMT1;1* cDNA, and was also different from *OsAMT1;2*. The gene was therefore designated as *OsAMT1;3* (Fig. 5.9 & 5.10). The polylinker (*SacI/XhoI/BamHI*) of vector  $\lambda$ EMBL-3 SP6/T7 was also present in this sequence. As expected, the sequence PISE48 with the forward primer did not show any identity with known ammonium transporter genes. Further sequencing was performed using internal primers as described previously. A total of 2987 bp sequences has been completed (Fig. 5.14, C). Several 5'-GAT(A/T)A-3' core sequences and a putative TATA box (TATAAAT) (at position -124) and CAAT (CAT), (at position -175) were identified in this sequence. Again a possible simple inverted repeat structure capable of forming a stable stem-loop structure was found downstream of the coding region of *OsAMT1;3*.

### 5.2.3 Comparison between Members of *OsAMT1* Gene Family

The coding regions of the three members of the *OsAMT1* gene family, *OsAMT1;1*, *OsAMT1;2* and *OsAMT1;3* were analyzed using the programs of the University of Wisconsin Genetics Computer Group (GCG) (Devereux *et al.*, 1984). Coding regions of all three genes were found to contain a single exon, with open reading frames

(ORFs) of 1590 bp, 1491 and 1485 in *OsAMT1;1*, *OsAMT1;2* and *OsAMT1;3*, respectively (Fig 5.14, A, B & C). The amino acid (aa) sequences deduced from these three ORFs consisted of 533, 498 and 496 residues, respectively. The deduced amino acid sequences of hypothetical *OsAMT1;1*, *OsAMT1;2* and *OsAMT1;3* proteins showed 95.6%, 73.0% and 80.8% sequence identity to that of *OsAMT1;1* cDNA. The pairwise similarities among the three putative ammonium transporters, presented in Table 5.1, ranged from 79 to 85%. Sequence divergence of *OsAMT1;2* from the other members is contained within the 5' and 3' ends of the coding region while *OsAMT1;3* showed divergence only at the 3' region. The pairwise comparison (using GCG program PHYLUP) of available plant ammonium transporter ORF sequences and the phylogenetic relationships are presented in Figure 5.11, A & B. In this phylogenetic analysis, the rice ammonium transporters emerged as a single cluster, separate to those from other ammonium transporters. Rice ammonium transporters are closer to *Arabidopsis AtAMT1* members (59-65% similarity) than those from tomato (50-60% similarity).

#### 5.2.4 Promoter::*uidA* Constructions.

To study the expression and regulation of ammonium transporter genes, predicted promoter region sequences from each *OsAMT1* member was cloned in front of the promoterless reporter gene *uidA* (*gus*).

Based on the presence of several promoter signals such as TATAA and CAAT, 2.4 kb of sequence upstream of the ATG codon was selected as a putative promoter region. A 3.5 kb region upstream of the ATG codon was amplified from the genomic clone pPIMP770 using primers SE74-668<sup>+</sup> and *OsAMT1;1-90'*, the latter designed to create a *NcoI* site spanning the ATG codon. The resulting 3.5 kb PCR fragment (Fig. 5.12, A) was digested with the restriction enzymes *HindIII* and *NcoI*. A 2.5 kb *HindIII/NcoI* fragment (Fig. 5.12, B) was recovered and cloned into the *HindIII/NcoI* digested pABCD (Bhattacharyya-Pakrasi *et al.*, 1993) containing the vector backbone sequences, *uidA* gene and *nos* terminator. The recombinant construct pPIMP920 (Fig. 5.12, C) shows the inserted promoter region in front of the reporter gene *uidA*.

A 2.5 kb *Ava*I (end filled) /*Hind*III fragment upstream of the start codon ATG of clone pPIMP321 (*OsAMT1;2*) was cloned into a *Nco*I(end filled)/*Hind*III site of pABCD vector to produce the recombinant plasmid pPIMP841 (Fig. 5.12, D).

Due to the lack of appropriate cloning sites, a 1.4 kb *Fok*I(end-filled)/*Eco*RI fragment from clone pPIMP834 (*OsAMT1;3*) was first cloned into an *Eco*RI/*Eco*RV digested pKr Bluescript SK (-) vector (Stratagene). A *Fok*I(end filled)/*Bam*HI fragment from the resulting recombinant plasmid pPIMP925 was cloned into a *Nco*I(end filled)/*Bgl*II fragment of pABCD vector (Fig. 5.12, E).

### 5.3 Discussions

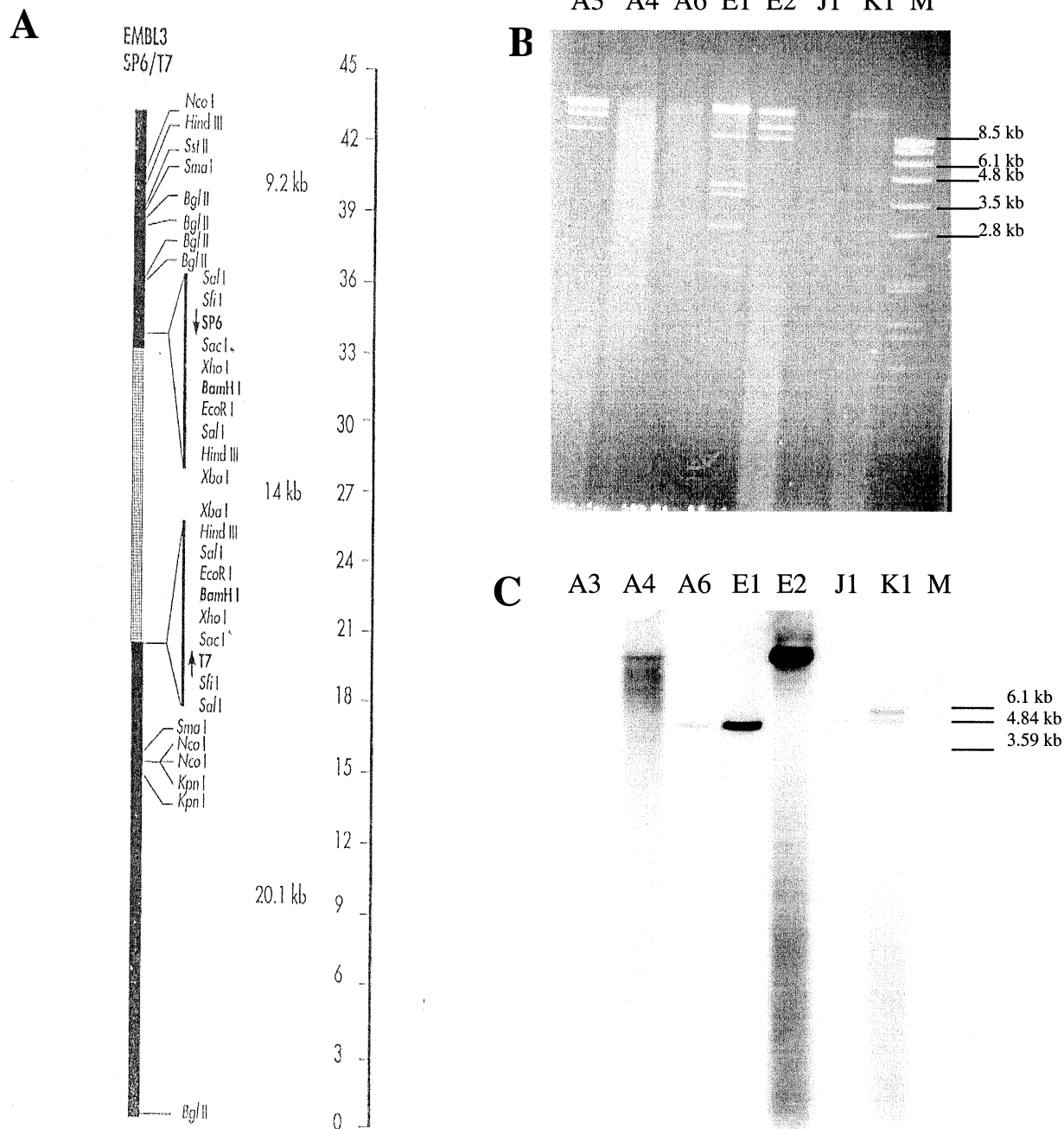
Ammonium transporter genes have been isolated from three different plant species and their expression studied under different nitrogen regimes to understand their role in root ammonium uptake. Evidence for their role in high affinity  $\text{NH}_4^+$  uptake comes from complementation and uptake kinetics studies in yeast mutants defective in  $\text{NH}_4^+$  transport (Lauter *et al.*, 1996; Ninnemann *et al.*, 1994). In tomato *LeAMT1;1* exhibited the highest expression in root hairs (Lauter *et al.*, 1996), whereas, *AtAMT1;1* showed its highest expression level in *Arabidopsis* roots (Gazzarrini *et al.*, 1999). Furthermore, expression levels of the *Arabidopsis AtAMT1;1* positively correlated with high affinity  $\text{NH}_4^+$  influx (Rawat *et al.*, 1999).

Three members of the *AtAMT1* family have now been isolated and characterized. Recently an additional family of ammonium transporters (*AtAMT2*) was reported in *Arabidopsis* (Gazzarrini *et al.*, 1999; Ninnemann *et al.*, 1994; Sohlenkamp *et al.*, 2000). It is conceivable that rice also has different families of ammonium transporters, as evident from the presence of at least 8-10 hybridizing bands on low stringency Southern blots probed with a full length *OsAMT1;1* cDNA. Using a high stringency hybridization approach, genomic clones of two more members of *OsAMT1* family were isolated in this project. Sequence data from these clones revealed that all three are intronless at least in coding regions. These represent the first genomic clones of ammonium transporters isolated from rice. The putative coding region sequences of *OsAMT1;1* cDNA and that of one the genomic clone (Clone pPIMP770) were 98.6% and 95.6% homologous at the nucleotide and amino acid levels, respectively. The differences were due to small deletions, additions and

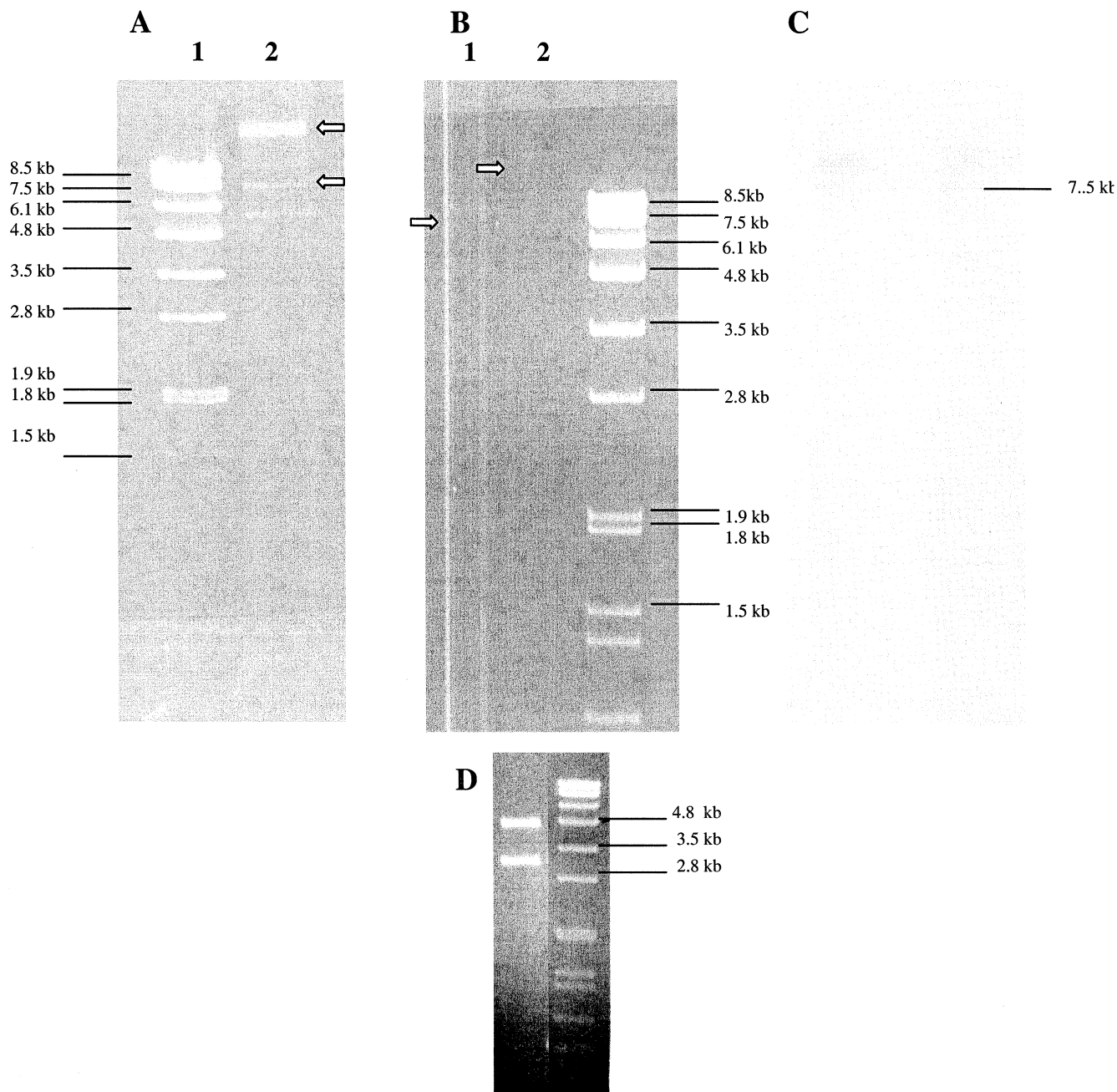
substitutions spread over the entire sequence. It is possible that these are actual sequence differences in *OsAMT1:1* from cultivar Nipponbare (from which the cDNA was obtained) and cv. IR36 (from which genomic library was made). It is also possible that single base substitutions were introduced during cDNA synthesis or sequencing. Sequence identities of the other two groups of clones with that of *OsAMT1:1* cDNA were 73% and 81%, respectively, suggesting that they are different members of the *OsAMT1* family. Phylogenetic analysis of all of the available ammonium transporters indicates that the rice ammonium transporters identified in this study belong to the *MEP/AMT1* super family (Gazzarrini *et al.*, 1999). Most members of this super family seem to be high affinity ammonium transporters (Sohlenkamp *et al.*, 2000). Some of which are constitutively expressed (Gazzarrini *et al.*, 1999; Lauter *et al.*, 1996) and others are responsive to levels of intermediate products (such as glutamine) of the nitrogen assimilation pathway (Rawat *et al.*, 1999). The existence of several ammonium transporters in rice suggests that each play unique roles in the plant nitrogen acquisition. Rice plants are usually exposed to changing level of ammonium supply from soil and fertilizers during their growth cycle and they usually achieve well balanced growth (Mae, 1997). This balance is probably maintained by the use of multiple transport systems with different kinetic properties and regulation, as observed in *Arabidopsis* (Gazzarrini *et al.*, 1999). The presence of  $\text{NH}_4^+$  strongly repressed both MEP1 and MEP2 transport activities in yeast cells indicating the involvement of nitrogen catabolite repression (Marini *et al.*, 1994). This repression mechanism acts at the level of permease synthesis and not on the carrier protein itself (Dubois and Grenson, 1979). Therefore, the expression of *AMT1* encoded protein is under the control of a nitrogen-regulated promoter, not only in plants but also in yeast.

One way to study the expression properties of ammonium transporters is to study the upstream control sequences in the promoter region. Nitrogen control of *MEP* genes in yeast is mediated by two GATA transcription factors, GLN3p and Nillp, which are also involved in the regulation of many other nitrogen-regulated genes (Marini *et al.*, 1997). These GATA factors activate yeast *MEP* gene transcription under poor nitrogen supply. Under adequate nitrogen, nitrogen catabolite repression reduces transcription of nitrogen regulated genes. GATA factors bind to the activating sequence 5'-GAT(A/T)A-3', which is often represented several times in

the upstream region of genes that respond to nitrogen control (Marini *et al.*, 1997). All three *OsAMT1* genes identified in this study possess several 5'-GAT(A/T)A-3' core sequences in their promoter regions. The presence of multiple 5'-GAT(A/T)A-3' sequence motifs in the rice *OsAMT1* genes indicate that higher plants possess a system of nitrogen regulation similar to that which exist in yeast. Other common control elements such as TATA box (TATAAAT) and CAAT (CAT) are also present in upstream sequences of all three genes. As a first step towards understanding the site and level of expression and regulation of these putative ammonium transporters, promoters from each of these genes were cloned in front of the reporter gene *uidA* (*gus*) and rice cv. Taipei 309 was transformed with these constructs. Expression pattern of GUS in the resulting transgenic lines will show the location and level of expression of the different *OsAMT* genes under different developmental or environmental conditions. Unfortunately due to time constraints, results from these experiments have not been included in this thesis.



**Figure 5.1** (A) Vector map of EMBL3 SP6/T7; (B) *SacI* digestion pattern of isolated genomic clones A3, A4, A6, E1, E2, J1 & K1. Lane M contains molecular wt. marker; (C) Southern blot of the digested clones probed with full length *OsAMT1;1*.



**Figure 5.2** (A)  $\lambda$ clone A3 was digested with restriction enzymes *Sfi*I and *Kpn*I and separated in an agarose gel (lane 2), ( $\Leftrightarrow$ ) indicates the band eluted from the gel and lane 1 is the mol. wt. marker; (B) eluted 7.5 kb (lane 1) and a mixture of 14 & 15 kb bands (lane 2) separated in an agarose gel; (C) Southern blot of B showing hybridization with the 7.5 kb fragment; (D) clone pPIMP 321 digested with *Sac*I liberating the 4.8 kb insert.