Molecular Physiology of Ammonium Transport in Rice

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the Australian National University

By

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Declaration

I hereby declare that the work presented in this thesis has been carried out by myself and does not incorporate any material previously submitted for another degree in any university. To the best of my knowledge and belief, it does not contain any material previously written by anther person, except where due reference is made in the text.

Date: 31/8/01

Moahammad Shamsul Hoque

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Abstract

The availability of nitrogen in the soil is one of the main limiting factors for plant productivity, biomass accumulation and crop yield. Well-aerated soils are relatively rich in NO_3^- and poor in NH_4^+, whereas in anaerobic soils of irrigated or rain-fed lowland rice, NH_4^+ is the most prevalent source of nitrogen. Ammonium uptake is presumed to be controlled by at least two gene families in plants, namely *AMT1* and *AMT2*. Each gene family consists of one to several members, some of which have been cloned in *Arabidopsis*, tomato and rice. As in *Arabidopsis*, rice is predicted to have multiple members for the *AMT1* gene family. Preliminary studies of an ammonium transporter (*OsAMT1;1*) in rice (cv. Taipei 309), presented in this thesis, revealed its expression in roots and mature leaves under different nitrogen regimes. Expression levels were generally higher in roots than in leaves. The project was aimed at providing an insight into rice *AMT* (*OsAMT*) genes, their regulation and their physiological roles in rice.

A transgenic approach was undertaken to investigate the role of *OsAMT1* in ammonium uptake and consequent ammonium assimilation under different nitrogen regimes. Two expression cassettes were made using the full-length cDNA of *OsAMT1;1* to have their sense or antisense expression driven by the maize ubiquitin promoter. Transgenic lines were produced from two rice cultivars, namely Taipei 309 and Jarrah (an Australian cultivar), by *Agrobacterium*-mediated transformation using these expression cassettes mounted on the binary vector pWBVec8 that contained a hygromycin resistance gene (*hph*) as the selectable marker. Only a small number of Taipei 309 transgenic lines could be regenerated with the antisense transgene, most of which were sterile and none showed any down-regulation of the endogenous *OsAMT1;1* mRNA. No antisense transgenic lines could be regenerated from cv. Jarrah

The *Ubi1(I)* promoter-driven *OsAMT1;1* sense transgene in cvs. Taipei 309 and Jarrah increased *OsAMT1;1* transcript levels, which positively correlated with transgene copy number. Under both ammonium-fed and ammonium-starved growth condtions, ammonium-induced depolarisation of root cell plasmamembrane electrical potentials (E_m) were substantially greater in *OsAMT1;1* over-expressing transgenic lines compared to wild type plants. These transgenic plants showed increased ammonium uptake and root ammonium content, however, they had decreased biomass especially when grown under high concentrations of NH_4^+.

A low stringency Southern blot hybridization of genomic DNA isolated from wild type Taipei 309 with radioactively-labelled full length *OsAMT1;1* cDNA showed 8-10 hybridizing bands. Using a similar low stringency hybridization approach, genomic clones of three members of the *OsAMT1* family, including the *OsAMT1;1*, were isolated. Sequence data from these clones revealed that all three were intronless, at least in the coding region. These represent the first genomic clones of ammonium transporters isolated from rice. The DNA sequences of the predicted coding regions of these genes showed 98.6%, 81% and 73% homology to that of *OsAMT1;1* from cv Nipponbare.

On the basis of the presence of several promoter signals, such as TATA and CAAT boxes, a 2.4 kb sequence upstream of the ATG codon from *OsAMT1;1,* a 2.5 kb fragment from clone *OsAMT1;2*, and a 1.4 kb fragment from clone *OsAMT1;3*, were selected as putative promoter regions and cloned in front of the reporter gene *UidA*. These constructs were then used to transform rice cv. Taipei 309 to study the expression and regulation of these ammonium transporter genes. Determination of the expression pattern of GUS in resulting transgenic lines would allow the location and level of expression of the different *OsAMT* genes under various developmental and environmental conditions to be measured. Due to time constrains, results from these experiments could not be included in this thesis.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Agronomical Aspects of Nitrogen Nutrition in Rice

Rice can grow in diverse agro-ecological conditions as far north as 49^o of latitude in Czechoslovakia and as far south as 35^o of latitude in Australia. The cultivated species of rice, *Oryza sativa*, has two subspecies, indica and japonica (Oka, 1982). The morphological and physiological characteristics, including nitrogen absorption and assimilation, are substantially different in these two subspecies. Indica varieties are more efficient in nitrogen absorption than japonica, while japonica is more efficient in the assimilation of nitrogen. Therefore, japonica varieties produce more yield increase than indica in response to fertilizer nitrogen (Augladette, 1965).

Rice is a diploid plant with a haploid chromosome number of 12 and a nuclear DNA content of about 5.6×10^8 bp (Walbot and Gallie, 1991). Because of its small genome size, the ease with which it can be genetically transformed and the similarity of its gene order and gene sequences with other cereals, rice has become the focus of cereal genome studies.

1.1.1 Rice Varieties

In the past four decades there has been a rapid increase in world-wide rice production due mainly to the introduction of modern high yielding rice varieties and only slightly to the increase in cultivated land area. Traditional tropical varieties that were cultivated and adapted for centuries were late maturing, photoperiod sensitive and high tillering. They have tall, thick but weak culms, long thin drooping and pale-green leaves, relatively large loose panicles, and they are not yield-responsive to N fertilizers (Evenson, 1977). The semi-dwarf modern rice varieties are believed to be derived from a natural mutant discovered in China in about 1900 (Greenland, 1997). This mutant was used in a breeding program in Taiwan, which led to the development of a semi-dwarf indica variety Dee-geo-woo-gen that shows profuse tillering property. (Barke *et al.*, 1985). In 1949, Dee-geo-woo-gen was crossed with a tall disease resistant variety Tsai-yuan-chunh and in 1956 Taichung Native 1 (TN1) was released from this cross (Yoshida, 1981). Thus, TN1 is considered as the first high yielding rice variety. In 1962, IRRI (International Rice Research Institute) rice breeders crossed Dee-geo-woo-gen with Peta, a popular tall, high-tillering variety grown in the Philippines. From this cross, IR8 was selected and released in 1966. IR8 is considered as the first high yielding indica variety adapted to tropical climate which responds to nitrogen. Since the introduction of IR8 by IRRI, there has been a continuous release of new high-yielding rice varieties, which are bred for the tropics. These varieties required large doses of N to achieve full yield potential (Dahlberg, 1997). Surprisingly, nitrogen requirements of most of the modern high yielding rice varieties are remarkably similar (Hsu, 1970; Murayama, 1970; Park, 1975). When grown under favourable N conditions the less leafy semi-dwarf plant types produce relatively high grain yields.

The two rice cultivars used in this study, Jarrah and Taipei 309, possess different genetic backgrounds. Jarrah is a modern high yielding, medium grain japonica variety bred at Yanco Agricultural Institute, Australia from the cross M7*2/Somewake. It was developed by Reinke, Lewin, and Blakeney, and released in 1993 (personal communication). The parent M7 is a japonica variety from California (Carnahan *et al.*, 1978) and the other parent Somewake is an old japonica variety from Japan (McDonald, 1994). The genetic background of the rice cultivar Taipei 309 is not fully known. It was developed in Taipei, Taiwan and is at least 30 years old (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 Taipei 309 is described as an intermediate between japonica and indica with 31-40% lodging. It has been widely used for tissue culture and rice transformation studies (Hamid *et al.*, 1996; Hiei *et al.*, 1994; Li *et al.*, 1993).

1.1.2 Rice Agroecosystems

Over the last 10,000 years rice has spread around the world and has been adapted to a wide range of environments. It is now grown from warm and humid lowlands to elevations of over 2700 m above sea, from the equator to as far north as 49 degree of latitude, and from areas that is subject to flooding to depths up to 5 meters to uplands where it is never flooded (IRRI, 1995). The following categories of rice have been defined based on these agroecological factors (IRRI, 1995) (1) the flood-prone rice, (2) rainfed lowland rice, (3) irrigated rice and (4) upland rice. The land of flood-prone rice is level to slightly sloping or lowland fields. During the growing season, rice fields are subject to more than 10 consecutive days of medium to very deep flooding (50 to more than 300 cm water). Most flood-prone rice is seeded immediately before the arrival of the floodwaters (Catling *et al.*, 1983). Soils are aerobic to anaerobic. For rainfed lowland rice, the land is level to slightly sloping, and subject to non-continuous flooding of variable depth and duration. Fields remain submerged for more than 10 consecutive days with depth of not more than 50 cm. Most lowland rice is transplanted into waterlogged soil and in some areas direct sowed into puddled or ploughed dry soil. Soil in this rice system alternates between aerobic and anaerobic, with variable period and duration. For irrigated rice, the fields are level and bounded with water control. Rice is transplanted or directly sowed in puddled soil. Soils remain in anaerobic conditions during the growth of the crop. With upland rice, fields are steeply slopping and rarely flooded. Rice is directly seeded on ploughed dry soil or dibbled in wet, non-puddled, mostly aerobic soils.

1.1.3 Forms of Nitrogen Available in Soil

Different forms of nitrogen are available for plants in common agricultural soils, including organic nitrogen associated with the soil humus and inorganic nitrogen, especially nitrate and ammonium. Inorganic nitrogen in the soil is derived from both natural, biochemical processes and industrial nitrogen fixation. Biogeochemical processes include biological nitrogen fixation, which produces approximately 170×10^6 t of reduced nitrogen annually (Burns and Hardy, 1975), and atmospheric oxidation of N_2 during lightening storms. Approximately one third to half of all nitrogen inputs into agricultural systems comes from industrial nitrogen fixation that produces ammonia via the Haber-Bosch process (Hauck, 1985b). Under normal agricultural conditions, soil NO_3^- concentrations can range between 0.5 and 10 mM, while NH_4^+ concentrations are often 10 to 1000 times lower, reaching the millimolar range only in exceptional cases such as after fertilization (Marschner, 1995). The NH_4^+ nitrogen in soil may be absorbed by soil microorganisms, or used in considerable amount by higher plants very rapidly, or fixed by clay minerals and organic matter and oxidised by soil microorganisms through the process of nitrification. Well-aerated soils are generally rich in NO_3^- and poor in NH_4^+ because of the activities of nitrifying bacteria (Kronzucker *et al.*, 1995). In contrast, in anaerobic and acidic soils, ammonium is often the predominant form of inorganic nitrogen due to inhibition of nitrification in these soils (Lovoie *et al.*, 1992; Sasakawa and Yamamoto, 1978). Different plant species show different preferences for inorganic N, although it is generally believed that NO_3^- is the preferred source of nitrogen for the growth and yield of most crop species (Haynes, 1986; Lewis, 1986). Nonetheless, there are many reports that NH_4^+ is the preferred nitrogen source for rice (Dijkshoorn and Ismunadji, 1972; Fried *et al.*, 1965; Sasakawa and Yamamoto, 1978; Wang *et al.*, 1993; Yoneyama and Kumazawa, 1974).

1.1.4 Nitrogen Fertilizers and Environmental Hazards

Although very abundant, gaseous N_2 cannot be used directly by plants. Gaseous N_2 is converted to forms available to plants by several ways. These include fixation by symbiotic bacteria such as *rhizobia,* fixation by free-living soil microorganisms, fixation by industrial processes (manufacturing of nitrogen fertilizers) and fixation by atmospheric electric discharge.

The Green Revolution resulted in a significant increase of worldwide cereal yield. Worldwide wheat yield increased from 1.2 t/ha in 1963-1965 to 2.5 t/ha in 1990-1992 and for rice this increase was from 2.1 t/ha in 1963-1965 to 3.5 t/ha in 1990-1992 (Anonymous). The Green Revolution involved mainly the cultivation of high yielding varieties and use of fertilizers, particularly nitrogen. The high yielding varieties were developed simply to convert applied fertilizer to grain yield because the availability of nutrients in the soil was sub-optimal. Therefore, without adequate dosages of nitrogen fertilizers high yielding varieties yielded lower than traditional varieties. Consequently, worldwide use of fertilizers increased several folds (Anonymous). According to one estimate 70% of the world rice is produced in rainfed or irrigated lowland systems in Asia (IRRI, 1997), where nitrogen fertilizer is a major limiting factor for yield. Consequently, each year large amounts of nitrogen fertilizer are used in this system. Most of this nitrogen is applied in the form of urea, which is converted to ammonium prior to being taken up by plants. Generally, added fertilisers are not completely used by the crop plants. Between 30 and 80 percent is taken up by crops depending on various factors (Craswell and Godwin, 1984). The percentage is often lower for flooded rice or for crops growing in high-rainfall areas (Hauck, 1985b). The rest of the fertilzer nitrogen is eventually lost to the surrounding environment by leaching of NO_3^- to the ground water and river systems or by loss of gaseous N_2, N_2O and NH_3 into the atmosphere (Mengel, 1992). Therefore, there are considerable economic and environmental costs involved in fertiliser production and use.

1.1.5 Nitrogen Nutrition, Growth and Development of Rice Plant

Depending on the genotype and environmental conditions, rice plants complete their developmental life cycle in 3-6 months from complete germination to maturity. Agronomically, the development of rice plant may be divided into three phases (Fig 1):

- The vegetative phase that refers to a period from germination to the initiation of panicle primordia. This stage is identified by active tillering, gradual increase in plant height, and leaf emergence at regular intervals.
- The reproductive phase, from panicle primordia initiation to flowering. The reproductive phase is characterised by stem elongation, emergence of the flag leaf, booting, heading and flowering.
- The ripening grain filling phase, from anthesis to maturity, is characterised by the development of seeds and the senescence of vegetative organs, leaves and roots.

At the vegetative stages (Fig.1) nitrogen mainly promotes early growth of the plant and increases the numbers of tillers. The initial number of tillers determines the potential panicle number and panicle primordia initiation starts at the maximum tillering stage (Yoshida, 1981). Nitrogen acquired during maximum tillering stage makes the most effective contribution to spikelet production (Yoshida, 1981). The rates of leaf area expansion and dry matter accumulation are also greatest from panicle primordia initiation up to the final spikelet initiation. During grain filling direct nitrogen uptake decreases and a large part of the nitrogen that accumulates in the grain is translocated from leaf blades and straw (Mae, 1997).

1.2 Physiology of Ammonium Acquisition by Rice

1.2.1 Sites of Nitrogen Uptake

Nitrogen is available in a range of forms to plants. Consequently, plants use different pathways for nitrogen uptake. Uptake of inorganic and organic forms of nitrogen from soils or from solution culture occurs through the plasma membrane of epidermal and cortical root cells (Larsson and Ingemarsson, 1989). The forms of absorbed nitrogen may play a role in the regulation of root growth and development (Bloom *et al.*, 1993).

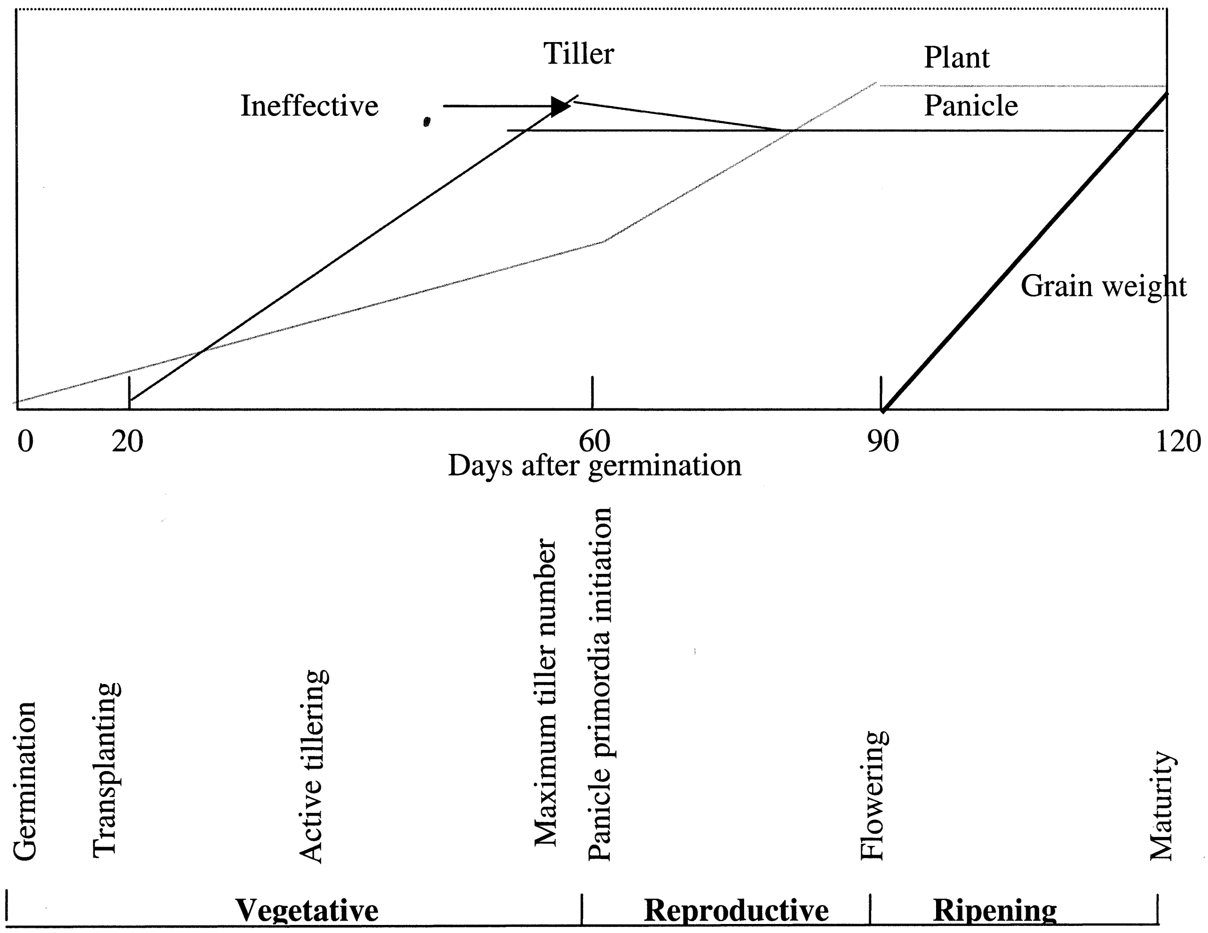

Figure 1. Developmental stages of a 120 d rice variety grown in the tropics under the transplanting cultivation system. (From Yoshida S, 1981)

Root hairs which cover apical root zone can make up 70-80% of the total root surface and are believed to play a major role in nutrient uptake (Marschner, 1995). There also appears to be N transporters at the plasma membrane of leaf cells. These transporters probably play a role in the recovery from N deficiency (chlorosis) following foliar application of NO_3^- or NH_4^+ (Bowman and Paul, 1992; Yin *et al.*, 1996).

1.2.2 Kinetics of Ammonium Transport

Ammonia (NH_3) is a weak base with a pKa value of 9.3.

$$NH_3 + H^+ \leftrightarrow NH_4^+$$

Therefore, at pH 7.3, 99% of ammonia is in the protonated form. At lower pH values, such as those found in the plant cell apoplast much less than 1 % of ammonia is in the neutral form. Nonetheless, there is physiological evidence that both forms, NH_3 and NH_4^+, are taken up independently by plant cells (Kleiner, 1981; MacFarlane and Smith, 1982; Smith and Waker, 1978; Walker *et al*, 1979). Uptake of ammonium into plant roots generally exhibits biphasic kinetics, which indicate the presence of two distinct transport systems for ammonium. One is a high affinity transport system, HATS, and the other is a low affinity transport system, LATS (Fried *et al.*, 1965; Kronzucker *et al.*, 1996; Ullrich *et al.*, 1984; Wang *et al.*, 1993). In rice the HATS operates at low external $[NH_4^+]_O$ (<1mM), shows saturation kinetics, is energy dependent, and leads to depolarization of the plasma membrane electrical potential during ammonium transport. At high external $[NH_4^+]_O$, (>1mM), the LATS predominates, although it is still not clear whether a transporter or simple diffusion of NH_3 is responsible for this second component of ammonium uptake. One interesting aspect of the HATS is that its K_m for ammonium appears to be dependent on prior ammonium provision. When rice plants were grown in 2 μm NH_4^+ the apparent K_m was 32.2 ± 2.1 μm and V_{max} was 12.8 ± 0.2 μm $g^{-1}FW\ h^{-1}$. On the other hand K_m increased to 188.1 μm and V_{max} decreased to 3.4± 0.2 μm $g^{-1}FW\ h^{-1}$ when plants were grown in 1000 μM of NH_4^+ (Wang *et al.*, 1993). These results illustrate a second feature, which is common to many species; namely, that total activity of the HATS is regulated by the N-status of the plant. This is considered further in the next section.

1.2.3 Regulation of Ammonium Uptake in Plants

1.2.3.1 Nitrogen Regulation of Ammonium Transport

Under normal agricultural conditions soil NH_4^+ concentrations are often 10 to 1000 times lower than NO_3^- concentrations (Marschner, 1995). Nonetheless, some plants preferentially take up NH_4^+ under limited nitrogen supply and in the presence of both nitrogen forms (Xu *et al.*, 1992). Plants have evolved a strict regulatory system for the uptake of NH_4^+ using both high affinity and low affinity transporters. Studies with bacteria, fungi and higher plants suggested that NH_4^+ uptakes in these organisms are regulated by the nitrogen status of cells and that uptake rates are higher in N limiting conditions than in conditions of adequate N supply (Crawford and Glass, 1998; Jayakumar *et al.*, 1985; Kleiner, 1981; Marini *et al.*, 1997). With this observation, it appears that there are similarities between nitrogen regulation of ammonium transporter (*AMT*) in bacteria, yeast and plants. However, the mechanisms involved often differ in different organisms.

In bacteria, regulation of ammonium transport and nitrogen assimilation is controlled by the global nitrogen regulatory system, Ntr (Jayakumar *et al.*, 1986; Kamberov *et al.*, 1995). The Ntr system consists of four proteins: a uridylyltransferase/uridylyl-removing enzyme UTase/UR, encoded by the *glnD* gene; the PII protein encoded by *glnB* gene; and a two component regulatory system composed of the histidine protein kinase NtrB and the response regulator NtrC (Merrick and Edwards, 1995). The Utase/UT and PII act together and provide a mechanism for sensing the intracellular nitrogen status and NtrB & C are involved as transcriptional activation components (Merrick, 1992). Under nitrogen starved condition NtrB protein catalyzes the phosphorylation of NtrC protein, while under nitrogen sufficient condition NtrB protein catalyzes the dephophorylation of NtrC protein. Phosphorylation of NtrC activates the transcription of genes involved in nitrogen acquisition and utilization, including the ammonium transporter *AmtB* in *Azospirillum brasilense* (Dommelen *et al.*, 1998).

The activity of the ammonium transport system in yeast appears to be controlled by the cell nitrogen status. All three *Mep* (*Mep1*, *Mep2* & *Mep3*) genes are subject to nitrogen catabolic repression when yeast cells are grown on good nitrogen sources including glutamine and high concentrations of ammonium (Marini *et al.*, 1997). On poor nitrogen sources such as proline, and in low concentration of ammonium, all three genes are induced. However, expression levels of the three genes are different, with *Mep2* expression being greater than that of *Mep1* or *Mep3* (Marini *et al.*, 1997). In poor nitrogen conditions, diploid cells of *Saccharomyces cerevisiae* differentiate into a filamentous pseudohyphal growth form that would predictably increase their surface area for nitrogen acquisition. Expression of *Mep2* is essential for pseudohyphal growth whereas expression of *Mep1* or *Mep3* is not. It has been suggested that *Mep2* is involved both as an NH_4^+ permease and as an NH_4^+ sensor to detect low concentrations of internal NH_4^+ in the cell (Lorenz and Heitman, 1998). Two GATA family transcription factors, GLN3p and Nillp, are believed to be involved in the regulation of *Mep* genes (Marini *et al.*, 1997). Under limited nitrogen supply, these GATA factors activate *Mep* gene transcription. Under excess nitrogen, nitrogen catabolite repression reduces transcription of nitrogen regulated genes (Marini *et al.*, 1997). 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.

Several molecular physiological studies have indicated that nitrogen deficiencies induce an enhanced capacity for nitrogen uptake in cereal plants (Lee and Rudge, 1986; Morgan and Jackson, 1988). The rate of NH_4^+ uptake is regulated by the feed back from the accumulated N (Glass and Siddiqi, 1995; Morgan and Jackson, 1988). The signals responsible for the negative regulation of influx in response to elevated NH_4^+ supply during growth are believed to be related to some N pool downstream from NO_3^- or NH_4^+ (Lee and Rudge, 1986). One recent study with *Arabidopsis* suggested that expression of *AtAMT1* gene is regulated by root glutamine concentrations rather than root NH_4^+ (Rawat *et al.*, 1999). There are indications that plants may have inherited parts of one or both of the nitrogen regulatory systems that operate in bacteria and yeast. Recently, a plant nuclear gene (*glB1*) was identified in *Arabidopsis* and castor bean, which encodes a homologue of the prokaryotic PII protein, a component of the bacterial Ntr system (Hsieh *et al.*, 1998). This suggests that a system analogous to Ntr may be present in plants. The promoter regions of the tomato *nia* gene and also rice ammonium transporters contain multiple 5'-GAT(A/T)A-3' sequence motifs (Jarai, 1992; this thesis, Chapter 5). It is still not clear exactly if and how this sequence is involved in nitrogen regulation in plants. Little is known about the molecular mechanisms underlying N regulation in plants, and it is now an open area for future research.

1.2.3.2 Regulation Due to Interaction between K^+ and NH_4^+

The potassium and ammonium ions are of similar size and charge densities (Burman *et al.*, 1989) and consequently there could be some interaction between them during their uptake by plants. At the transport level the uptake of K^+ is significantly reduced by the presence of NH_4^+ in the solution (Deane-Drummond and Glass, 1983; Morgan and Jackson, 1989; Wang *et al.*, 1996). On the other hand, there appears to be no consensus regarding the influence of K^+ on NH_4^+ uptake. Some observed that the uptake of NH_4^+ by plant roots is independent of K^+ levels in the uptake solution and the K^+ status in plants (Mengel *et al.*, 1976; Rufty *et al.*, 1982) while others observed K^+ reducing the NH_4^+ uptake (Scherer *et al.*, 1984; Vale *et al.*, 1988; Wang *et al.*, 1996). The K^+ channel (KAT 1) isolated from *Arabidopsis* had a conductance for NH_4^+ that was 30% of its K^+ conductance (Schachtman *et al.*, 1992). In one experiment, it was found that withholding N from barley plants for 3 days increased not only NH_4^+ uptake but also K^+ uptake (Lee and Rudge, 1986). These observations have been rationalised by assuming that K^+ and NH_4^+ may share a common transport system (Schachtman *et al.*, 1992; Wang *et al.*, 1996). However, NH_4^+ induced membrane depolarization generally decreases K^+ uptake. Ayling (1993) found no direct competition between the two ions which suggests different transporters for the two ions.

1.2.3.3 Regulation Due to Interaction with NO_3^-

Some species of plants prefer NO_3^- for growth and development while others show increased preferences for NH_4^+ over NO_3^-, indicating different uptake regulatory systems for these two forms of N. Kinetic studies of ammonium and nitrate uptake in different plant species indicate that the presence of $N0_3^-$ has little effect on the kinetic parameters for NH_4^+ uptake (Wijk and Prins, 1993), while nitrate uptake was almost totally suppressed by NH_4^+ (Boxman and Relofs, 1988; Ingestad, 1979; Marschner, Haussling, and George, 1991; Wijk and Prins, 1993). It has been demonstrated in rice that net NO_3^- acquisition in the presence of NH_4^+ was 2.2 times less than that with provision of NO_3^- alone (Kronzucker *et al.*, 1999). The reduced NO_3^- acquisition was due to the repression of NO_3^- influx, not enhancement of NO_3^- efflux (Kronzucker *et* *al.*, 1999). Similar results were also observed in maize. When nitrogen starved seedlings were transferred to $^{15}NO_3^-$ solution, net uptake of $^{15}NO_3^-$ was depressed by the presence of ammonium, whereas no effect of ammonium presence was observed on the efflux of $^{14}NO_3^-$ (MacKown *et al*, 1982). On the other hand, uptake of NH_4^+ can even be stimulated by NO_3^-. In rice, net NH_4^+ acquisition was increased by as much as 50% by NO_3^- compared with the NH_4^+ only control (Kronzucker *et al.*, 1999). Similar stimulatory effect of NO_3^- on NH_4^+ acquisition has also been observed in soybean (Rideout *et al.*, 1994).

The presence of ammonium in the growth medium also inhibits the activity of nitrate reductase in roots. In different maize genotypes the presence of ammonium resulted in depressed nitrate reductase (NR) synthesis that in turn, led to reduced incorporation of ^{15}N into the soluble fraction in roots (Pan *et al.*, 1985). In roots of *Phaseolus vulgaris*, the presence of ammonium inhibited nitrate reduction as determined by an *in vivo* assay (Breteler and Nissen, 1982). However, it could not be ruled out that products of ammonium assimilation, rather than ammonium *per se*, were responsible for the observed inhibition (Breteler and Nissen, 1982). The uptake and reduction of nitrate are two separate processes, and there may be a separate effect of ammonium on each of these. Indeed, these separate effects of ammonium have been demonstrated in an experiment with maize. Uptake was less affected by ammonium in the 5 d old seedlings than in the 8 d old seedlings, but reduction was equally affected in both cases (Pan, *et al.*, 1985).

1.3 Biochemistry of Nitrogen Assimilation

In plants, all NO_3^- nitrogen is first reduced to NH_4^+ before being incorporated into an organic nitrogen form (Crawford and Arst, 1993). NO_3^- is reduced to NH_4^+ in two different reactions. The first reaction is catalyzed by nitrate reductase (NR), an enzyme that transfer two electrons to ammonium from NADH or, in a few species, NADPH.

$$NO_3 + NADH + H^+ \rightarrow NO_2 + NAD^+ + H_2O$$

This reaction occurs in the cytosol. Nitrite produced in the cytosol from nitrate reductase action is transported into chloroplast in leaves or proplastids in roots where subsequent reduction to NH_4^+ is catalyzed by nitrite reductase (NI). Ammonium absorbed directly from the soil or produced from nitrate reduction is assimilated either by GDH or Glutamine synthetase/Glutamine 2-oxoglutarate aminotransferase (GS/GOGAT) pathways. Available evidence suggests, including (i) the presence of the necessary enzymes, (ii) the incorporation pattern of labelled nitrogen, and (iii) the use of specific inhibitors, suggest that GS and GOGAT are the main route for NH_4^+ incorporation into organic compounds (Miflin and Lea, 1976). NH_4^+ derived from photorespiration is also assimilated via GS/GOGAT pathways (Fernandes *et al.*, 1985). GS catalyses the addition of an NH_2 group from NH_4^+ to the carboxyl group of glutamate. There are two forms of GS in plants; a cytoplasmic and a plastidic form both of which have affinities for ammonium, with K_m ranging from 10-20 μM (Stewart *et al.*, 1980).

During assimilation of nitrate, ammonium is formed in the plastids, the site of nitrite reductase, and here it is liable to immediate incorporation via the plastid form of GS (Vezin *et al.*, 1987). In leaves, plastidic GS play a predominant role in the assimilation of ammonium derived from photorespiration (Lam *et al.*, 1996). Ammonium that is not assimilated in the plastid can serve as a substrate for GS in the cytosol. On the other hand, when the external N-source is ammonium, on crossing the plasma membrane this nutrient will initially enter the cytosol where it will be exposed to cytosolic GS. Thus the cytosolic form of GS, rather than the plastidic form, is likely to play a dominant role in the assimilation of externally supplied ammonium. In fact, this distinction between plastidic and cytosolic reactions may underlay one of the characteristic differences in gross composition between nitrate and ammonium grown plants, namely their amide glutamine and asparagine content. For example, when maize plants were grown in NH_4^+ as sole nitrogen source, root glutamine and asparagine concentrations were 17 and 21 $\mu M\ g^{-1}$ fresh root weight, respectively. In NO_3^- grown plants, on the other hand, root glutamine and asparagine concentrations were both 1 $\mu M\ g^{-1}$ fresh root weight (Lee *et al.*, 1992). The vacuole is a site for amide storage when amide concentrations in the cytoplasm rise, such as in ammonium-grown plants (Lee *et al.*, 1992). During growth with nitrate as N source, there may be little glutamine in the root cytosol, because glutamine is rapidly metabolized to glutamate by the plastid form of GOGAT (Fig 1.1). Hence there is little opportunity for glutamine to enter the vacuole and accumulate there and glutamine levels in the tissue as a whole remain low.

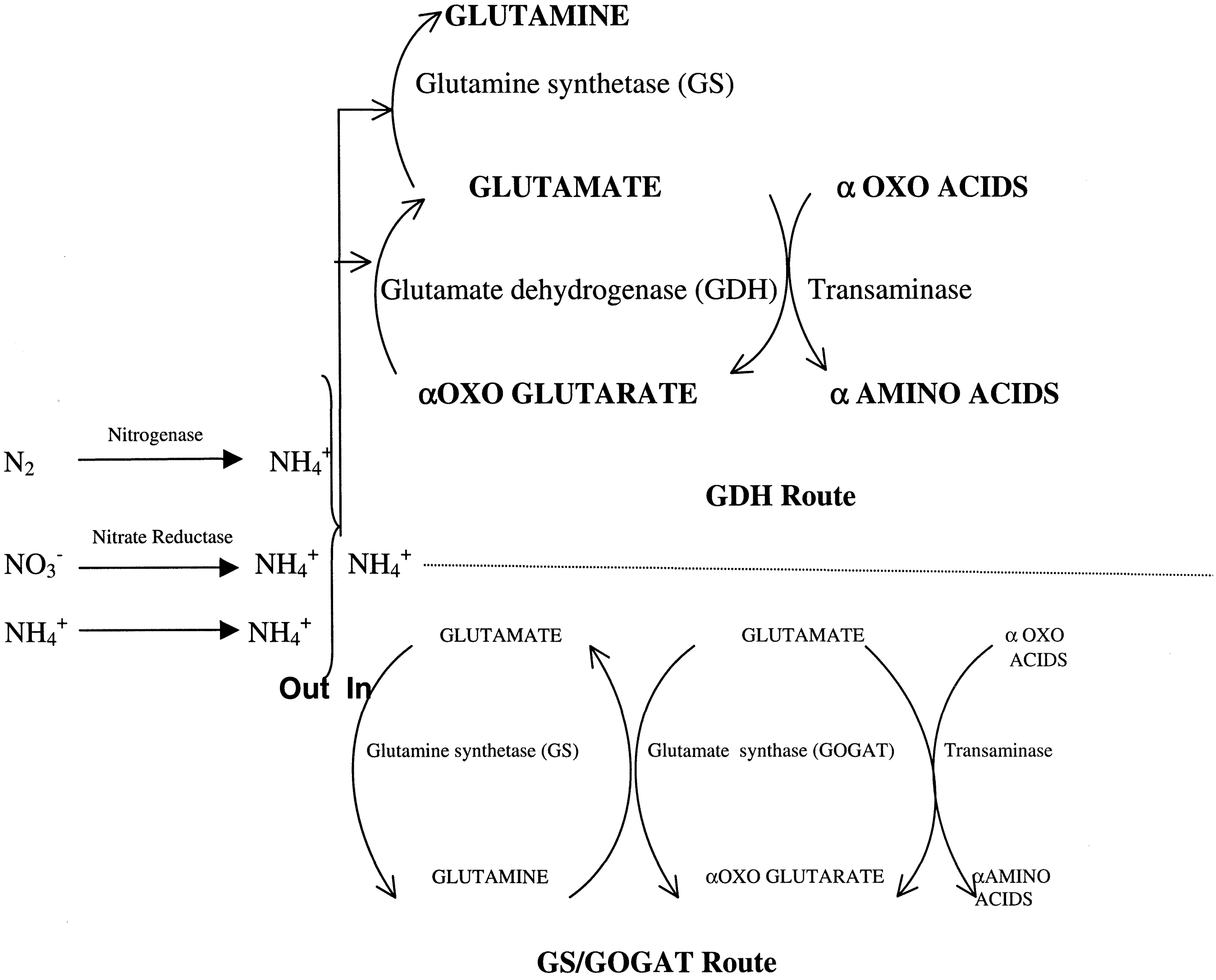

Figure 1.1 Pathways of nitrogen assimilation via the GDH and GS/GOGAT route (Miflin and Lea, 1976)

1.4 Molecular Biology of Ammonium Transport in Plants

1.4.1 Isolation of *AMT1* Genes

The application of molecular biological tools has enabled the isolation of a number of ammonium transporters. Three genes in *S. cerevisiae,* namely *Mep1*, *Mep2* and *Mep3*, which are thought to encode two high-affinity transport systems and a low affinity - system, respectively, have been cloned (Dubois and Grenson, 1979; Marini *et al.*, 1997; Marini *et al.*, 1994). The *Arabidopsis AtAMT1;1* was the first high affinity ammonium transporter identified in plants (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, related root-specific ammonium transporters have also been isolated from other plant species: *LeAMT1* from tomato (Lauter *et al.*, 1996), *AtAMT1;2* and *AtAMT1;3* from *Arabidopsis* (Gazzarrini *et al.*, 1999), and *OsAMT1;1* from rice (Von Wiren *et al.*, 1997). Recently, a second family of ammonium transporters having considerable sequence divergence from *AtAMT1* has been discovered in *Arabidopsis* with the cloning of *AtAMT2;1* (Sohlenkamp *et al.*, 2000).

1.4.2 Regulation of Transcription and Proposed Physiological Roles of *AMT1* Proteins

Expression studies of *AtAMT1;1* showed that the gene is transcribed in all major tissues, with the highest expression level in roots (Ninnemann *et al.*, 1994). The *LeAMT1;1* in tomato also displayed highest expression in root hairs and exhibited constitutive expression in roots (Lauter *et al.*, 1996). Nitrogen starvation led to a rapid increase in *AtAMT1;1* transcript levels in roots which concurrently showed high HATS activity. Following resupply of high nitrogen to N-starved plants, *AtAMT1;1* transcript level and HATS activity both decreased rapidly (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999). The decline in *AtAMT1;1* mRNA was blocked by methionine sulfoximine (MSX), which inhibits the conversion of ammonium to glutamine (Gln) (Gazzarrini *et al.*, 1999; Rawat *et al.*, 1999). This led to the proposal that Gln or other products of NH_4^+ assimilation, but not NH_4^+ was responsible for the down regulation of *AtAMT1;1* expression in *Arabidopsis* (Rawat *et al.*, 1999). A similar finding has also been reported previously in maize, where unidirectional influx of NH_4^+ and NO_3^-

were reduced when intracellular levels of *gln* or *asn* were increased by specific treatments (Lee and Ratcliffe, 1991). Treatments, which reduced the levels of gln or asn, had the opposite effect on NO_3^- and NH_4^+ influx. Thus, it appears that not only NH_4^+, but also NO_3^- uptake are regulated by products of ammonium assimilation (Lee *et al.*, 1992; Lee and Rudge, 1986). However, ammonium itself may also regulate ammonium uptake, at least in maize (Causin and Barneix, 1993; Ryan and Waker, 1994).

Regulation of transcription of other *AtAMT1* family members has also been examined. It has been found that although *AtAMT1;1* is expressed in all major tissues, *AtAMT1;2* and *AtAMT1;3* are expressed only in roots (Gazzarrini *et al.*, 1999). NH_4^+ transporters in leaves may be involved in the accumulation of NH_4^+ from the vascular system across the mesophyll plasma membrane. NH_4^+ concentration in the xylem can rise up to 2.6 mM under excess NH_4^+ supply or even up to 300 μM in the absence of supplied NH_4^+ (Cramer and Lewis, 1993). In mesophyll cells NH_4^+ transporters might also be involved in the recovery of photorespiratory NH_4^+. If reassimilation of NH_3 is absent or inhibited, even under ambient CO_2 concentrations, plants suffer N stress and ultimately exhibit chlorosis (Keys *et al.*, 1978). All three *AtAMT1* family members displayed diurnal variation in transcription levels in roots, with *AtAMT1;3* showing the most marked change. Like *LeAMT1;1* in tomato, *AtAMT1;2* showed the most stable expression levels in all nitrogen conditions, pointing to a possible role in constitutive high-affinity uptake of NH_4^+ at various external concentrations (Gazzarrini *et al.*, 1999). Thus, there are several pieces of indirect evidence that proteins of *AMT1* gene family play a role in root ammonium uptake in plants. Over and under expression of individual transporter genes using transgenic approach may provide an insight into specific roles of these ammonium transporters in rice.

1.5 Rice Transformation

The success of foreign gene delivery, integration and expression in rice was mainly due to the emergence of efficient rice tissue culture and regeneration technologies in the mid 1980s (Kishor and Reddy, 1993). Transgenic rice is now used as a tool to study various aspects of gene expression and regulation (McElroy and Brettell, 1994). Efficient tissue culture, transformation methods, and subsequent regeneration of fertile plants have now been perfected in rice (Upadhyaya *et al.*, 1998; Upadhyaya *et al.*, 2000).

The most commonly used gene delivery systems for rice transformation include polyethylene glycol (PEG) treatment of protoplasts, electroporation of protoplasts, biolistics or microprojectile bombardment and *Agrobacterium*-infection of callus material. Most of the parameters for successful rice transformation such as promoters, selectable marker (SM) genes (*nptII*, *hph*, *Als*, or *bar*), reporter genes, selective agents and selection regime have been worked out from the early efforts on protoplast-based transformation, (Tyagi *et al.*, 1999). In the biolistic or microprojectile bombardment method, microprojectiles (gold or tungsten) coated with DNA are used to bombard the target tissue. The most common approach is to co-bombard the plasmid containing the gene of interest (GOI) with that containing the SM gene (mostly CaMV35S promoter driven *hph* gene encoding hygromycin resistance). It is assumed that the two plasmids can concatamerize before being integrated into the rice genome. Thus, a certain proportion of plants produced under hygromycin selection will also contain the GOI.

The target material (mature or immature embryo-derived calli), SM gene (CaMV35S driven *hph* gene), and the tissue culture and regeneration conditions used in the biolistic method of Taipei 309 transformation (Li *et al.*, 1993; Upadhyaya *et al.*, 1996) can also be used for high frequency *Agrobacterium*-mediated transformation (Upadhyaya *et al.*, 2000; Wang *et al.*, 1997). The super-virulent *Agrobacterium* strain AGL1 (Lazo, Stein, and Ludwig, 1991), in combination with improved binary vectors containing an intron-interrupted *hph* gene, have been used to achieve high frequency transformation (up to 40%) of a number of japonica varieties (Wang *et al.*, 1997; Upadhyaya *et al.*, 1998; Wang *et al.*, 1998; Upadhyaya *et al.*, 2000). Recently, by using the same method with improved promoters and selectable markers, the Australian rice cultivars Jarrah and Amaroo have also been transformed (Upadhyaya *et al.*, 2000).

1.6 Objective of the Present Project

Around 70% of the world rice is cultivated in irrigated lowland system in Asia (IRRI, 1997) where nitrogen fertilizer is the main limiting factor for yield. Consequently, large amounts of fertilizer nitrogen are used mainly in the form of urea, which is converted to ammonium before being taken up by plants. As mentioned earlier, there are many reports that for lowland rice NH_4^+ is the preferred and most available source of nitrogen (Dijkshoorn and Ismunadji, 1972; Fried *et al.*, 1965; Sasakawa and Yamamoto, 1978; Wang *et al.*, 1993; Yoneyama and Kumazawa, 1974). It has always been a problem to raise the N utilization rate of the rice plant, and to increase the efficiency of absorbed N for grain production, irrespective of the amount of N supplied. Rice plants generally use less than 50% of the applied fertlizer, the rest being eventually lost into the environment (Hauck, 1985a).

It should be possible to decrease the loss of fertiliser N to the environment by increasing the rate at which soil ammonium is utilised by rice plants. This might be achieved by increasing the number and /or activity of ammonium transporters in root cell membrane or by increasing the affinity of these transporters for their substrate. Of course, it might also be necessary to increase the strength of the metabolic sink for ammonium in roots or shoots so that ammonium taken up is not accumulated to toxic levels, or simply lost by efflux back to the soil. These could be achieved by genetic transformation of rice with ammonium transporter genes.

The aims of this project were therefore as follows:

- to isolate and characterise *AMT1* ammonium transporters from rice
- to modify the expression of *AMT1*gene in rice
- to perform molecular and physiological analyses of transgenic plants to determine if they are altered in ammonium uptake, assimilation, growth and development.

It was hoped that, in addition to adding to our fundamental knowledge of ammonium transport in plants, this project would also contribute novel germplasm for breeding programs aimed at increasing N-use efficiency in rice.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains and Plasmids

2.1.1 Bacterial Stains

Bacterial strains used in this study are listed and described in Table 2.1. *Escherichia coli* strains were grown overnight on LB agar medium with appropriate selection at 37°C. For short-term storage, bacterial cultures were stored on LB agar plates at 4°C. For long-term storage, liquid cultures were grown in 2x YT liquid medium with selection to the stationary phase, mixed with an equal volume of 50% (v/v) glycerol and stored at -70°C.

2.1.2 Plasmids

Plasmids and recombinant plasmid constructs used in the course of this study are listed in Tables 2.2 and 2.3.

2.2 Media and Stock Solutions

2.2.1 Bacterial Media, Solutions and Reagents

Standard bacterial media, stock solutions and reagents detailed in Sambrook *et al.*, (1989) were used in all molecular cloning and recombinant DNA work.

2.2.2 Tissue Culture Media, Solutions and Reagents

N6 macro-elements (20X)

	g/L
$(NH_4)_2SO_4$	9.3
KNO_3	56.6
KH_2PO_4	8.0

$MgSO_4.7H_2O$	3.7
$CaCl_2.2H_2O$	3.3

N6 micro-elements (1000X)

	mg/100 mL
$MnSO_4.4H_2O$	440
$ZnSO_4.7H_2O$	150
H_3BO_3	160
KI	80

N6 Vitamins (100X)

	mg/100 mL
Glycine	20
Thiamine-HCl	10
Pyridoxine-HCl	5
Nicotinic acid	5

B5 micro-elements (100X)

	mg/L
$MnSO_4.4H_2O$	1000
$Na_2MoO_4.2H_2O$	25
H_3BO_3	300
$ZnSO_4.7H_2O$	200
$CuSO_4.5H_2O$	3.87
$CoCl_2.6H_2O$	2.5
KI	75

B5 vitamins (100X)

Gamborg's vitamin solution (1000x)

(Sigma cell culture Cat G 2519)

FeEDTA(200X)

	g/200 mL
Ferric-Sodium salt	1.47

(Sigma cell culture Cat. 64H0339)

2,4-D dichloro-phenoxyacetic acid (2,4-D) 1 mg/mL stock

Dissolve 100 mg of 2,4-D in 1mL absolute ethanol, add 3 mL of 1N KOH, adjust to pH 6 with 1N HCl and store at 4°C.

6-benzyl amino purine (BAP) 1 mg/mL stock

(Sigma cell culture solution Cat. B 3274) and store at 4°C.

Naphthalene acetic acid (NAA) 1 mg/mL stock

(Sigma cell culture solution Cat. N1641) and store at 4°C.

Abscisic acid (ABA) 2.5 mg/mL stock

Dissolve 250 mg of ABA in 2 mL of 1M NaOH. Make to 100 mL with (sterile) distilled water.

Hygromycin 50 mg/mL stock

Roche Diagnostics Cat # 843 555.

Timetin 150 mg/mL stock

Dissolve 3100 mg of Timetin in 20.66 mL of sterile water to a final concentration of 150 mg/mL. Aliquot 500 µL in each Eppendorf tubes and store at -20°C.

MS salts

Murashige minimal organic medium (Sigma).

Callus induction N6D media

	amount/L
N6 macro (10X)	100 mL
N6 micro (1000X)	1 mL
N6 vitamins (1000X)	1 mL
MS iron/EDTA	5 mL
Myo-inositol	100 mg
Casamino acid	300 mg
Proline	2.9 g
2,4-D (1mg/mL)	2 mL
Sucrose	30 g

Adjust pH to 5.8 with 1M KOH

Phytagel	3 g

Sub culture media NB (Li L et al., 1993)

	amount/L
N6 macro-elements (20X)	50 mL
B5 micro-elements (100X)	10 mL
B5 vitamins (100X)	10 mL
FeEDTA (200X)	5 mL
2,4-D (1 mg/mL)	2 mL
Sucrose	30 g
Proline	500 mg
Glutamine	500 mg
Casein enzymatic hydrolysate (CEH)	300 mg

Adjust pH to 5.8-5.85 with 1M KOH

Phytagel	3 g

Osmotic pre-treatment media NBO

NB with 30 g/L mannitol and 30 g/L sorbitol added to media before pH adjustment.

Selection media NBHT30 (for first selection)

NB with 30 mg/L hygromycin and 150 mg/mL Timentin added to the media after autoclaving and just before pouring.

Selection media NBHT50 (for subsequent selection)

NB with 50 mg/L hygromycin and Timentin 150 mg/mL added to media after autoclaving and just before pouring.

Pre-regeneration media PRHT50

NB media without **2,4-D**

	amount/L
*BAP	2 mg
*NAA	1mg
*ABA	5 mg
*Hygromycin	50 mg
*Timentin	150 mg

Regeneration media RHT50

NB media without **2,4-D**

	amount/L
*BAP	3 mg
*NAA	0.5 mg
*Hygromycin	50 mg
*Timentin	150 mg

Rooting media ½ MS

	amount/L
MS salts and vitamin mixture	2.21 g
Sucrose	10 g
Phytagel	2.5 g
*NAA	0.05 mg
*Timentin	150 mg

* Added after Autoclaving

2.3 Rice Cultivars and Plant Growth Conditions

Two rice (*Oryza sativa*) cultivars, Taipei 309 and Jarrah, were used in this study. Unless stated otherwise, rice seedlings were grown hydroponically. Seeds were dehusked, surface sterilized in sodium hypochlorite (1% v/v) for 15 min and rinsed in sterile water. They were then germinated on filter paper, wetted with sterile water (for wild type plants) or sterile water containing 75 mg/L hygromycin (for transgenic plants). After four or five days, germinated seeds were transferred to hydroponic culture containing modified Johnson's nutrient (MJN) solution (Epstein, 1972) containing different nitrogen concentrations as specified for individual experiments.

2.4 Rice Tissue Culture and Transformation

2.4.1 Callus Induction

Mature seeds (cvv. Taipei 309 and Jarrah) were dehusked and washed with 70% ethanol for 1 minute followed by a single wash with sterile distilled water. Seeds were then sterilized for 30 min in 1-2 % sodium hypochlorite (3:1 or 1:1 v/v water and commercial bleach depending on the contamination load in the seeds) with gentle mixing. Seeds were washed several times in sterile water to remove all traces of bleach.

Surface sterilized seeds were blotted with sterile blotting paper and placed embryo side up on NB solid medium and incubated in the dark at 28°C. Embryos were excised from the germinated seeds after 4-5 days and placed back on to NB medium in the dark at 28°C. After three weeks, primary calli were excised and placed onto solid fresh NB medium. Embryogenic calli (1-2 mm size), subcultured every two weeks for not more than four times, were used as target tissue for *Agrobacterium* co-cultivation.

2.4.2 Rice Transformation

2.4.2.1 Agrobacterium infection

Agrobacterium strain AGL1 (Lazo *et al.*, 1991) was used for transformation. Binary vectors containing respective gene expression cassettes were introduced into AGL1 by triparental mating (Ditta *et al.*, 1980). *Agrobacterium*-mediated transformation was performed according to Wang *et al.*, (1997) with minor modifications. In short, AGL1 containing each binary vector construct was grown on LB plates with 25 mg l^{-1} rifampicin and 25 mg l^{-1} spectinomycin for 2 d at 28°C. The lawn of bacterial cells was harvested in 10 mL liquid NB broth supplemented with 100 µM acetosyringone. Embryogenic calli were immersed in the *Agrobacterium* suspension for 10 min. The bacterial suspension was drained and calli were placed on plates of NB medium containing 100 µM acetosyringone and co-cultivated at 25°C for 48 h in the dark. Calli were then washed thoroughly but gently in water containing 150 mg l^{-1} Timentin with at least five changes of washing solution.

2.4.2.2 Selection of Transformed Callus lines

After co-cultivation with *Agrobacterium* and subsequent washing, transformed calli were blotted on sterile filter paper and placed on the first selection media, NBTH30 (NB + 150 mg l^{-1} Timentin + 30 mg l^{-1} hygromycin), and incubated at 28°C in the dark for 3-4 weeks. This induced the transformed calli to produce new buds that were excised and plated onto the second selection media, NBTH50 (NB + 150 mg l^{-1} Timentin + 50 mg l^{-1} hygromycin), and allowed to proliferate for 4 weeks at 28°C in the dark.

2.4.2.3 Regeneration of Transgenic Plants

Actively growing calli were placed on to pre-regeneration medium PRTH50 (see section 2.3.2), and incubated at 27°C for two weeks. Healthy calli were transferred to regeneration medium, RTH50 (see section 2.3.2), and incubated at 25°C in the light (20-40 µE m^{-2} s^{-1}) under a 16 h photoperiod. Regenerated shoots were then placed onto half strength MS medium (Murashige and Skoog, 1962) containing sucrose (10 g l^{-1}), NAA (0.05 mg l^{-1}) and hygromycin (50 mg l^{-1}) in Magenta pots. They were placed under lights with a 16 h photoperiod for one to two weeks.

2.4.2.4 Raising of Transgenic Plants

Well rooted plantlets were replanted in Jiffy pots containing a mixture of soil, perlite, sand and peat moss (50:25:15:10 by volume). They were kept in a mist chamber in a naturally illuminated glasshouse with 28°C day and 21°C night temperatures for 1 week before transfer to 15 cm plastic pots with the same potting mix, kept in water tubs (10-12 cm water depth).

2.5 Nucleic Acid Extraction Methods

2.5.1 Rice Genomic DNA Extraction

Rice genomic DNA was isolated from plants using a commercial nucleic acid isolation kit (Puregene, Gentra systems Inc, Minneapolis). Samples were frozen in liquid nitrogen immediately after collection. Approximately 50 mg of young rice leaves were crushed in liquid nitrogen, then added to 600 µl of cell lysis solution (Tris-HCl 10mM, EDTA 1mM, pH 8.8 with 1% SDS), vortexed and incubated at 65°C for 60 min. 3 µl of RNAse (5 mg/mL) was then added, mixed, and incubated for 15 min at 37°C. The mixture was then spun at 12,000 rpm for 3 min to pellet plant debris, and the supernatant was collected in a new tube. To the supernatant, 200 µl of protein precipitation solution (6M Ammonium Acetate) was added, it was vortexed for 20 seconds, and spun at 12,000 rpm for 3 min. The supernatant was again collected into a new tube and 600 µl of isopropanol was added and mixed thoroughly by inverting at least 50 times. The mixture was then centrifuged at 12,000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 70% ethanol, air dried and resuspended in 50 µl of TE (Tris-HCl 10 mM, EDTA 0.1 mM).

2.5.2 Bacterial Plasmid DNA Extraction

Small-scale preparations of plasmid DNA were obtained by using a QIAprep kit (QIAGEN 1995). A single bacterial colony was streaked on to a LB plate with the appropriate antibiotic and incubated overnight at 37°C. With a sterile yellow pipette tip, the overnight grown bacterial culture was scooped up and suspended in 200 µl of solution P1 (50 mM Tris-HCl, 10 mM EDTA, pH 8.0, 100 µg/mL RNAse A). Cells were lysed by gentle mixing with 200 µl of P2 solution (200 mM NaOH, 1% SDS) until the solution became viscous and slightly clear. The solution was then neutralized by mixing with 200 µl of P3 buffer (3M KOAc pH 5.5) and kept on ice for 15 min. The supernatant was collected after centrifugation at 12,000 rpm for 15 min. Plasmid DNA was precipitated with 0.7 volume of isopropanol, washed with 70% ethanol, vacuum dried and dissolved in 40 µl of TE.

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^{TM} 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
    GCGCCGGCGp
```

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^{TM} 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^{TM} 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^{TM} DNA labelling system (Amersham^{TM}). 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^{TM}), 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^{TM} (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^{TM} *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 *recal 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, *EcoR*V, *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)

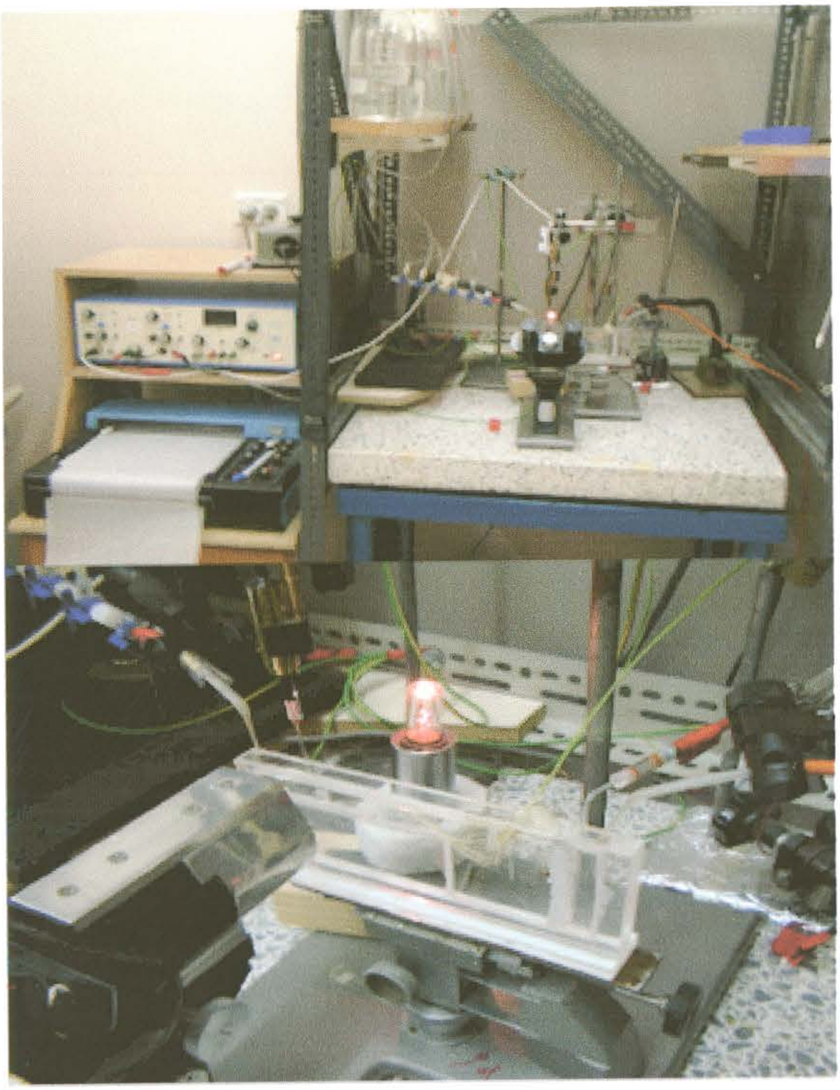

Figure 2.1 Experimental setup to measure rice root E_m.

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.

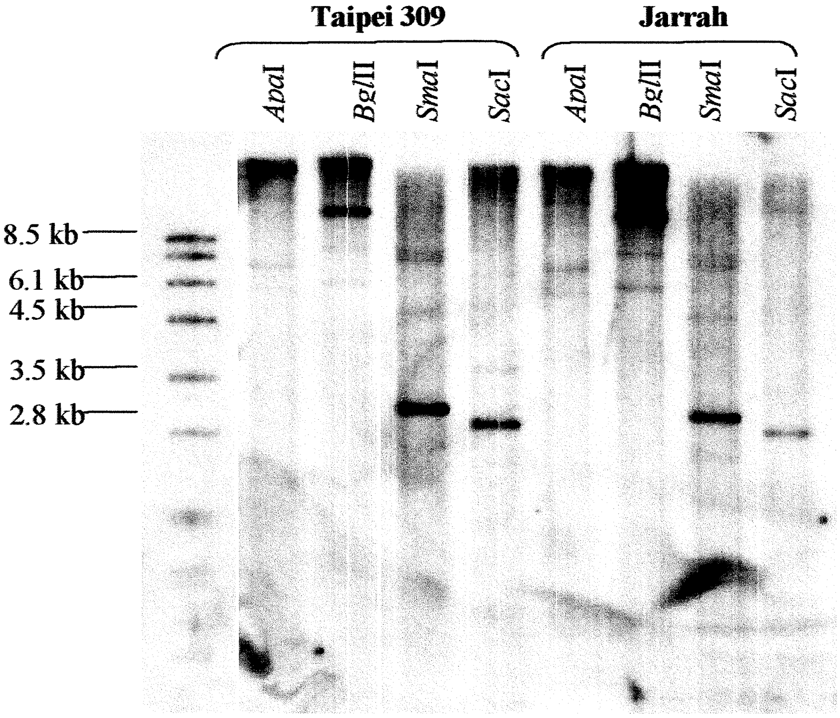

Figure 3.1 Southern blot showing *OsAMT1* copy number in rice cvv. Taipei 309 (lane 2 to 5) and Jarrah (lane 6 to 9). Genomic DNA (2 μg) from each cultivar was digested with *Apa*I, *Bgl*II, *Sma*I and *Sac*II and probed with 604 bp 3' end fragment of EST (Accession D39189). Lane 1 is the molecular marker.

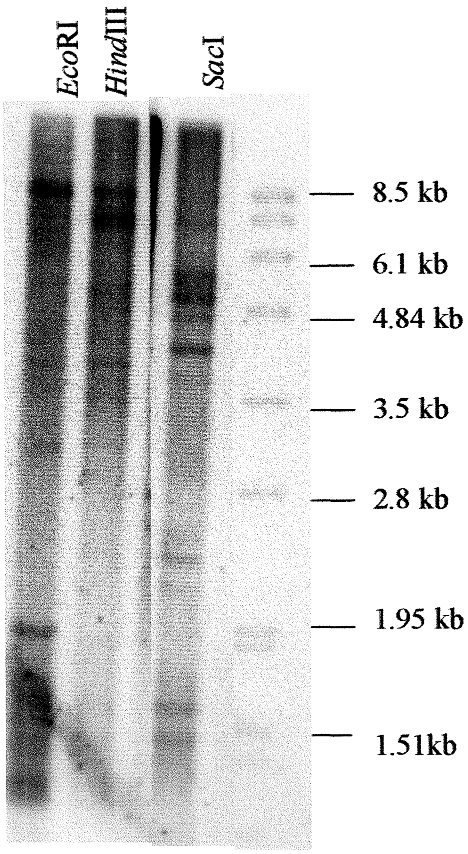

Figure 3.2 Southern blot showing *OsAMT1* copy number in rice cv. Taipei 309. Genomic DNA (2 µg) was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2) and *Sac*I (lane 3) and probed with full length of EST (Accession D39189). Lane 4 is the molecular marker.

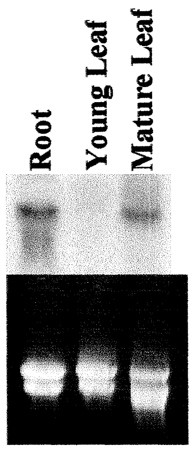

Figure 3.3 Northern blot showing expression of *OsAMT1;1* in different organs of rice. **(A)** Northern blot (upper panel) probed with rice EST (GeneBank Accession No. D39189) and ethidium bromide staining (lower panel) of electrophoretically separated total RNA from three week rice roots (lane 1), young 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).

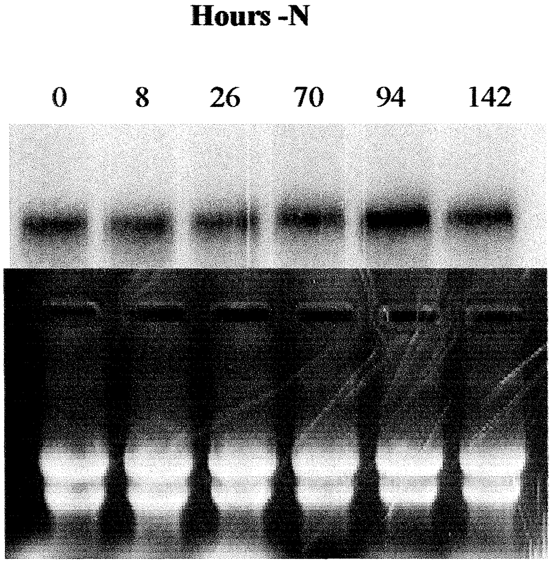

Figure 3.4 Northern blot showing the effect of nitrogen starvation on *OsAMT1;1* expression in rice. Northern blot (upper panel) probed with rice EST (Accession D39189) and ethidium bromide staining (lower panel) of electrophoretically separated total RNA from three week rice roots from plants grown 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).

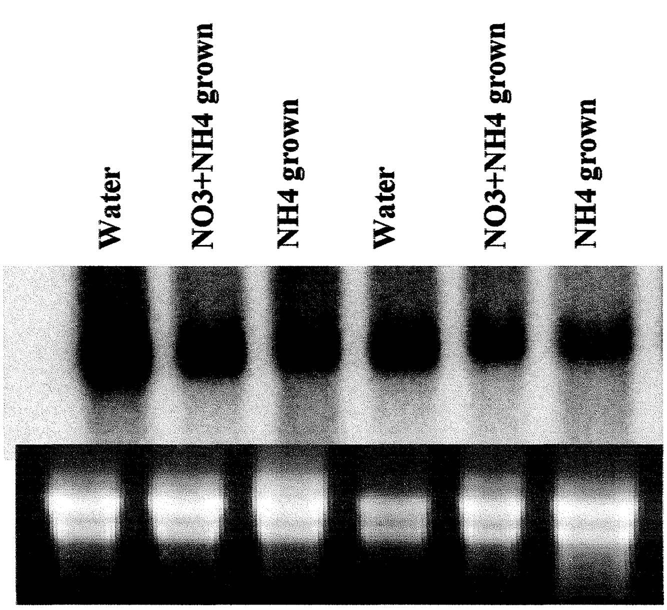

Figure 3.5 Northern blot showing the effect of different nitrogen sources and age of seedlings on *OsAMT1;1* expression in rice. Northern blot (upper panel) probed with rice EST (Accession D39189) and ethidium bromide staining (lower panel) of electrophoretically separated total RNA from from roots of 6 d old seedlings geminated in water (lane1), 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).

```
                1171                                                                                           1260
pPIMP1          ~~~~~~~~~g gccgcccagc tccacggcgg gtgcggcgcg tgggggatcc tcttcaccgc gctcttcgcg aggcagaagt acgtcgagga
pPIMP2          ~~~~~~~~~~ ~~cgcccagc tccacggcgg gtgcggcgcg tgggggatcc tcttcaccgc gctcttcgcg aggcagaagt acgtcgagga
pPIMP3          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
OsAMT1;1        cccgctcgag gcggcccagc tccacggcgg gtgcggcgcg tgggggatcc tcttcaccgc gctcttcgcg aggcagaagt acgtcga.ga
Consensus       *********- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                1261                                                                                           1350
PpPIMP1         gatctacggc gccggccggc cgtacggcct gttcatgggc ggcggcggca agctgctcgc cgcgcacgtc atccagatcc tggtcatctt
pPIMP2          gatctacggc gccggccggc cgtacggcct gttcatgggc ggcggcggca agctgctcgc cgcgcacgtc atccagatcc tggtcatctt
pPIMP3          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
OsAMT1;1        gatctacggc gccggccg.. cgtacggcct gttcatg... ggacgccgca agctgctcgt tggcgctgtc atccagatcc tggtcatctt
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                1351                                                                                           1440
ppPIMP1         cgggtgggtc agctgcacca tgggacctct cttctacggg ctcaagaagc tcggcctgct ccgcatctcc gccgaggacg agacgtccgg
ppPIMP2         cgggtgggtc agctgcacca tgggacctct cttctacggg ctcaagaagc tcggcctgct ccgcatctcc gccgaggacg agacgtccgg
ppPIMP3         ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
OsAMT1;1        cgggtgggtc agctgcacca tgggacctct cttctacggg ctcaagaagc tcggcctgct ccgcatctcc ggccgagacg agacgtccgg
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                1441                                                                                           1530
pPIMP1          catggacctg acacggcacg gcgggttcgc gtacgtctac cacgacgagg acgagcacga caagtctggg gttggtgggt tcatgctccg
pPIMP2          catggacctg acacggcacg gcgggttcgc gtacgtctac cacgacgagg acgagcacga caagtctggg gttggtgggt tcatgctccg
pPIMP3          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
OsAMT1;1        catggacctg acacggcacg gcgggttcgc gtacgtctac cacgacgagg acgagcacga caagtctggg gttggtgggt tcatgctccg
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                1531                                                                                           1620
pPIMP1          gtccgcgcag acccgcgtcg agccggcggc gg.cggctgc ctccaacagc aacaaccaag tgtaaccaat ccagaacgaa cgacgtcaca
pPIMP2          gtccgcgcag acccgcgtcg agccggcggc gg.cggctgc ctccaacagc aacaaccaag tgtaaccaat ccagaacgaa cgacgtcaca
pPIMP3          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
OsAMT1;1        gtccgcgcag acccgcgtcg agccggcggc ggccggctgc ctccaacagc aacaaccaag tgtaaccaat ccagaagcaa gcacgtcaca
Consensus       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

                1621                                                                                           1710
pPIMP1          gcgaaggaag aaatcacGGG TTTCTCTCCC TCTCCGATCT CGATCGTCAC GTCATAAATT TGATCCCCAT ATTTGATTGC CAGTTTCTGT
pPIMP2          gcgaaggaag aaatcacGGG TTTCTCTCCC TCTCCGATCT CGATCGTCAC GTCATAAATT TGATCCCCAT ATTTGATTGC CAGTTTCTGT
pPIMP3          ~~~~~~~~~~ ~~~~~~tGGG TTTCTCTCCC TCTCCGATCT CGATCGTCAC GTCATAAATT TGATCCCCAT ATTTGATTGC CAGTTTCTGT
OsAMT1;1        gcgaaggaag aaatcacGGG TTTCTCTCCC TCTCCGATCT CGATCGTCAC GTCATAAATT TGATCCCCAT ATTTGATTGC CAGTTTCTGT
Consensus       ---------- -------*** ********** ********** ********** ********** ********** ********** **********

                1711                                                                                           1800
pPIMP1          TTGGGCCAAA TGCTTTTGCC GCTCTCTCTG GTGTTGCAAG ACTGTAAAAA CACTGTAGGA TGGACGAGTG TCTTTCACTT TTG.CTGGGC
pPIMP2          TTGGGCCAAA TGCTTTTGCC GCTCTCTCTG GTGTTGCAAG ACTGTAAAAA CACTGTAGGA TGGACGAGTG TCTTTCACTT TTG.CTGGGC
pPIMP3          TTGGGCCAAA TGCTTTTGCC GCTCTCTCTG GTGTTGCAAG ACTGTAAAAA CACTGTAGGA TGGACGAGTG TCTTTCACTT TTG.CTGGGC
OsAMT1;1        TTGGGCCAAA TGCTTTTGCC GCTCTCTCTG GTGTTGCAAG ACTGTAAAAA CACTGTAGGA TGGACGAGTG TCTTTCACTT TTGcCTGGGC
Consensus       ********** ********** ********** ********** ********** ********** ********** ********** ***-******

                1801                                                                                           1890
pPIMP1          TTCTCTTGTG TACAGGCATG CGTACGTGTC TTAGAATGTG TGGTGTGAAG G..tGGGAAG AATCAGAGGT TAGGGTTTAA TTTTCTTTTG
pPIMP2          TTCTCTTGTG TACAGGCATG CGTACGTGTC TTAGAATGTG TGGTGTGAAG G..tGGGAAG AATCAGAGGT TAGGGTTTAA TTTTCTTTTG
pPIMP3          TTCTCTTGTG TACAGGCATG CGTACGTGTC TTAGAATGTG TGGTGTGAAG G..tGGGAAG AATCAGAGGT TAGGGTTTAA TTTTCTTTTG
OsAMT1;1        TTCTCTTGTG TACAGGCATG CGTACGTGTC TTAGAATGTG TGGTGTGAAG GgtgGGGAAG AATCAGAGGT TAGGGTTTAA TTTTCTTTTG
Consensus       ********** ********** ********** ********** ********** *---****** ********** ********** **********

                1891                                                                                           1980
pPIMP1          CACAATGGTT ACTGCTATTA TTGTTTTATT TTGTGGTCGA ATTTTATCGT CA~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
pPIMP2          CACAATGGTT ACTGCTATTA TTGTTTTATT TTGTGGTCGA ATTTTATCGT CA~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
pPIMP3          CACAATGGTT ACTGCTATTA TTGTTTTATT TTGTGGTCGA ATTTTATCGT CATAAGGGTG TGGTGGAATG GTGGTCAAGA TAGGTGGCTG
OsAMT1;1        CACAATGGTT ACTGCTATTA TTGTTTTATT TTGTGGTCGA ATTTTATCGT CATAAGGGTG TGGTGGAATG GTGGTCAAGA TAGGTGGCTG
Consensus       ********** ********** ********** ********** ********** ********** ********** ********** **********

                1981                                                                                           2070
pPIMP1          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
pPIMP2          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
pPIMP3          TGCAGGGCTC AAAGACTTTG CGTGGGTCCT TTTGTCCTGC AGTGCTCTAC CTCTCTATCA AAACTTTGGC TTatttcctg gaatctagtg
OsAMT1;1        TGCAGGGCTC AAAGACTTTG CGTGGGTCCT TTTGTCCTGC AGTGCTCTAC CTCTCTATCA AAACTTTGGC TT~~~~~~~~ ~~~~~~~~~~
Consensus       ********** ********** ********** ********** ********** ********** ********** ********** **********

                2071                                          2116
pPIMP1          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~
pPIMP2          ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~
pPIMP3          gtttgagagt gtttgtttta tactcagttc tgcattatgt ttacga
OsAMT1;1        ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~
Consensus       ********** ********** ********** ********** *****
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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 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 μM 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 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 NH_4^+ fed plants than 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 $[NH_4^+]_o$ (< 1mM) is due to carrier-mediated $NH4^+$ 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 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 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 NH_4^+ blocks ATP production and reducing 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 NH_4^+ (2 mM). The reduction in biomass when plants were grown at high (2 mM) 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 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 NH_4^+. There were also differences in flowering time within each cultivar under different NH_4^+ nutrition. Flowering in Jarrah was delayed at least 4-5 days when grown in 2 mM NH_4^+ compared to 10 μM of 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 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 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 $[NH_4^+]_o$ 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 then 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 $[NH_4^+]_o$. For example, addition of 100 μM $NH4^+$ into -N solution induced an average depolarization of 53.4±10.4 (mV) in the case of wild type plants while in transgenic plants (line 46) this value was 108.6±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 *e 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_{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_{max} values in wild type plants grown under different nitrogen conditions differed. For example, the V_{max} value (35 ± 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 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_{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.

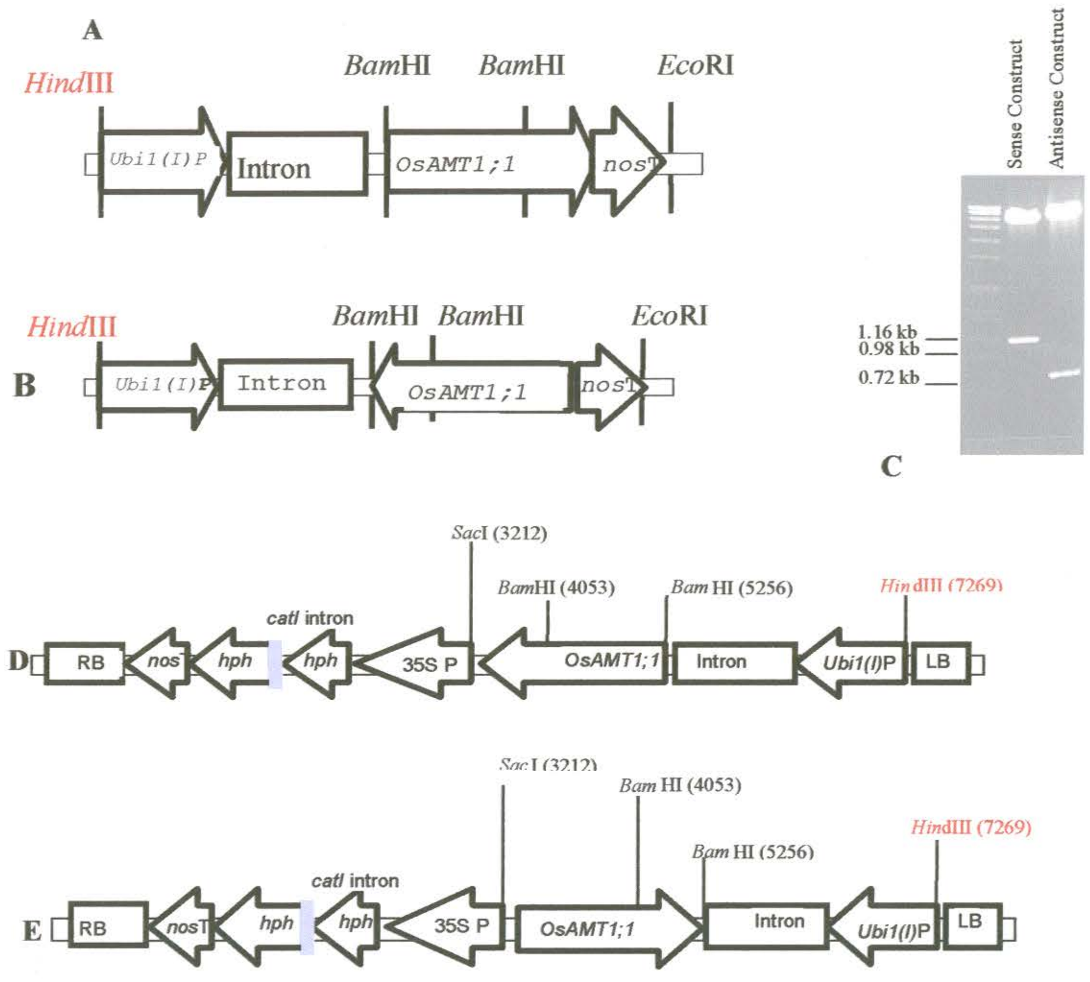


Figure 4.1. *OsAMT1;1* gene construction. **(A)** Construct pPIMP56 containing the 2.04 kb *OsAMT1;1* cDNA inserted in sense orientation between the maize ubiquitin promoter (*Ubi1(I)*) and the nopaline synthase gene terminator (*nos*T); **(B)** construct pPIMP58 containing the 2.04 kb *OsAMT1;1* cDNA 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/*Hin*dIII fragment) inserted into the binary vector pWBvec8 (*Xba*I-endfilled/*Hin*dIII 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.

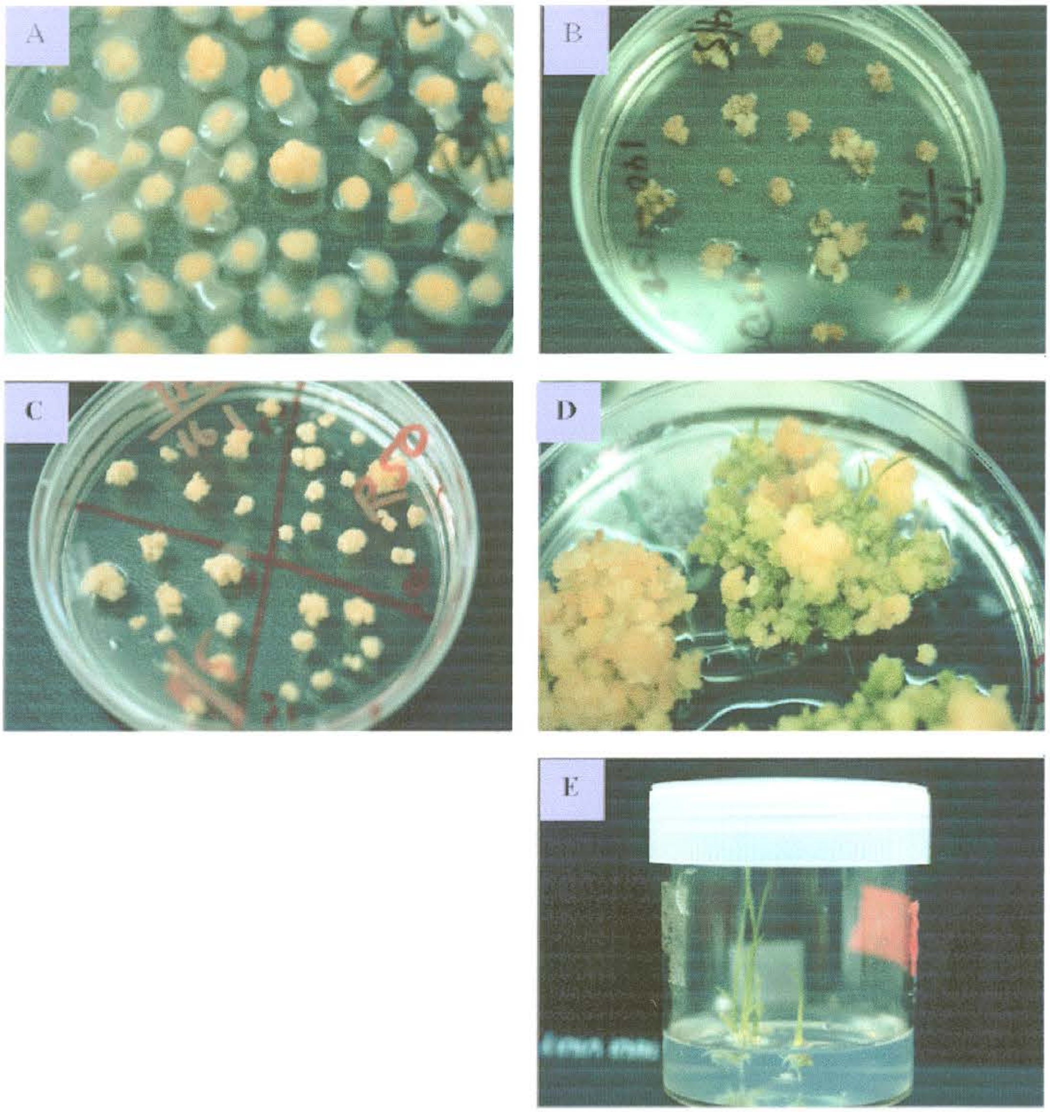


Figure 4.2. Stages in the production of fertile transgenic plants from rice cvv.Taipei 309 and Jarrah. **(A)** *Agrobacterium* (containing binary vector construct with *Ubi1(I)P-OsAMT1;1* and CaMV35SP-*hph* gene expression cassettes) and rice embryogenic calli co-cultivation; **(B)** *Agrobacterium* 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.

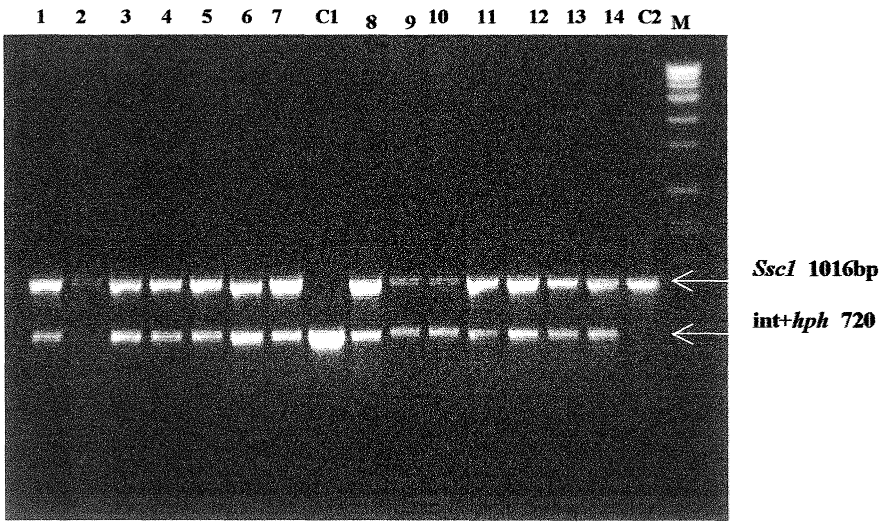
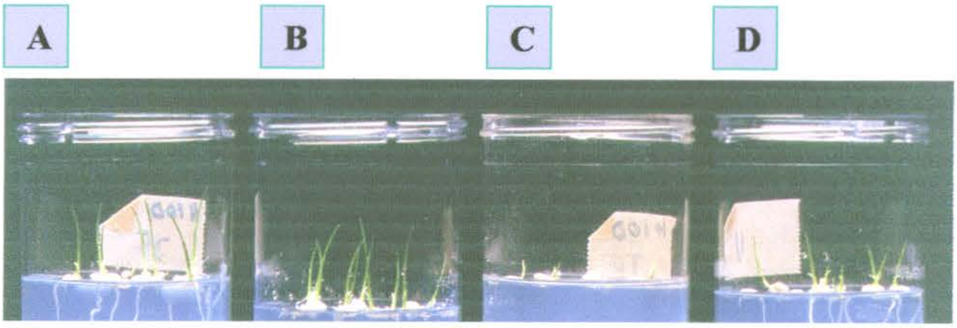


Figure 4.3 Preliminary analysis of transgenic lines by polymerase chain reaction (PCR). Genomic DNA was isolated from primary transgenic plants (T_0) and used in co-amplification of *hph* and *SSs1* (internal control) DNA fragments by PCR. PCR products from DNA of various transgenic lines having *hph*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



Transgenic Plants WT -Hygromycin WT +Hygromycin Transgenic Plants

Figure 4.4 Segregation analysis with progeny of transgenic lines. Sterile seeds of wild type (WT) and T_1 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).

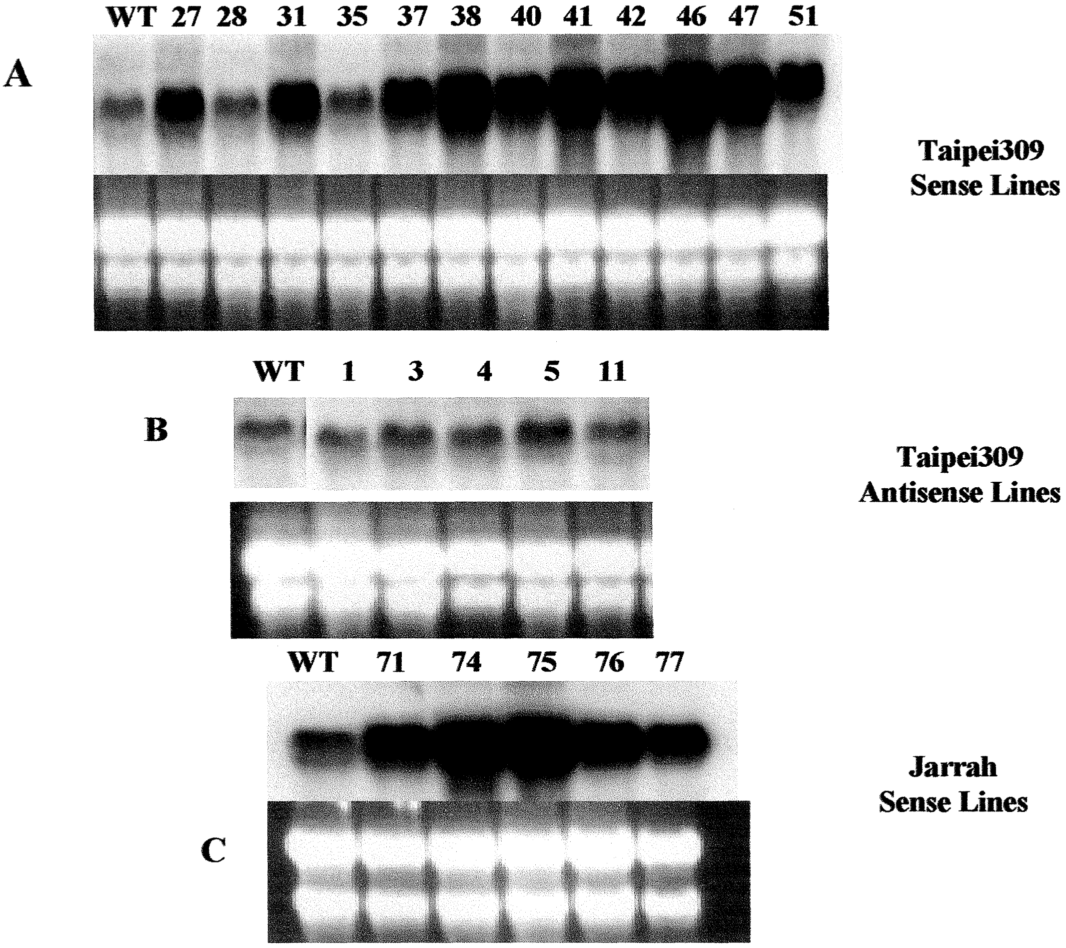


Figure 4.5. Northern blot analysis of *OsAMT1;1* expression in rice roots. Total RNA was isolated from the roots of hydroponically grown plants and 25 µg was loaded in each lane. **(A)** Northern blot hybridization (upper panel) and ethidium bromide stained gel (lower panel) of total RNA from Taipei 309 (lane 1) and various T_1 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 form Taipei 309 (lane1), and various T1 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 form Jarrah (lane1) and various T_1 Jarrah *OsAMT1;1* over-expressing lines (71, 74, 75, 76 & 77). The blots were exposed to autoradiography using phosphor screens (Molecular dynamics) overnight.

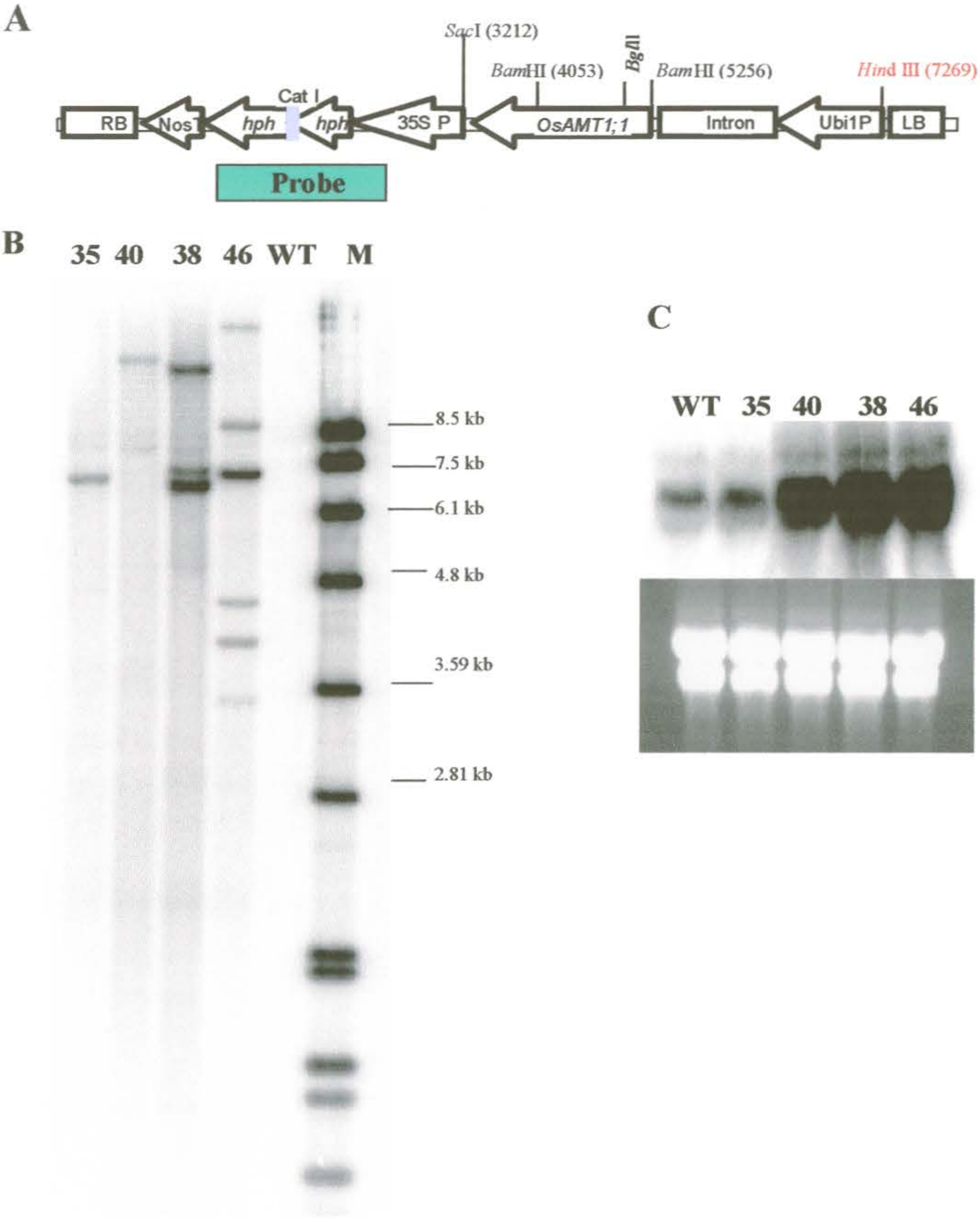


Figure 4.6. Estimation of *OsAMT1;1* transgene copy number in Taipei 309 T_1 transgenic lines by Southern blot analysis and corresponding transgene expression levels. **(A)** Construct showing *hph* gene used as a probe; **(B)** genomic DNA from Taipei T_1 transgenic lines (indicated by code number) and Taipei 309 (lane 5) digested with *Bgl*II, separated on a 0.8% agarose gel, blotted onto nylon membrane and hybridized with ^{32}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.

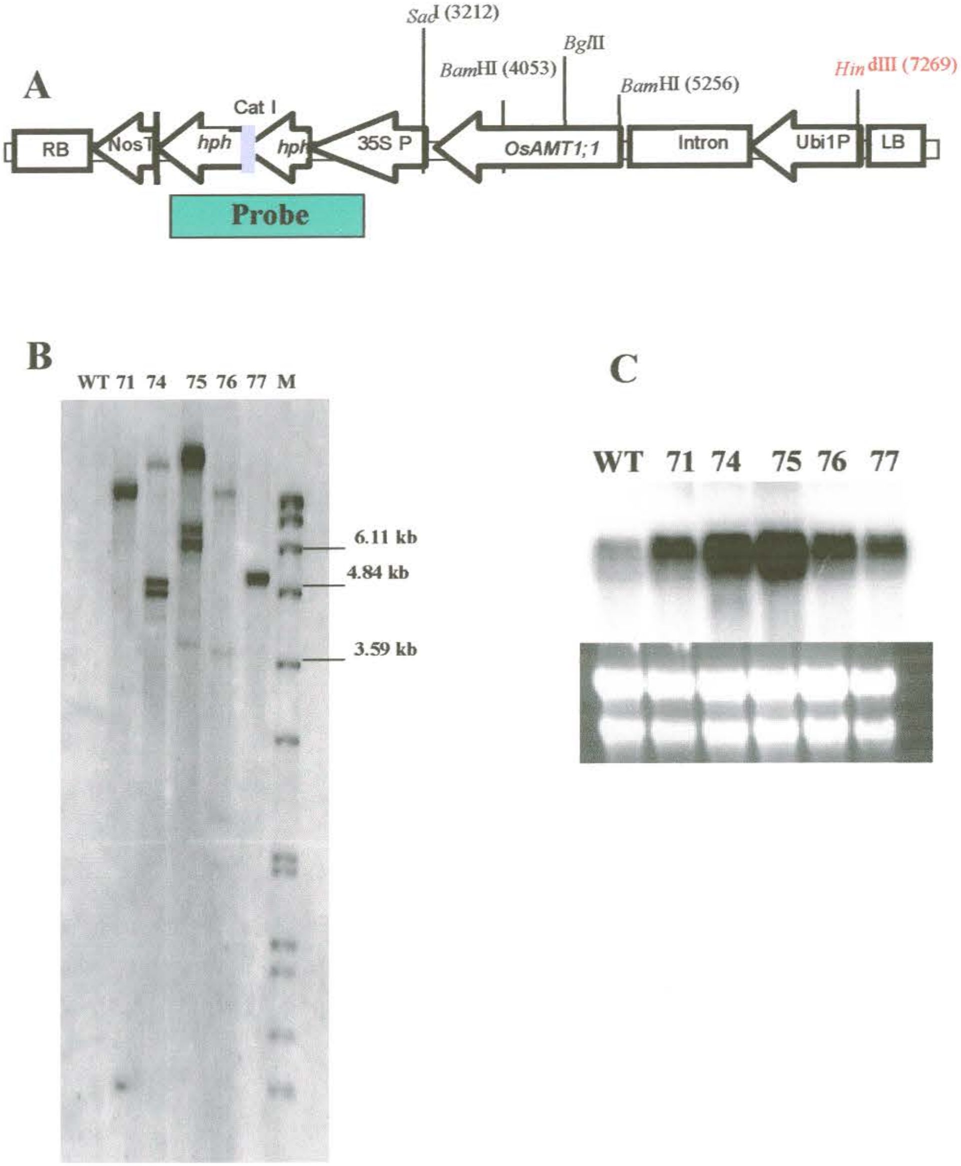


Figure 4.7. Transgene copy number and expression in Jarrah T_1 transgenic lines. **(A)** Construct showing *hph* gene used as a probe; **(B)** genomic DNA from Jarrah T_1 transgenic lines (indicated by code number) and Jarrah (lane 1), digested with *Bgl*II, separated on a 0.8% agarose gel, blotted onto nylon membrane and hybridized with ^{32}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.

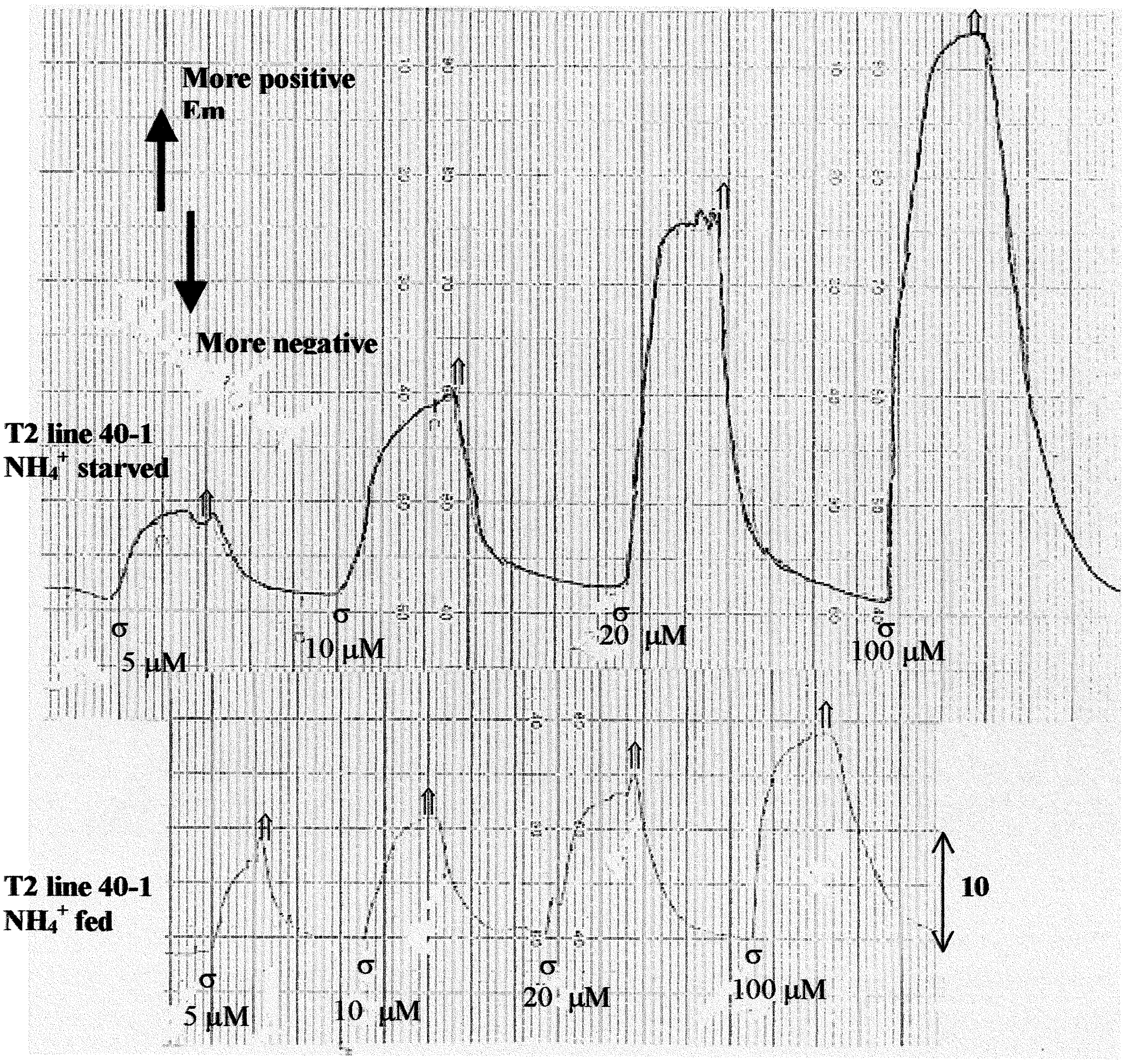


Figure 4.8. Representative traces from T_2 line 40-1 showing the depolarization of root cell E_m induced by adding various concentrations of NH_4Cl. (σ), various concentrations of NH_4Cl was added into the nutrient solution. NH_4Cl was withdrawn from the nutrient solution where indicated (⇑).

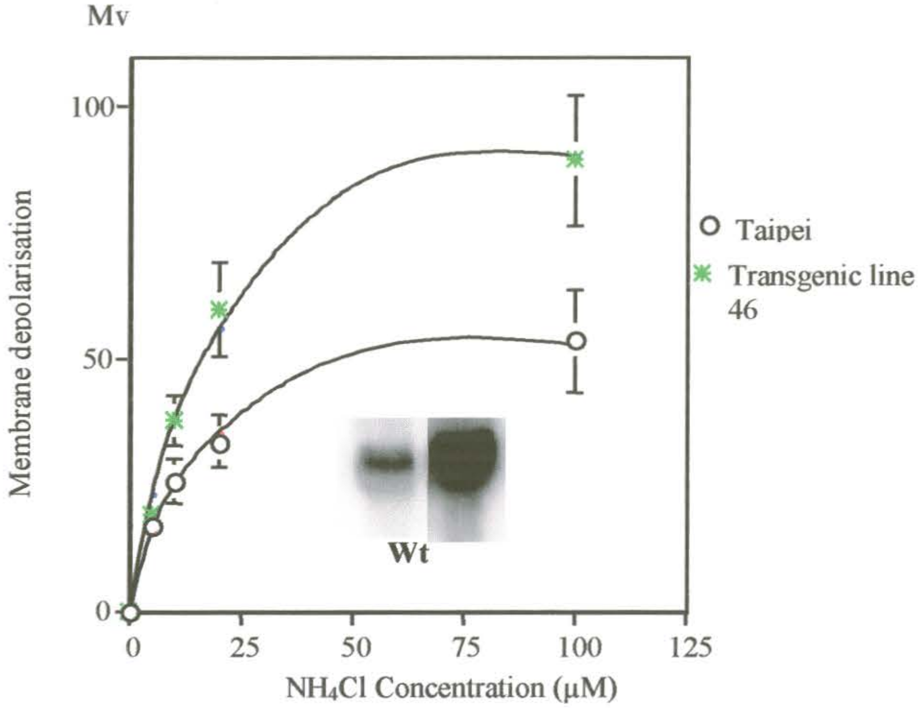


Figure 4.9. Membrane depolarization of transgenic and wild type rice root cells in response to increasing concentrations of NH_4Cl. Plants were grown in modified Johnson's nutrient solution (Epstein, 1972) with 2.0 mM NO_3^- and 1.0 mM of NH_4^+ for three weeks. For the nitrogen starvation treatment 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).

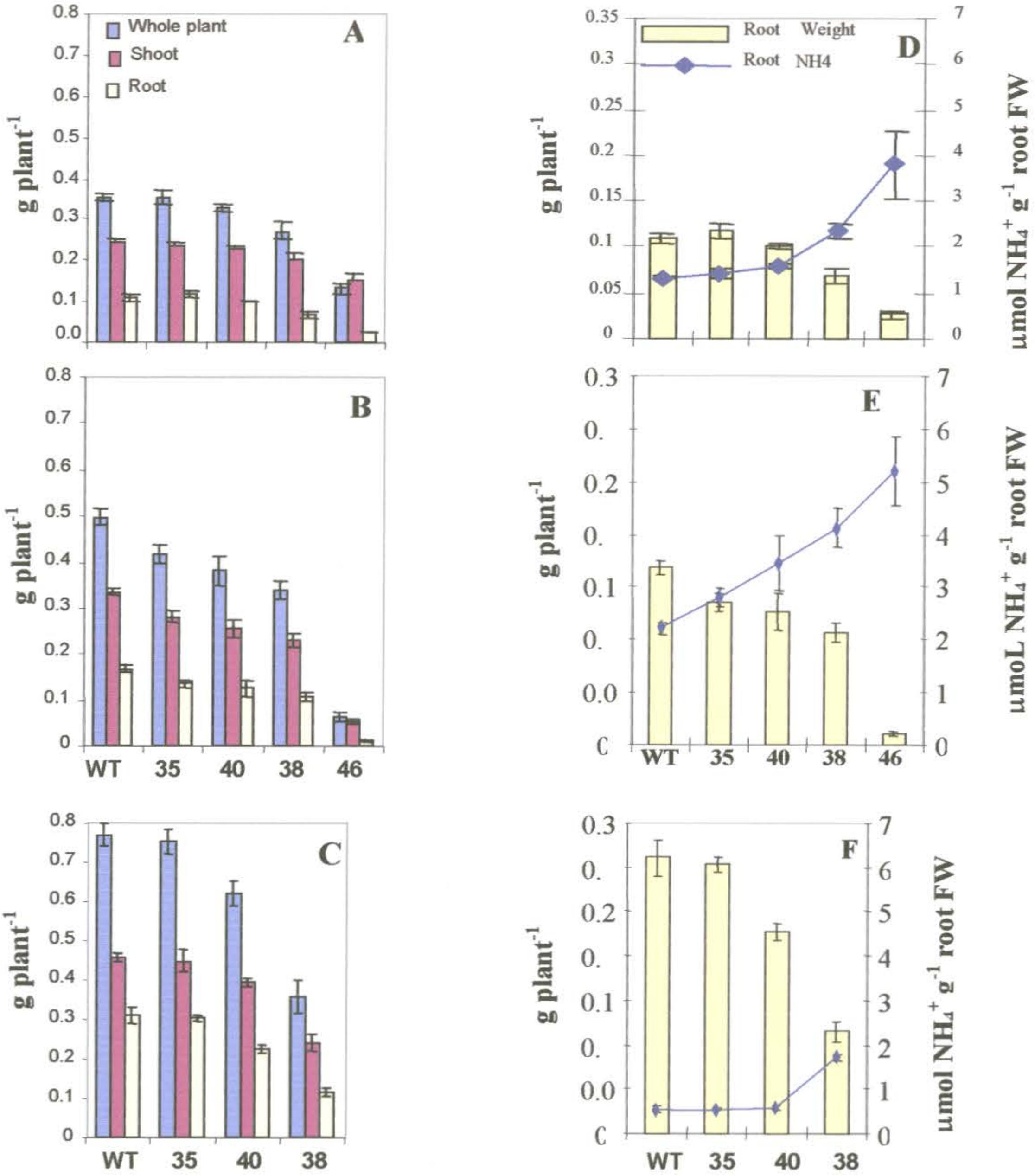


Figure 4.11 Whole plant, shoot and root weights and root ammonium content of wild type (Taipei-309) and transgenic plants. Rice seedlings (wild type and transgenic) were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM NO_3^- and 1.0 mM NH_4^+ for 16 d. First set, 16 d old, grown in the 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 µM 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.

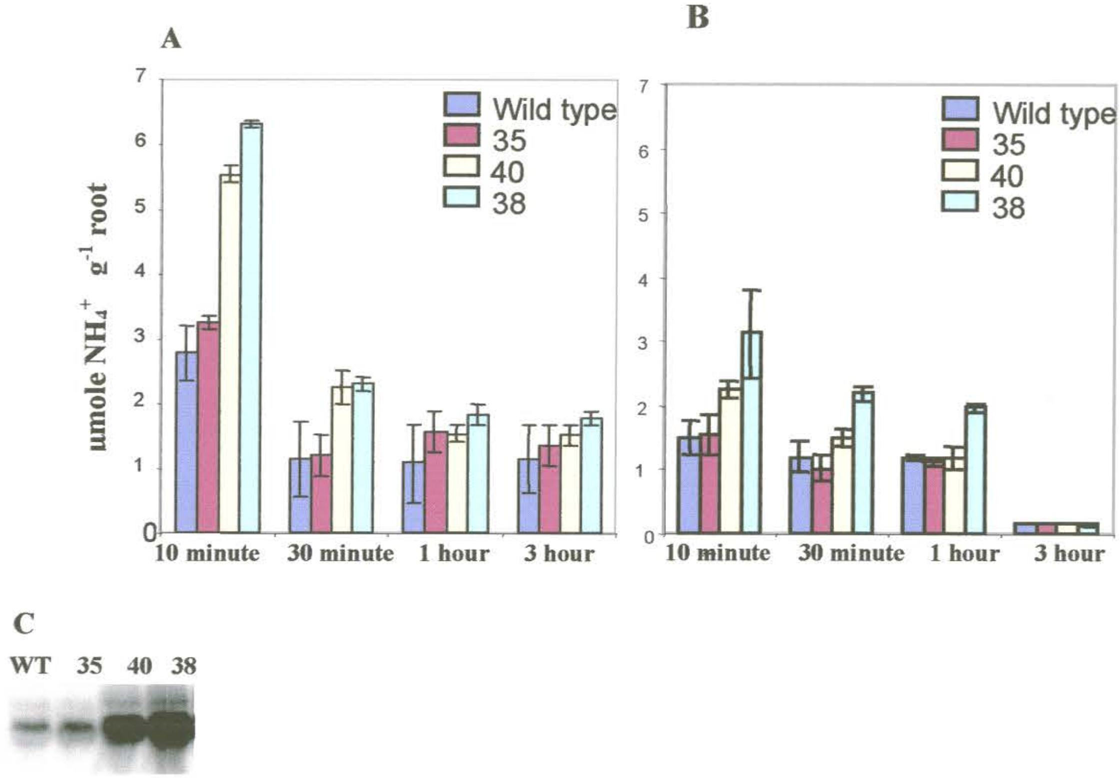


Figure 4.12. Ammonium uptake rate of transgenic and wild type (Taipei-309) rice plants. Rice seedlings were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM NO_3^- and 1.0 mM of NH_4^+ for 16 d. **(A)** 17 d old seedlings, last 1 d grown in -N solution; **(B)** 20 d old seedlings, last two 2 d grown in -N solution. Five seedlings from each treatment were placed in a tube containing 80 mL of nutrient solution with 75μM of 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 μmol (g fresh root wt. 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.

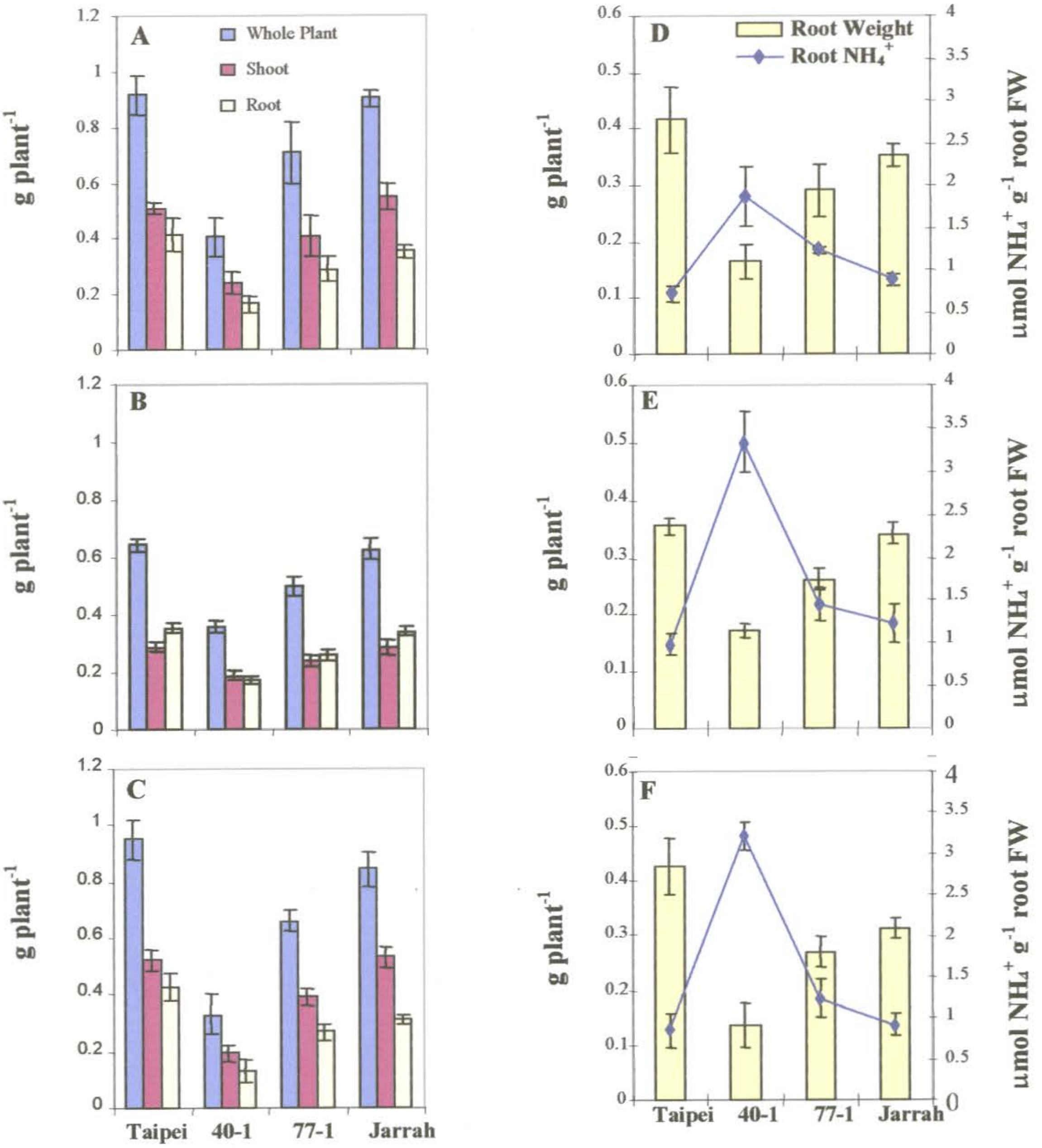


Figure 4.13 Whole plant, shoot and root fresh weights, and root ammonium content of wild type and transgenic plants. Rice seedlings (wild type and transgenic) were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM NO_3^- and 1.0 mM NH_4^+ for two week. The 1st set was grown in the same 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_3^- containing solution (C & F). After three hours of NH_4^+ uptake in 75 µM NH_4^+, 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_4^+ uptake experiments (D, E, & F). Error bars indicated the standard error of the mean.

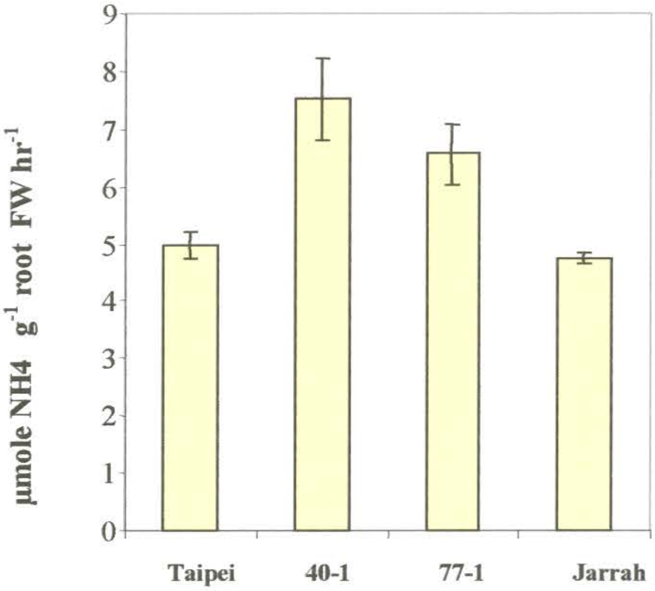


Figure 4.14. Ammonium uptake rate of wild type and transgenic rice plants from nutrient solution during the 3 h period of growth. Rice seedlings (wild type and transgenic) were grown hydroponically in MJN solution (Epstein, 1972) with 2.0 mM NO_3^- and 1.0 mM of NH_4^+ for two weeks. Then transferred 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_4^+ as the only nitrogen source at zero time. Tubes were gently aerated to ensure the uniform distribution of NH_4^+ 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^{-1}). Error bars indicate the standard error of the mean.

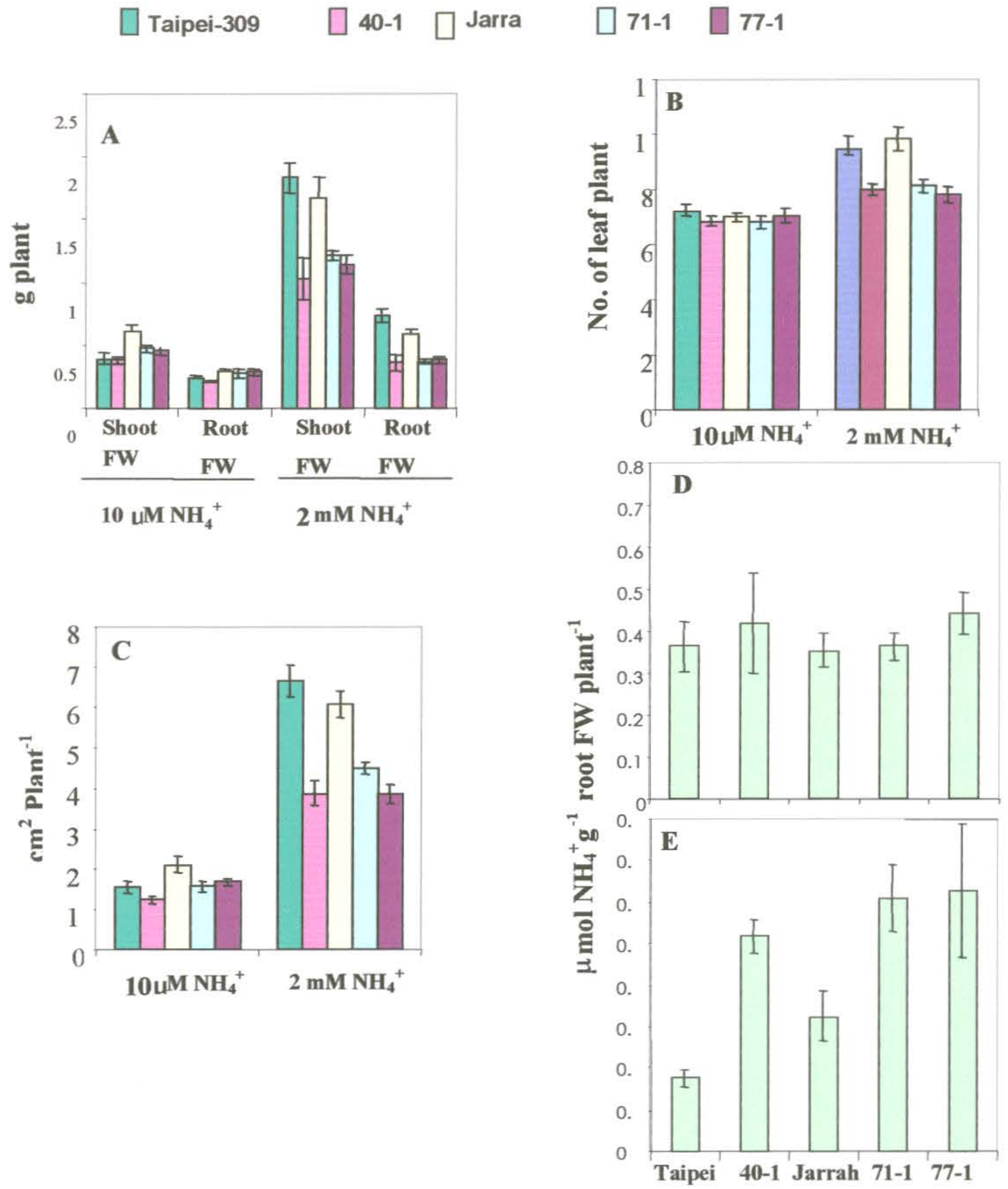


Figure 4.15 Shoot and root fresh weights of 6 week old wild type and transgenic plants grown in 10 µM and 2 mM NH$_4^+$ (A). Number of leaves plant^{-1} (B) and leaf area (C) of 6 week old wild type and transgenic plants grown in 10 µM and 2 mM NH$_4^+$. Root NH$_4^+$ content of 6 week old wild type and transgenic plants grown in 10 µM (D) and 2 mM NH$_4^+$ (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_o) plant lines	Number of transgenic (T_1) 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
Antisense lines (Taipei 309)						
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
Sense lines (Taipei 309)						
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_2 populations.

Pedigree	Cultivar Used	Transgene Copy Number In T_o 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_1 line 46	T_2 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.

		Km (μM)	V_{max} (mV)
Experiment 1			
Ammonium fed plants	Taipei 309	4.1 ± 1.1	35 ± 8
	T_1 line 46	16.2 ± 1.4	71 ± 14
One day nitrogen starved	Taipei 309	10 ± 5	26 ± 3
	T_1 line 46	9 ± 3	50 ± 3
Two day nitrogen starved	Taipei 309	13 ± 4	52 ± 4
	T_1 line 46	20 ± 1	86 ± 10
Experiment 2			
Ammonium fed plants	Taipei 309	4 ± 12	4 ± 1
	T_2 line 40	8 ± 11	5 ± 1
Seven day nitrogen starved	Taipei 309	22 ± 7	43 ± 6
	T_2 line 40	25 ± 5	62 ± 6

Table 4.6 Segregation analysis in the T_1 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 λEMBL-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 *Sau*3A1 and resulting fragment were cloned into the *Bam*H1 site in the vectorλEMBL-3 SP6/T7. The titre of the library was ~ 1.8 x 10^6 pfu ml^{-1} (~ 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 (~5 kb) in A3, A6, and E1, one different (~12 kb) band in E2, and two bands in both J1 (~5 kb and 4.5 kb) and K1 (~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*1 and *Kpn*1 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*1 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®-7Zf$^+$(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 ~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^{r}Bluescript SK(-) vector (Stratagene, USA) to produce pPIMP770 (Fig. 5.3, B).

Fragments generated by *Sac*I digestion of clones J1 and K1 were subcloned into pK^{r}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 *Sac*1/*Xho*1/*Bam*H1 restriction recognition sequences from the cloning vector λEMBL-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$^+$) and the 3' end (*OsAMT1;1*-2058$^-$) (Refer to Table 3.1 and section 3.2). Using a modified Expand^{TM} Long template PCR system (Boehringer Mannheim, Australia), a 2.04 kb PCR product was obtained and cloned into a pGEM®-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) seaquakes (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 *Sac*1/*Xho*1/*Bam*H1 sequence from the cloning site of vector λEMBL-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 (*Sac*I/*Xho*I/*Bam*HI) of vector λEMBL-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 phylogenic 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$^+$ and *OsAMT1;1*-90$^-$, the latter designed to create a *Nco*I site spanning the ATG codon. The resulting 3.5 kb PCR fragment (Fig. 5.12, A) was digested with the restriction enzymes *Hin*dIII and *Nco*I. A 2.5 kb *Hind*III/*Nco*I fragment (Fig. 5.12, B) was recovered and cloned into the *Hind*III/*Nco*I 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 NH_4^+ uptake comes from complementation and uptake kinetics studies in yeast mutants defective in 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 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 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 posses 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 constrains, results from these experiments have not be included in this thesis.

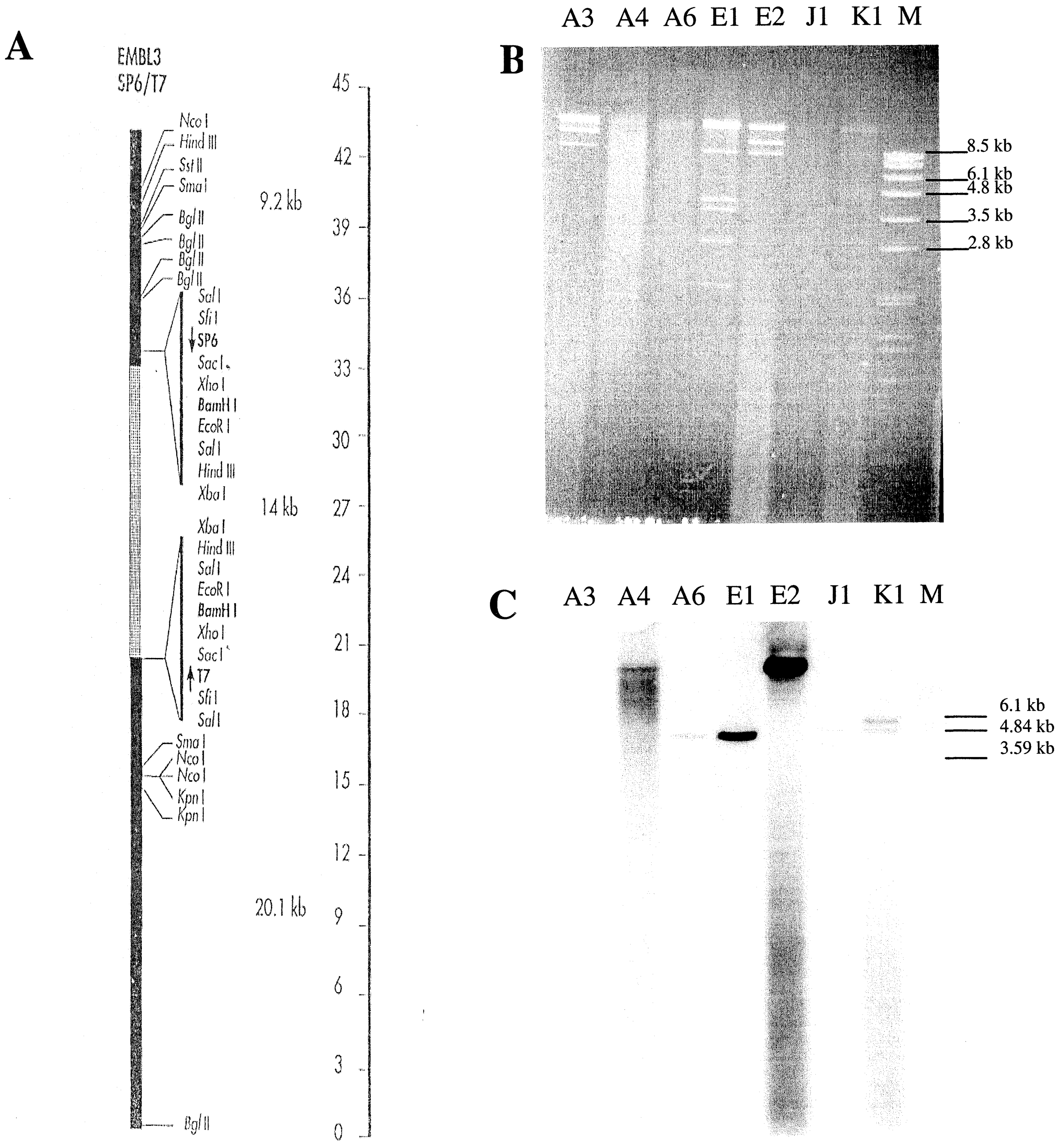


Figure 5.1 (A) Vector map of EMBL3 SP6/T7; (B) *Sac*I digestion pattern of isolated genomic clones A3, A4, A6, E1, E2, J1 & K1. Lane M contains molecular wt. marker; (C) Southern blot of the digested clones probed with full length *OsAMT1;1*.

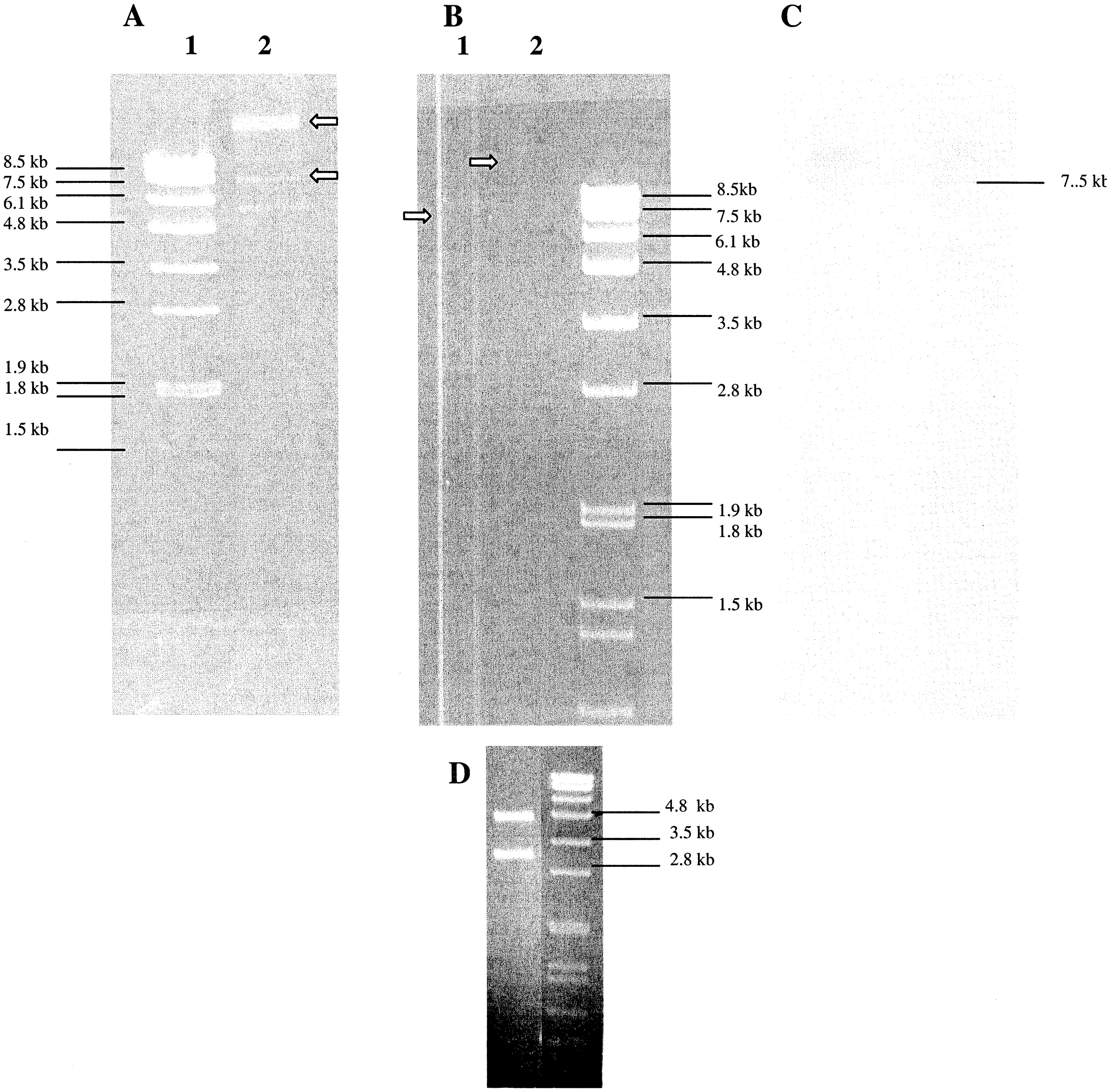


Figure 5.2 (A) λclone A3 was digested with restriction enzymes *Sfl*I and *Kpn*I and separated in an agarose gel (lane 2), (⇐ indicates the band eluted from the gel and lane 1 is the mol. wt. marker; (B) eluted 7.5 kb (lane 1) and a mixutre of 14 & 15 kb bands (lane 2) separated in an agarose gel; (C) 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.

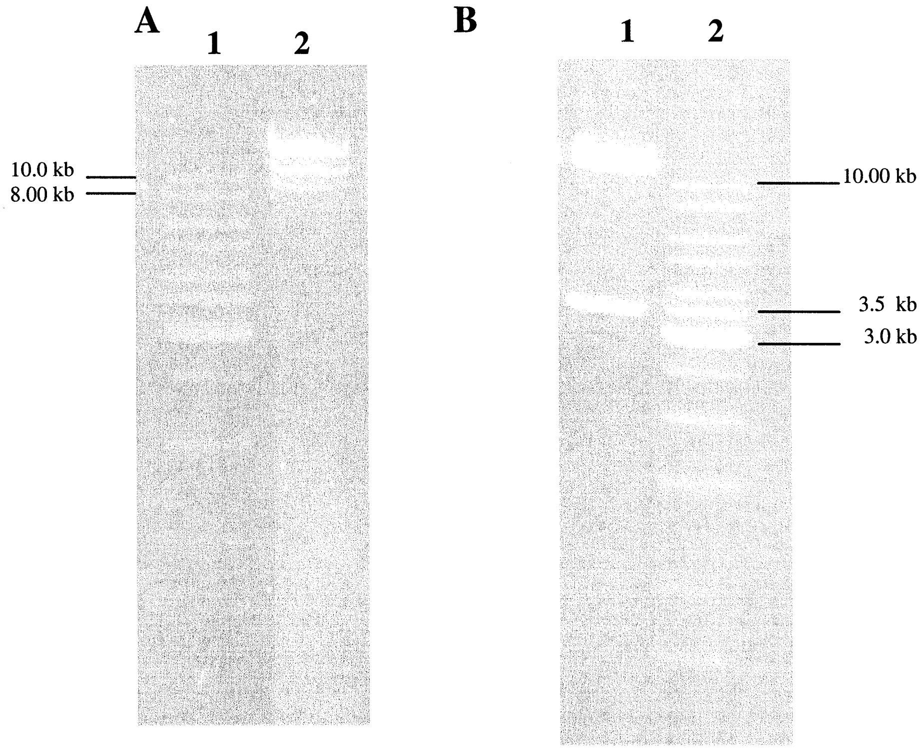


Figure 5.3 (A) Genomic λclone E2 digested with *Sac*I (lane 2) and Marker (lane 1); (B) clone pPIMP 770 digested with *Sac*I liberating the 12 kb insert (lane 1).

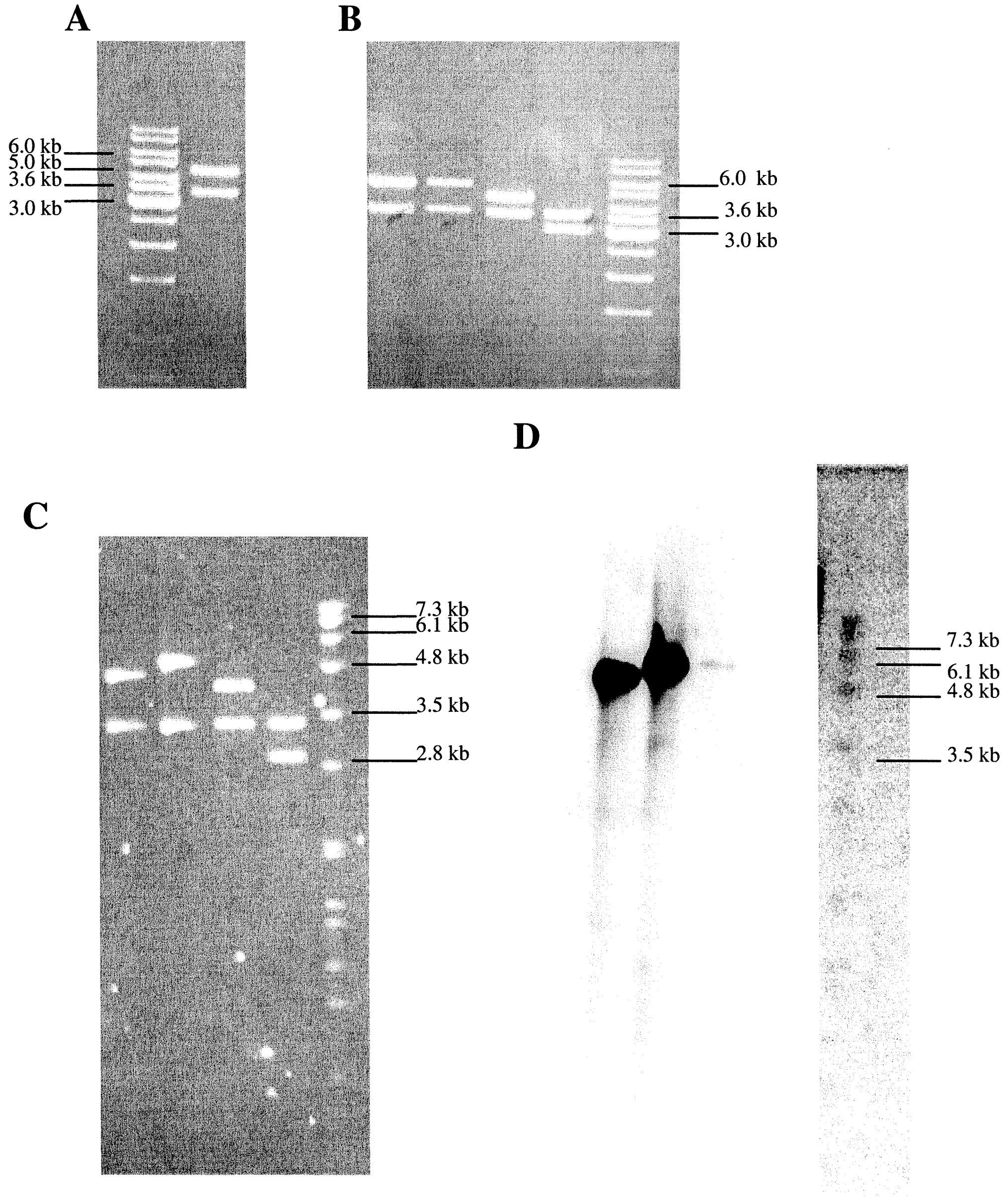


Figure 5.4 (A) pPIMP 827 digested with *Sac*I showing 4.8 kb insert (lane 2), and marker (lane 1); (B) pPIMP 833 (lane 1), pPIMP 834 (lane 2), pPIMP 936 (lane 3) and pPIMP 937 (lane 4) digested with *Sac*I showing different sized inserts and marker (lane 5); (C) pPIMP 827 (lane 1) pPIMP 834 (lane 2), pPIMP 936 (lane 3), pPIMP 937 (lane 4) digested with *Sac*I and marker (lane 5) separated in agarose gel for Southern blotting; (D) Southern blot probed with *OsAMT1;1* showing respective hynbridizing bands in pPIMP 827 (lane 1) and pPIMP 834 (lane 2).

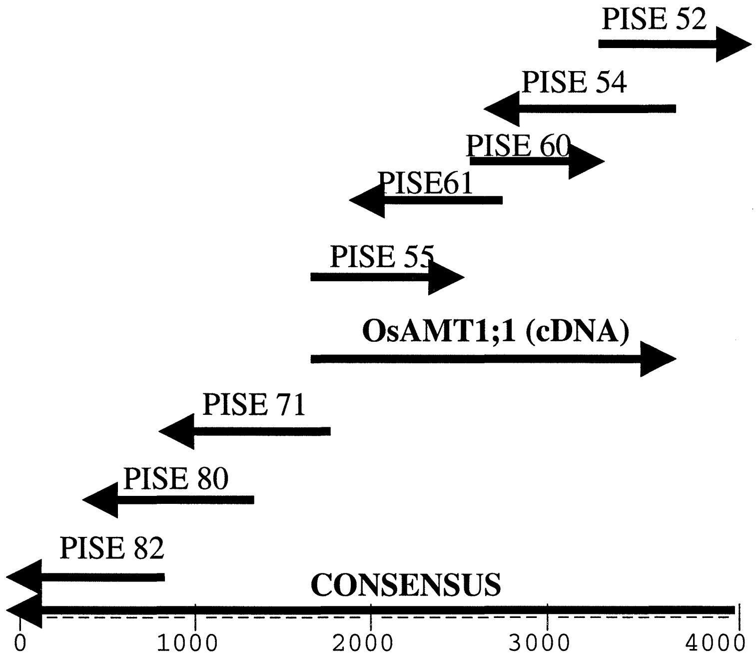


Figure 5.5. Sequencing strategy for *OsAMT1;1* (λclone E_2) from clone pPIMP770. Sequence PISE 52 with internal primer OsAMT1; 1 -1698$^+$, PISE 54 with internal primer OsAMT1; 1 -2058$^-$, PISE 60 with internal primer OsAMT1;1-864$^+$, PISE 61 with internal primer OsAMT1;1-1143$^-$, PISE 55 with internal primer OsAMT1;1-44$^+$ of *OsAMT1;1* cDNA, PISE 71 with internal primer OsAMT1;1-90$^-$(*Nco*1 overhang), PISE 80 with internal primer SE71-414$^+$ and PISE 82 with internal primer SE79 -597$^+$.

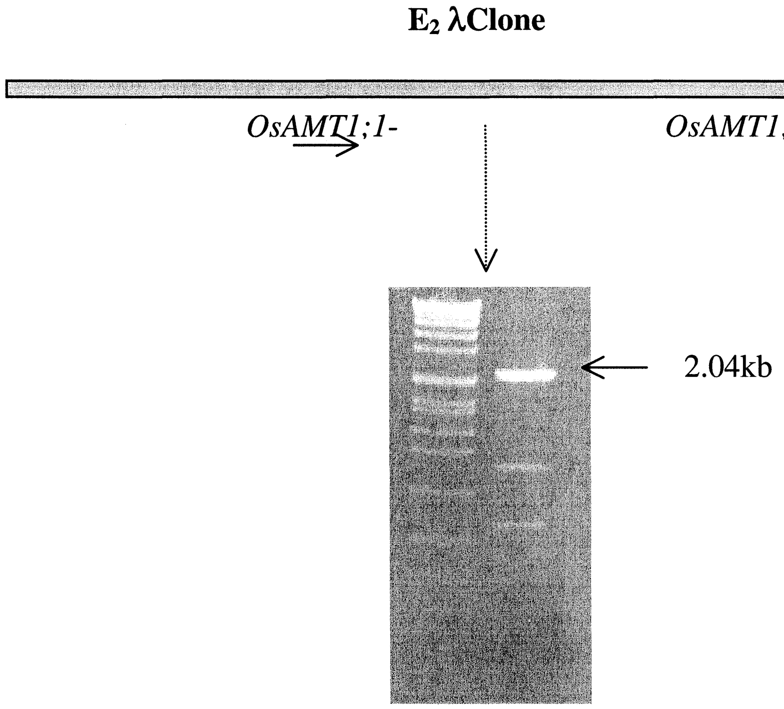


Figure 5.6 PCR amplification of an *AMT1* gene product from clone E2. A PCR product of 2.04 kb was amplified from λ E2, using OsAMT1;1-44$^+$ forward primer and OsAMT1;1-2058$^-$ reverse primer.

Figure 5.7 Evaluation of 5' and 3' sequences of pPIMP577.

(A). ***Oryza sativa*** **putative ammonium transporter** ***OsAMT1*****p (*****OsAMT1*****) mRNA, complete cds, Length = 2046.**
Score = 599 bits (302), Expect = e-169 Identities = 311/314 (99%) Strand = Plus / Plus

```
PISEQ30    77  aacgagggatcgtagagaggagggtttggtgagggagggaggaagatggcgacgtgcgcg 136
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1   44  aacgagggatcgtagagaggagggtttggtgagggagggaggaagatggcgacgtgcgcg 103

PISEQ30   137  gcggacctggcgccgctgctggggccggtggcggcgaacgcgacggactacctgtgcaac 196
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1  104  gcggacctggcgccgctgctggggccggtggcggcgaacgcgacggactacctgtgcaac 163

PISEQ30   197  cggttcgccaacacgacgtcggcggtggacgcgacgtacctgctcttctcggcgtacctc 256
               ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1  164  cggttcgccgacacgacgtcggcggtggacgcgacgtacctgctcttctcggcgtacctc 223

PISEQ30   257  gtgttcgccatgcagctcgggttcgcgatgctctgcgccgggtcggtgcgggccaagaac 316
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1  224  gtgttcgccatgcagctcgggttcgcgatgctctgcgccgggtcggtgcgggccaagaac 283

PISEQ30   317  acgatgatcatcatgctcaccagcgtgctcgacgccgcggccggggcgctcttctactac 376
               ||||||| |||||||||||||| |||||||||||||||||||||||||||||||||||||
OsAMT1;1  284  acgatgaacatcatgctcaccaacgtgctcgacgccgcggccggggcgctcttctactac 343

PISEQ30   377  ctcttcggcttcgc 390
               ||||||||||||||
OsAMT1;1  344  ctcttcggcttcgc 357
```

Score = 353 bits (178), Expect = 6e-95 Identities = 284/308 (92%), Gaps = 13/308 (4%)Strand = Plus / Plus

```
PISEQ30   402  gcacgccgtccaacggcttcatcggaagcagttcttcggcctcaagcacatgccg--ca 459
               |||||||||||||||||||||||||||||||||||||||||||||||||||||||   |
OsAMT1;1  378  gcacgccgtccaacggcttcatcggaagcagttcttcggcctcaagcacatgccggcaa 437

PISEQ30   460  gaccgggtt-cgactacgacttcttcctcttccagtgggccttcgccatcgccgcccgcc 518
               ||||||||| |||||||||||||||||||||||||||||||||||||||||||| |||||
OsAMT1;1  438  gaccgggttccgactacgacttcttcctcttccagtgggccttcgccatcgccg-ccgcc 496

PISEQ30   519  gggatcacgtngggctccatcgccgagaggccgcagttcgtngcctacctcatctacttc 578
               |||||||||| ||||||||||||||||||| |||||||||| ||||||||||||||||  
OsAMT1;1  497  gggatcacgtcgggctccatcgccgagaggacgcagttcgtcgcctacctcatctact-- 554

PISEQ30   579  ccgccttcctcnccgggtttcgtctaccccggtggtgntccactggatctgntccgcccg 638
               ||||||||||| ||||| |||||||| ||||||||||  |||||||||||| |||| |||
OsAMT1;1  555  ccgccttcctcaccggg-ttcgtcta-cccggtggtgtcccactggatctggtccg-ccg 611

PISEQ30   639  atgggtgggcctcttgccttcccgcacgtncgganctcttgctgttcggcttccggtgtn 698
               ||||||||||||| |||| |||||||||| |||| ||| ||||||||||| |||||||| 
OsAMT1;1  612  atgggtgggcctc-tgcc-tcccgcacgtccggacctc-tgctgttcggc-tccggtgtc 667

PISEQ30   699   atcgactt 706
                ||||||||
OsAMT1;1668     atcgactt 675
```

(B). ***Oryza sativa*** **putative ammonium transporter** ***OsAMT1*****p (*****OsAMT1*****) mRNA, complete cds Length = 2046 Score = 1090 bits (550), Expect = 0.0 Identities = 594/605 (98%), Gaps = 4/605 (0%) Strand = Plus / Minus**

```
PISE31   102    aagccaaagttttgatagagaggtagagcactgcaggacaaaggacccacgcaaagtct 161
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 2046   aagccaaagttttgatagagaggtagagcactgcaggacaaaggacccacgcaaagtct1987

PISE31   162    ttgagccctgcacagccacctatcttgaccaccattccaccacacccttatgacgataaa 221
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1986   ttgagccctgcacagccacctatcttgaccaccattccaccacacccttatgacgataaa1927

PISE31   222    attcgaccacaaaataaaacaataatagcagtaaccattgtgcaaaagaaaattaaaccc 281
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1926   attcgaccacaaaataaaacaataatagcagtaaccattgtgcaaaagaaaattaaaccc1867

PISE31   282    taacctctgattcttccca--ccttcacaccacacattctaagacacgtacgcatgcctg 339
                ||||||||||||||||||   |||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1866   taacctctgattcttccccacccttcacaccacacattctaagacacgtacgcatgcctg1807

PISE31   340    tacacaagagaagcccag-caaaagtgaaagacactcgtccatcctacagtgtttttaca 398
                |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1806   tacacaagagaagcccaggcaaaagtgaaagacactcgtccatcctacagtgtttttaca1747

PISE31   399    gtcttgcaacaccagagagagcggcaaaagcatttggcccaaacagaaactggcaatcaa 458
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1746   gtcttgcaacaccagagagagcggcaaaagcatttggcccaaacagaaactggcaatcaa1687

PISE31   459    atatggggatcaaatttatgacgtgacgatcgagatcggagagggagagaaacccgtgat 518
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1686   atatggggatcaaatttatgacgtgacgatcgagatcggagagggagagaaacccgtgat1627

PISE31   519    ttcttccttcgctgtgacgtcgttcgttctggattggttacacttggttgttgctgtcgg 578
                ||||||||||||||||||||  ||  ||||||||||||||||||||||||||||||| ||
OsAMT1;1 1626   ttcttccttcgctgtgacgtgcttgcttctggattggttacacttggttgttgctgttgg1567

PISE31   579    aggcagcccgccgccgccggctcgacgcgggtctgcgcggaccggagcatgaacccacca 638
                |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 1566   aggcagccggccgccgccggctcgacgcgggtctgcgcggaccggagcatgaacccacca1507

PISE31   639    accccagacttgtcgtgctcgtcctcgtcgtggtagacgtacgccgaacccgccgtgccg 698
                ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
OsAMT1;1 1506   accccagacttgtcgtgctcgtcctcgtcgtggtagacgtacg-cgaacccgccgtgccg1448

PISE31   699    tgtca 703
                |||||
OsAMT1;1 1447   tgtca 1443
```

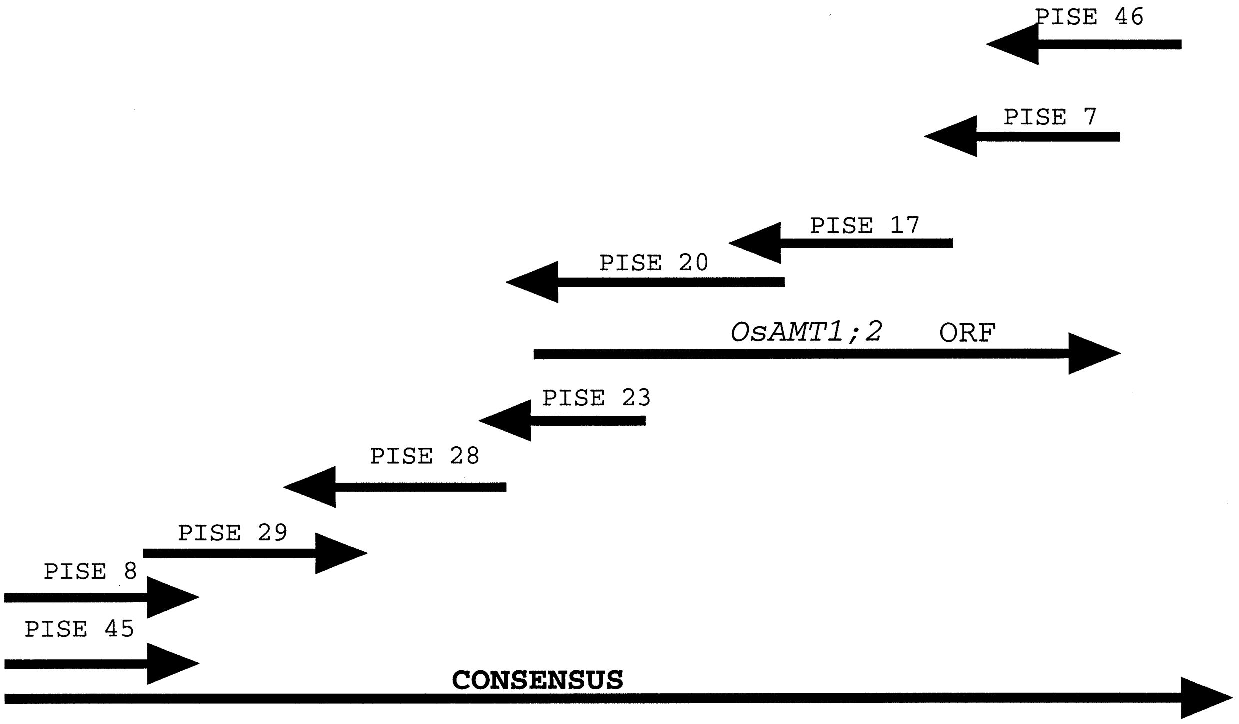


Figure 5.8 Sequencing strategy for *OsAMT1;2* (λclone A3 & J) from the subclones pPIMP321 and pPIMP827. Sequence PISE 46 with universal reverse primer, PISE7 with M13 universal forward primer, PISE17 with internal primer SE7-696$^+$, PISE 20 with internal primer SE17-658$^+$, PISE23 with internal primer SE 20-649$^+$, PISE 28 with internal primer SE 23-423+, PISE 29 with internal primer S 8-559+, PISE 8 with M13 universal reverse primer and PISE 45 with universal forward primer.

Figure 5.9 The sequences of isolated genomic clones of *OsAMT1* gene family, methionine codons are in bold and italicised.

```
            1                                                    50
{OsAMT1_1}  ATGGCgACGT GCgcGGcgga CCTgGcgCCG CTgCTgGGgc cgGtGGCG..
{OsAMT1_3}  ATGGCaACGT GCgcGGatac CCTcGgcCCG CTgCTgGGca cgGcGGCG..
{OsAMT1_2}  ATGGCgACGT GCttGGacag CCTcGggCCG CTtCTcGGcg gcGcGGCGaa
 Consensus  *****_**** **__**____ ***_*__*** **_**_**__ __*_****__

            51                                                  100
{OsAMT1_1}  ....gCgaAC GCGaCggACT ACcTgTGCAA CcgGTTCgCc GACACgaCgT
{OsAMT1_3}  ....gCgaAC GCGaCggACT ACcTgTGCAA CcaGTTCgCg GACACcaCgT
{OsAMT1_2}  ctccaCcgAC GCGgCcaACT ACaTcTGCAA CagGTTCaCg GACACctCcT
 Consensus  -----*--** ***_*__*** **_*_***** *__****_*_ *****__*_*

            101                                                 150
{OsAMT1_1}  CgGCgGTGGA CgCGACGTAC CTGCTCTTCT CGGCgTACCT CGTGTTCGCC
{OsAMT1_3}  CgGCcGTGGA CtCGACGTAC CTGCTCTTCT CGGCgTACCT CGTGTTCGCC
{OsAMT1_2}  CcGCgGTGGA CgCGACGTAC CTGCTCTTCT CGGCcTACCT CGTGTTCGCC
Consensus   *_**_***** *_******** ********** ****_***** **********

            151                                                 200
{OsAMT1_1}  ATGCAGCTCG GgTTCGCgAT GCTCTGCGCc GGgTCgGTgC GgGCCAAGAA
{OsAMT1_3}  ATGCAGCTCG GcTTCGCcAT GCTCTGCGCc GGgTCcGTcC GcGCCAAGAA
{OsAMT1_2}  ATGCAGCTCG GgTTCGCcAT GCTCTGCGCg GGcTCcGTcC GcGCCAAGAA
Consensus   ********** *_*****_** *********_ **_**_**_* *_********

            201                                                 250
{OsAMT1_1}  CaCgATGAAC ATCATGCTcA CCAACGTGcT CGACGCCGCg GCCGGgGCGC
{OsAMT1_3}  CaCcATGAAC ATCATGCTtA CCAACGTGcT CGACGCCGCc GCCGGcGCGC
{OsAMT1_2}  CtCaATGAAC ATCATGCTcA CCAACGTGtT CGACGCCGCc GCCGGcGCGC
Consensus   *_*_****** ********_* ********_* *********_ *****_****

            251                                                 300
{OsAMT1_1}  TCTTCTACTA CCTCTTCGGC TTCGCtTcgc ttcgcgattg ccttcgGcaC
{OsAMT1_3}  TCTTCTACTA CCTCTTCGGC TTCGCcT... .......tcg ccttcgGggC
{OsAMT1_2}  TCTTCTACTA CCTCTTCGGC TTCGCtTcgc gtc....... ......GgaC
Consensus   ********** ********** *****_*___ __________ ______*__*

            301                                                 350
{OsAMT1_1}  GCCGTCCAAc GGCTTCATCG GGAAGCAgTT CTTCGGcCTc AAGCAcaTgC
{OsAMT1_3}  GCCGTCCAAc GGCTTCATCG GGAAGCAcTT CTTCGGcCTc AAGCAggTcC
{OsAMT1_2}  GCCGTCCAAg GGCTTCATCG GGAAGCAgTT CTTCGGgCTg AAGCAcaTgC
Consensus   *********_ ********** *******_** ******_**_ *****__*_*

            351                                                 400
{OsAMT1_1}  cggCaagacc gGGtTcCGAC TACgaCTTCT TCCTCTTCCA GTGGGCCTTC
{OsAMT1_3}  ...Cacaggt cGGcTtCGAC TACagCTTCT TCCTCTTCCA GTGGGCCTTC
{OsAMT1_2}  ...Cgcagac aGGgTaCGAC TACgaCTTCT TCCTCTTCCA GTGGGCCTTC
Consensus   ---*------ _**_*_**** ***__***** ********** **********

            401                                                 450
{OsAMT1_1}  GCCATCGCCG CCGCcGGgAT CACGTCgGGc TCCATCGCCG AgaGGACgCa
{OsAMT1_3}  GCCATCGCCG CCGCgGGcAT CACGTCcGGc TCCATCGCCG AgcGGACcCa
{OsAMT1_2}  GCCATCGCCG CCGCcGGcAT CACGTCcGGt TCCATCGCCG AacGGACgCg
Consensus   ********** ****_**_** ******_**_ ********** *__****_*_
```

```
               451                                                500
{OsAMT1_1}     gTTCgtcGCc TAcCTCATCT ACTCCGCCTT CCTCACCGGg TTCGTcTACC
{OsAMT1_3}     gTTCgtgGCg TAcCTCATCT ACTCCGCCTT CCTCACCGGc TTCGTcTACC
{OsAMT1_2}     cTTCagcGCg TAtCTCATCT ACTCCGCCTT CCTCACCGGg TTCGTgTACC
Consensus      _***___**_ **_******* ********** *********_ *****_****

               501                                                550
{OsAMT1_1}     CGGTGGTGTC cCACTGGaTC TGGTCCgCCG AtGGGTGGGC cTCtGCctcC
{OsAMT1_3}     CGGTGGTGTC cCACTGGaTC TGGTCCgCCG AcGGGTGGGC cTCgGCttcC
{OsAMT1_2}     CGGTGGTGTC gCACTGGtTC TGGTCCaCCG AcGGGTGGGC tTCgGCcggC
Consensus      ********** _******_** ******_*** *_******** _**_**___*

               551                                                600
{OsAMT1_1}     CGcacGtCcG GacCtcTGCT gTTCggcTCc GGtGTCATCG ACTTCGCCGG
{OsAMT1_3}     CGaacGtCgG GgtCgcTGCT cTTCgggTCc GGcGTCATCG ACTTCGCCGG
{OsAMT1_2}     CGgctGaCgG GtcCgtTGCT gTTCaagTCg GGcGTCATCG ACTTCGCCGG
Consensus      **___*_*_* *__*__**** _***___**_ **_******* **********

               601                                                650
{OsAMT1_1}     cTCcGGcGTc GTCCAcaTGG TcgGcgGtgt cGCcGGgCTc TGGGGcGCgc
{OsAMT1_3}     gTCaGGgGTt GTCCAcaTGG T..GgcGgcg tGCcGGaCTc TGGGGcGCcc
{OsAMT1_2}     cTCcGGcGTc GTCCAtcTGG TcgGtgGcat tGCtGGcCTg TGGGGtGCct
Consensus      _**_**_**_ *****__*** *__*__*___ _**_**_**_ *****_**__

               651                                                700
{OsAMT1_1}     TCATCGAGGG CCCCCGCATc gGGaGgTTCG ACcaCGCCGG CCGatCGGTG
{OsAMT1_3}     TCATCGAGGG CCCCCGCAT. tGGcGgTTCG ACcaCGCCGG CCGctCGGTG
{OsAMT1_2}     TCATCGAGGG CCCCCGCATc gGGcGcTTCG ACgcCGCCGG CCGcaCGGTG
Consensus      ********** *********_ _**_*_**** **__****** ***__*****

               701                                                750
{OsAMT1_1}     GCGcTcaagG GcCACAGCGC gTCgCTcGTC GTGCTtGGCA cCTTCCTGCT
{OsAMT1_3}     GCGcTgcgcG GcCACAGCGC gTCgCTcGTC GTGCTcGGCA gCTTCCTGCT
{OsAMT1_2}     GCGaTgaaaG GgCACAGCGC cTCaCTgGTC GTGCTcGGCA cCTTCCTGCT
Consensus      ***_*____* *_******** _**_**_*** *****_**** _*********

               751                                                800
{OsAMT1_1}     GTGGTTCGGc TGGTaCGGaT TcAACCCcGG gTCgTTCacC ACCATCctCA
{OsAMT1_3}     GTGGTTCGGg TGGTaCGGgT TtAACCCcGG cTCgTTCctC ACCATCctCA
{OsAMT1_2}     GTGGTTCGGg TGGTtCGGcT TcAACCCgGG gTCcTTCacC ACCATCtcCA
Consensus      *********_ ****_***_* *_*****_** _**_***__* ******__**

               801                                                850
{OsAMT1_1}     AgacgTACGG CccGgCcGGc ggcATCaACG GGCAGTGGTC cGgaGTcGGc
{OsAMT1_3}     AatccTACGG CccGcCcGGt agcATCcACG GGCAGTGGTC gGcgGTgGGa
{OsAMT1_2}     AgatcTACGG CgaGtCgGGc acgATCgACG GGCAGTGGTC gGcgGTgGGc
Consensus      *____***** *__*_*_**_ ___***_*** ********** _*__**_**_

               851                                                900
{OsAMT1_1}     CGCACCGCCG TGACgACgaC cCTgGCcGGC AGCgtgGCgG CGC.TcACcA
{OsAMT1_3}     CGCACCGCCG TGACcACcaC cCTcGCcGGC AGCacgGCgG CGC.TcACgA
{OsAMT1_2}     CGCACCGCCG TGACgACgtC gCTgGCgGGC AGCgtcGCcG CGCtTaACcA
Consensus      ********** ****_**__* _**_**_*** ***___**_* ***_*_**_*

               901                                                950
{OsAMT1_1}     CGCTgTtCGG gAAGcGgctc CaGACGGGGC ACTGGAACGT GgtCGACGTC
{OsAMT1_3}     CGCTcTtCGG gAAGaGgctc CaGACGGGGC ACTGGAACGT GatCGACGTC
{OsAMT1_2}     CGCTgTaCGG cAAGaGatgg CtGACGGGGC ACTGGAACGT GacCGACGTC
Consensus      ****_*_*** _***_*____ *_******** ********** *__*******
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            951                                               1000
{OsAMT1_1}  TGCAACGGcC TCCTCGGCGG gTTCGCcGCc ATCACCGCcG GgTGCagCGT
{OsAMT1_3}  TGCAACGGcC TCCTCGGCGG cTTCGCgGCg ATCACCGCcG GtTGCtcCGT
{OsAMT1_2}  TGCAACGGtC TCCTCGGCGG gTTCGC.GCg ATCACCGCgG GcTGCtcCGT
Consensus   ********_* ********** _*****_**_ ********_* *_***__***

            1001                                              1050
{OsAMT1_1}  cGTCGACCCG TGGGCcgCGa TcATCTGCGG GTTCGTcTCG GCGTGGGTgC
{OsAMT1_3}  cGTCGACCCG TGGGCcgCGa TcATCTGCGG GTTCGTcTCG GCGTGGGTgC
{OsAMT1_2}  gGTCGACCCG TGGGCgtCGg TgATCTGCGG GTTCGTgTCG GCGTGGGTcC
Consensus   _********* *****__**_ *_******** ******_*** ********_*

            1051                                              1100
{OsAMT1_1}  TCATCGGCct CAACgcGCTc GCcgcGcgcC TCAAGTTCGA cGACCCGCTc
{OsAMT1_3}  TCATCGGCct CAACgcGCTg GCggcGaggC TCAAGTTCGA cGACCCGCTc
{OsAMT1_2}  TCATCGGCtg CAACaaGCTg GCgctGatgC TCAAGTTCGA tGACCCGCTg
Consensus   ********__ ****__***_ **___*___* ********** _********_

            1101                                              1150
{OsAMT1_1}  GAGGCcgCcC AGCTcCACGG CGGGTGCGGC GCGTGGGGGa TCcTCTTCAC
{OsAMT1_3}  GAGGCggCgC AGCTgCACGG CGGGTGCGGC GCGTGGGGGg TCaTCTTCAC
{OsAMT1_2}  GAGGCgaCgC AGCTgCACGG CGGGTGCGGC GCGTGGGGGa TCaTCTTCAC
Consensus   *****__*_* ****_***** ********** *********_ **_*******

            1151                                              1200
{OsAMT1_1}  cGCGCTcTTC GCGaGgcAGa AGTACGTcGA ggaGATCTaC GGc...gCcG
{OsAMT1_3}  gGCGCTgTTC GCGcGcaAGg AGTACGTgGA ccaGATCTtC GGccagcCcG
{OsAMT1_2}  cGCGCTgTTC GCGcGcaAGg AGTACGTcGA gctGATCTaC GGggtgcCgG
Consensus   _*****_*** ***_*__**_ *******_** ___*****_* **_____*_*

            1201                                              1250
{OsAMT1_1}  GccGgCCGTA cGGcCTGTTC ATGGGCGGCG GCGGcaaGCT gCTCGccGCG
{OsAMT1_3}  GgcGcCCGTA tGGgCTGTTC ATGGGCGGCG GCGGccgGCT gCTCGggGCG
{OsAMT1_2}  GgaGgCCGTA cGGgCTGTTC ATGGGCGGCG GCGGgagGCT tCTCGcgGCG
Consensus   *__*_***** _**_****** ********** ****___*** _****__***

            1251                                              1300
{OsAMT1_1}  CACgTcaTcc agATCCTGGT cATCttcGgG TGGGTcAGCt gCACCATGGg
{OsAMT1_3}  CACaTagTgg taATCCTGGT cATCgcgGcG TGGGTgAGCt tCACCATGGc
{OsAMT1_2}  CACaTcgTgc agATCCTGGT gATCgtcGgG TGGGTcAGCg cCACCATGGg
Consensus   ***_*__*__ __******** _***___*_* *****_***_ _********_

            1301                                              1350
{OsAMT1_1}  acCtCTcTTC tacGgGCTca AgAaGcTcGG ccTGCTcCGC aTCTCcgCCg
{OsAMT1_3}  gcCgCTgTTC ctgGtGCTca AcAaGcTgGG atTGCTgCGC aTCTCggCCg
{OsAMT1_2}  gaCgCTcTTC tacGtGCTgc AcAgGtTcGG gcTGCTcCGC gTCTCgaCCt
Consensus   __*_**_*** ___*_***__ *_*_*_*_** __****_*** _****__**_


            1351                                              1400
{OsAMT1_1}  aGgacGAGAc GtccGGCATG GACCtGaCac GgCACGGCGG GTTCGcGTAC
{OsAMT1_3}  aGgacGAGAt GgccGGCATG GACCaGaCgc GcCACGGCGG GTTCGcGTAC
{OsAMT1_2}  cGacaGAGAt GgaaGGCATG GACCcGtCgt GcCACGGCGG GTTCGgGTAC
Consensus   _*___****_ *___****** ****_*_*__ *_******** *****_****

            1401                                              1450
{OsAMT1_1}  GtctACcAcG ACGAgGaCga GcaCgaCaag tctGggttg gtgggttcat
{OsAMT1_3}  GcgtACcAcG ACGAcGaCgc GagCggCaag ccgGaccgca gcttcggcgg
{OsAMT1_2}  GtggACgAgG ACGAaGgCca GcgCcgCgtc aggGccaagt cggcggcgga
Consensus   *___**_*_* ****_*_*__ *__*__*___ ___*______ __________
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             1451                                               1500
{OsAMT1_1}   GctccggtcC gcGcaGaCcc gcgtcgaGCc GGcggcgGCC Ggctgcctcc
{OsAMT1_3}   GttcatgctC aaGtcGgCgc acggcacGCa GGtcgccGCC Gagatgggag
{OsAMT1_2}   GacggctcgC gtGgaGcCca gaaagtcGCc GGagcaaGCC Gcggcgggcc
Consensus    *--------* --*--*-*-- -------**- **-----*** *---------

             1501                                               1550
{OsAMT1_1}   aacagcaaca accaagtgta accaatccag aacgaacgac gtcacagcga
{OsAMT1_3}   gccatgtc~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
{OsAMT1_2}   agttggtg~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
 Consensus   ---------- ---------- ---------- ---------- ----------

             1551                                               1600
{OsAMT1_1}   aggaagaaat cacgggtttc tctccctctc cgatctcgat cgtcacgtca
{OsAMT1_3}   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
{OsAMT1_2}   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
Consensus    ---------- ---------- ---------- ---------- ----------
```

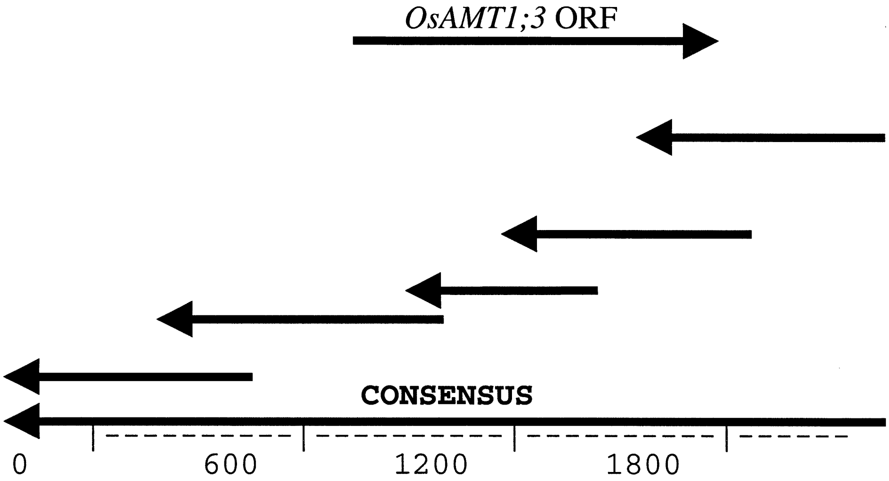
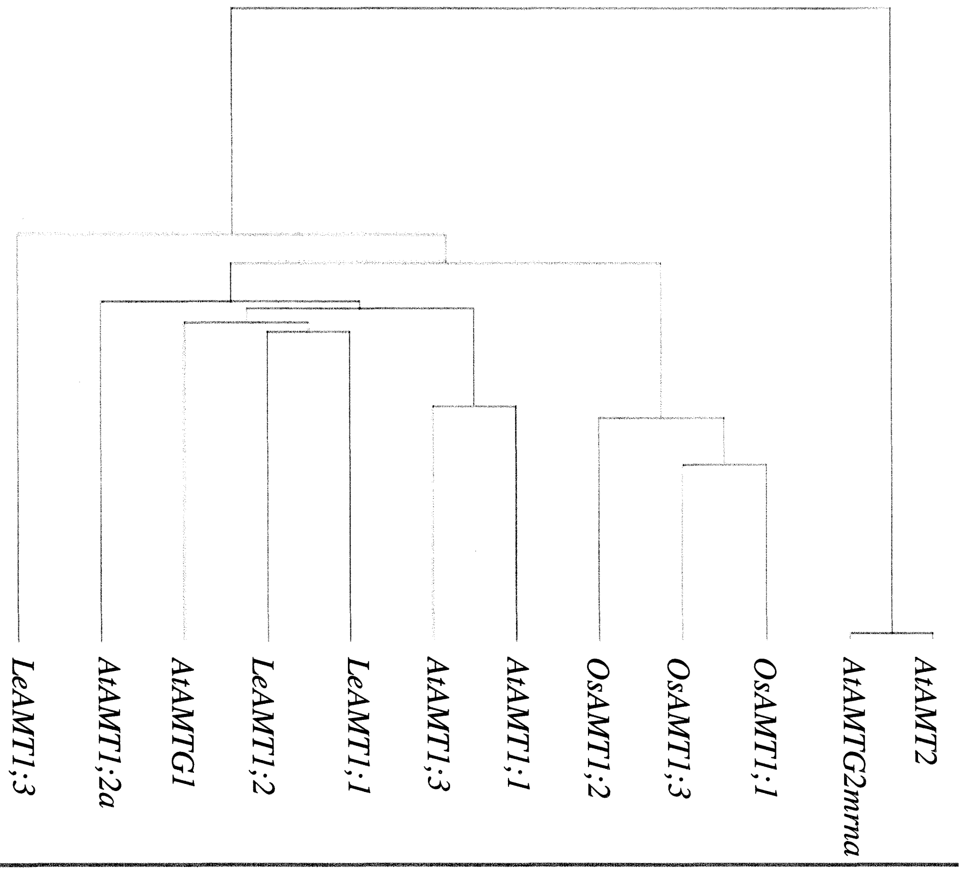


Figure 5.10 Sequencing strategy for *OsAMT1;3*. (λclone K) from the sub clone pPIMP834. Sequence PISE47 with M13 universal reverse primer, PISE 57 with clone K internal primer SE47- 538^{+}, PISE 62 with internal primer SE57-565^{+}, PISE 69 with internal primer SE62- 624^{+} and PISE 75 with internal primer S69-579^{+}.

A.



B.

	OsAMT1;2	OsAMT1;3	AtAMT1;1	AtAMT1;2	AtAMT1;3	AtAMT2	AtAMTG1	AtAMTG2	AtAMTG3	LeAMT1;1	LeAMT1;2	LeAMT1;3
OsAMT1;1	0.76	0.81	0.63	0.61	0.59	0.31	0.57	0.31	0.28	0.59	0.55	0.50
OsAMT1;2		0.76	0.61	0.60	0.59	0.33	0.59	0.33	0.28	0.58	0.56	0.51
OsAMT1;3			0.65	0.62	0.61	0.32	0.59	0.32	0.29	0.60	0.58	0.52
AtAMT1;1				0.64	0.75	0.33	0.64	0.33	0.31	0.67	0.65	0.58
AtAMT1;2					0.62	0.33	0.64	0.33	0.30	0.63	0.65	0.57
AtAMT1;3						0.33	0.63	0.33	0.31	0.64	0.63	0.59
AtAMT2							0.33	0.99	0.31	0.31	0.33	0.34
AtAMTG1								0.33	0.31	0.65	0.66	0.61
AtAMTG2									0.31	0.31	0.33	0.34
LeAMT1;1											0.67	0.60
LeAMT1;2												0.62

Figure 5.11 A. Phylogenetic tree of plant ammonium transporters. ORF were aligned using PILEUP. The gene bank accession numbers for the sequences used are as follows: *At AMT1;1* (X75879), *AtAMT1;2* (AF083036), *AtAMT1;3* (AF083035), *AtAMT2* (AF 182039), *AtAMT2G* (AC004683), *AtAMT3G* (AL035709), *AtAMTG1* (ATF 16A16), *LeAMT1;1* (X92854), *LeAMT1;2* (X95098), *LeAMT1;3* (AF 118858) *OsAMT1;1* (This thesis), *OsAMT1;2* (This thesis), *OsAMT1;3* (This thesis). B. Comparison of the known plant *AMT* ORF sequences. ORF sequence similarities between all pairs of available Ammonium transporter sequences.

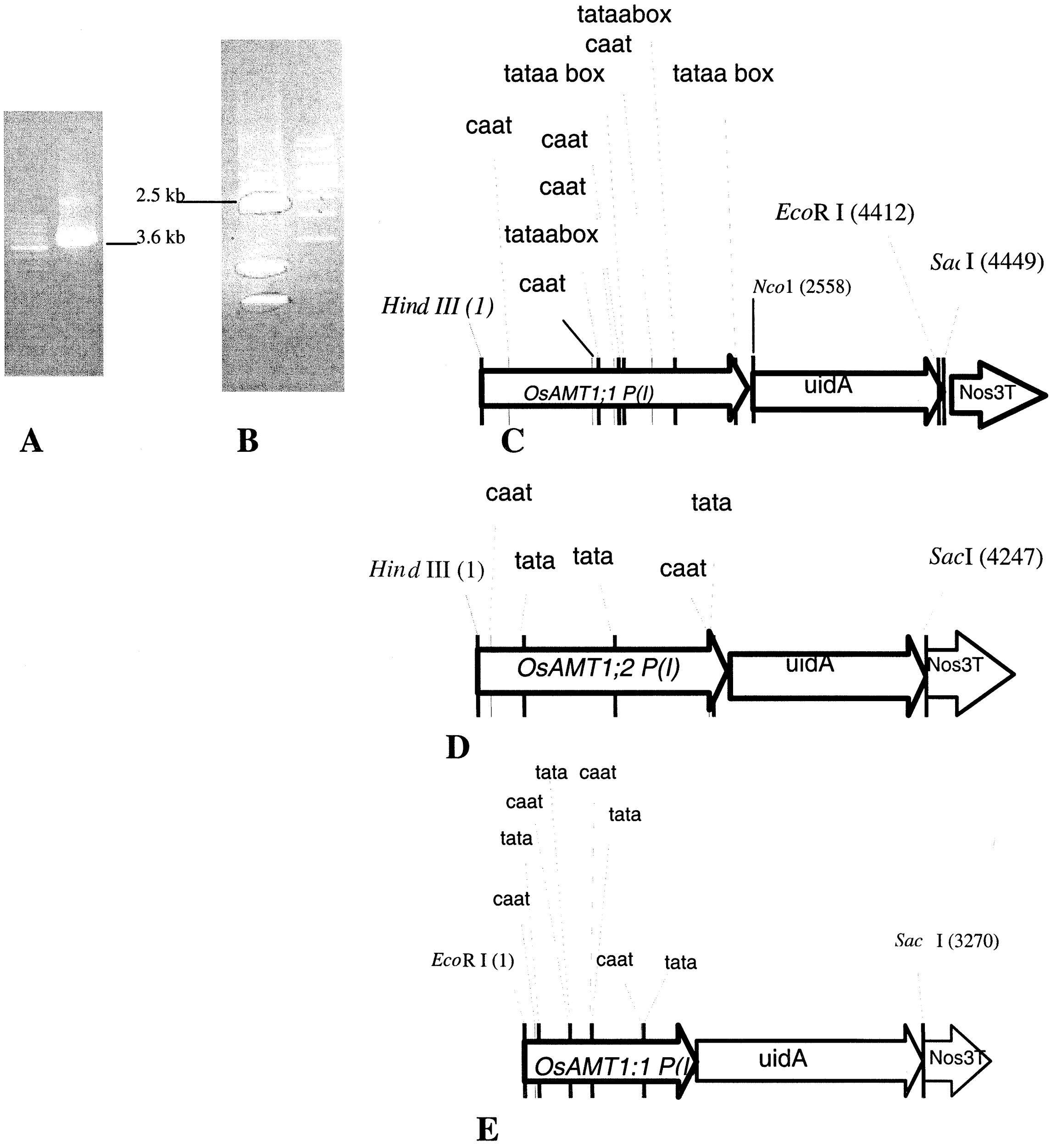


Figure 5.12 Construction of promoter-*gus* fusions. (A) A 3.6 kb PCR product amplified with the forward primer SE74-668$^+$ and the reverse primer *OsAMT1;1*-90$^-$; (B) PCR product was digested with *Hind*III and *Nco*I to produce three fragments including a 2.5 kb *Hind*III/*Nco*I fragment; (C) construct pPIMP920 showing the 2.5 kb promoter region from *OsAMT1;1* gene linked to *uidA*; (D) construct pPIMP841 showing 2.38 kb promoter region from *OsAMT1;2* gene linked *uidA*; (E) construct pPIMP934 showing the 1.4 kb promoter region from *OsAMT1;3* gene linked to *uidA*.

Figure 5.13. The nucleotide sequences of the rice ammonium transporter gene *OsAMT1;1* (A), *OsAMT1;2* (B) *OsAMT1;3* (C). The deduced amino acid sequence is shown underneath in single letter code. The symbol * indicates the termination codons. Proposed ATG initiation codons are bold and italics. Putative GATA sequences are underlined, TATA and CAAT sequences are in bold and underlined, and possible transcription termination sequences (arrow and underlined) are indicated. The GenBank accession number for these sequences are Bankit No. 348508, 348512 and 348516 for *OsAMT1;1, OsAMT1;2* and *OsAMT1;3* respectively.

(A). *OsAMT1;1*

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GATAACCAAATCGGACGCTGACCTTGCTGGGCGAACTGGGTGATCATCGATGGCGATGCGAGACATCACCCAACTGCGTCGGGTCTCCAC   90
AGGAGGGAGTTGCTTGTGCTTGGTCCGTTTGGGGATCGTTAACTTAAACACTTTTACGGCGACCTCGACACAGCTAAACCCTAAACTAAT   180
TGCGAGTTAGAGGCTTATCTCGATCTCTTCTATGCAGATGTTTGACAACTTGGGAGTAGTTTACTGCTGGTTTGGAGTATCTTCTCAACT   270
TGCAATTTGATTATGTTTAAACGGGGAGTGCATGATTGGTGTTCGCATGGTTTTAAATCAGATTTTATAAACTGATGCTCGTCAAGAGAC   360
GACAAGGGGCCAGATTAGGGCAGCAGAGTACGTGCTTGCTTGAATTCTGAAGCATGTACGAAATAAATACGATAGAAATTTCTTAAGAAA   450
TTAGGTATTTTTCTGACCCTCCAATAAGATCGCGTGGTTGCCAGTATTGCACGTCGACTACTACATATCTGAATTCAGAACAATCCAAAA   540
GAGAAGTTACTGTTGATATTTCTACGTATAAAAAAAACATCAAAATGCTTTGTATATTACGAAAACAGAGCGAGTTCCCTTATTGACCAG   630
AGCAAAAAGGTTGAGCCTGATTAAACAAAGTCTATGAGCTTGCAGGATGCGTCTCTTCCCAAATTTATTCACACCAAAGTCCTCTTCGAT   720
GACATCGCCCTATTTGAATCTTATCGTTGACATTGCTCATTTTGCTCTTTAGTTAATCTGGGCAAATGATTGGCGGTGGTACTTCGTGAT   810
GTGGAACAGTGAAACTGTTTGTCAATCTGTGCGCTCGAGGTACAACCAGGTCGGTTCCTTTGCTGTTTTATTAATAAAAGGAGCATAAAT   900
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GAACAGAATAGGATATGTATTAAAAAAACAGAATGGAAGAAAAATCCTGAGTCACAAGCACGAAAAATGAAGGCGAGATTAATTCGAAAC   1350
CATACACATCATCATCCACATCTCGTCGTTTGTCTCACAGGACATGACACAGGGAGCGAAAACCACATCATTAATCGCGGCCTACAGCTA   1440
CACATCCAGATTCTCCCGGGATCCCCGAAACGGCTCCCACCCCGCAACCGCCGCAAGCCGACCCAGCCAAAGGAGATCCCCCTCCACCAC   1530
GGAAGATTCACTGCGCGGTGGGCCCCGCCGCCAAAAACCAAAACGACGAAACCATTCCGCGTCATCTCTCCCGCACGGCGAGCGAGCGAG   1620
CGAGCGACCTGACCTCCTCCTCCTATAAATCCGGCGCCAGCGTATCTCCCCAACCTCCCACGCCCAATCCTGCCGCCGTTTCAGCAGCTC   1710
TAGTTTGAACGAGGGATCGTAGAGAGGAGGGTTTGGTGAGGGAGGGAGGAAGATGGCGACGTGCGCGGCGGACCTGGCGCCGCTGCTGGG   1800
                                                     M  A  T  C  A  A  D  L  A  P  L  L  G   13
GCCGGTGGCGGCGAACGCGACGGACTACCTGTGCAACCGGTTCGCCGACACGACGTCGGCGGTGGACGCGACGTACCTGCTCTTCTCGGC   1890
 P  V  A  A  N  A  T  D  Y  L  C  N  R  F  A  D  T  T  S  A  V  D  A  T  Y  L  L  F  S  A    43
GTACCTCGTGTTCGCCATGCAGCTCGGGTTCGCGATGCTCTGCGCCGGGTCGGTGCGGGCCAAGAACACGATGAACATCATGCTCACCAA   1980
 Y  L  V  F  A  M  Q  L  G  F  A  M  L  C  A  G  S  V  R  A  K  N  T  M  N  I  M  L  T  N    73
CGTGCTCGACGCCGCGGCCGGGGCGCTCTTCTACTACCTCTTCGGCTTCGCCTTCGCCTTCGGCACGCCGTCCAACGGCTTCATCGGGAA   2070
 V  L  D  A  A  A  G  A  L  F  Y  Y  L  F  G  F  A  F  A  F  G  T  P  S  N  G  F  I  G  K    103
GCAGTTCTTCGGCCTCAAGCACATGCCGCAGACCGGGTTCGACTACGACTTCTTCCTCTTCCAGTGGGCCTTCGCCATCGCCGCCGCCGG   2160
 Q  F  F  G  L  K  H  M  P  Q  T  G  F  D  Y  D  F  F  L  F  Q  W  A  F  A  I  A  A  A  G    133
GATCACGTCGGGCTCCATCGCCGAGAGGACGCAGTTCGTCGCCTACCTCATCTACTCCGCCTTCCTCACCGGGTTCGTCTACCCGGTGGT   2250
 I  T  S  G  S  I  A  E  R  T  Q  F  V  A  Y  L  I  Y  S  A  F  L  T  G  F  V  Y  P  V  V    163
GTCCCACTGGATCTGGTCCGCCGATGGGTGGGCCTCTGCCTCCCGCACGTCCGGACCTCTGCTGTTCGGCTCCGGTGTCATCGACTTCGC   2340
 S  H  W  I  W  S  A  D  G  W  A  S  A  S  R  T  S  G  P  L  L  F  G  S  G  V  I  D  F  A    193
CGGCTCCGGCGTCGTCCACATGGTCGGCGGTGTCGCCGGGCTCTGGGGCGCGCTCATCGAGGGCCCCCGCATCGGGAGGTTCGACCACGC   2430
 G  S  G  V  V  H  M  V  G  G  V  A  G  L  W  G  A  L  I  E  G  P  R  I  G  R  F  D  H  A    223
CGGCCGATCGGTGGCGCTCAAGGGCCACAGCGCGTCGCTCGTCGTGCTTGGCACCTTCCTGCTGTGGTTCGGCTGGTACGGATTCAACCC   2520
 G  R  S  V  A  L  K  G  H  S  A  S  L  V  V  L  G  T  F  L  L  W  F  G  W  Y  G  F  N  P    253
CGGGTCGTTCACCACCATCCTCAAGACGTACGGCCCGGCCGGCGGCATCAACGGGCAGTGGTCCGGAGTCGGCCGCACCGCCGTGACGAC   2610
 G  S  F  T  T  I  L  K  T  Y  G  P  A  G  G  I  N  G  Q  W  S  G  V  G  R  T  A  V  T  T    283
GACCCTGGCCGGCAGCGTGGCGGCGCTCACCACGCTGTTCGGGAAGCGGCTCCAGACGGGGCACTGGAACGTGGTCGACGTCTGCAACGG   2700
 T  L  A  G  S  V  A  A  L  T  T  L  F  G  K  R  L  Q  T  G  H  W  N  V  V  D  V  C  N  G    313
CCTCCTCGGCGGGTTCGCCGCCATCACCGCCGGGTGCAGCGTCGTCGACCCGTGGGCCGCGATCATCTGCGGGTTCGTCTCGGCGTGGGT   2790
 L  L  G  G  F  A  A  I  T  A  G  C  S  V  V  D  P  W  A  A  I  I  C  G  F  V  S  A  W  V    343
GCTCATCGGCCTCAACGCGCTCGCCGCGCGCCTCAAGTTCGACGACCCGCTCGAGGCCGCCCAGCTCCACGGCGGGTGCGGCGCGTGGGG   2880
 L  I  G  L  N  A  L  A  A  R  L  K  F  D  D  P  L  E  A  A  Q  L  H  G  G  C  G  A  W  G    373
GATCCTCTTCACCGCGCTCTTCGCGAGGCAGAAGTACGTCGAGGAGATCTACGGCGCCGGCCGGCCGTACGGCCTGTTCATGGGCGGCGG   2970
 I  L  F  T  A  L  F  A  R  Q  K  Y  V  E  E  I  Y  G  A  G  R  P  Y  G  L  F  M  G  G  G    403
CGGCAAGCTGCTCGCCGCGCACGTCATCCAGATCCTGGTCATCTTCGGGTGGGTCAGCTGCACCATGGGACCTCTCTTCTACGGGCTCAA   3060
 G  K  L  L  A  A  H  V  I  Q  I  L  V  I  F  G  W  V  S  C  T  M  G  P  L  F  Y  G  L  K    433
GAAGCTCGGCCTGCTCCGCATCTCCGCCGAGGACGAGACGTCCGGCATGGACCTGACACGGCACGGCGGGTTCGCGTACGTCTACCACGA   3150
 K  L  G  L  L  R  I  S  A  E  D  E  T  S  G  M  D  L  T  R  H  G  G  F  A  Y  V  Y  H  D    463
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GTCACGTCATAAATTTGATCCCCATATTTGATTGCCAGTTTCTGTTTGGGCCAAATGCTTTTGCCGCTCTCTCTGGTGTTGCAAGACTGT   3420
 S  R  H  K  F  D  P  H  I  *                                                                533
AAAAACACTGTAGGATGGACGAGTGTCTTTCACTTTTGCTGGGCTTCTCTTGTGTACAGGCATGCGTACGTGTCTTAGAATGTGTGGTGT   3510
            <---------           --------->
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GTCATAAGGGTGTGGTGGAATGGTGGTCAAGATAGGTGGCTGTGCAGGGCTCAAAGACTTTGCGTGGGTCCTTTTGTCCTGCAGTGCTCT   3690

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CTGATCTTTACAACGACCGCTGTATAGAGAACAGACTATATCAAGGTTTTGGGTCCGTGGTCTTCTTTTTGGG   4123
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(B). *OsAMT1;2*

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ACCAGCTGCTGCGCAACCGGCCAACCGCCCAGCCGTGCAGCTGTGGTGGAGTGACCACGGCCACGACTCCGTGCGCGCGGGTGGACGTAA    180
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TACACATACAACAAGTAACGTAGAGAGATCCCCAAATCAGCTGCGGCAGAAGGCAGAAACCGTGACTAGTACATCTCATAAACTTAACGA    630
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CAGAGTGTAAAAATTAGATACGCAGCATCTGCAAGTCATACTTGCAAGGAAGAAGCAATTGCGTCCCTATCCACCACATCTTATCCAGTC   1080
TTGTCAGAGTTTTGACCTAAGGAATTGCTTCATCATCTGAATTTATTCTACTGGAAGAAATTACCTACTGCTATGCCAAGAAGTAAGAAT   1170
ACATGGTTAATCTATGTTGACACCCTCATTTCTGAAGACAAACAACATAAATTTGCAGTGAGTTAAAACATATAGTTTCAGTGAAAATAT   1260
CGTACAGGTTAATGTAGCCAGACCAACACAGAGATCTGATTGACAATTACAGAGTACTCAGTAGTCAGCAAGCAGGTTTAGCATGGACTA   1350
CCTGTTGATTACATGGTTTCAGTCAGACACGAGTTCTTCAGGGAGGCAAATTAATCACAAGGTTCTTCCAAGACAGAAGCCGCCGGTAAG   1440
GTATGGAAGCAAATGGGTTTATCTCCGTTTGTGCCAATAACCTTATCGAATATCTAATTGTTCGCTAAGTACGCATTGCACACATCATAT   1530
ACCATCTCGATTGATGAGTTTTGTCGCCATGTTCTGCTAGGTACGACCTATCCGCTGCTGGGTTTACATGCATGCCTGAAGAACTTAAAC   1620
AGTTAATCAACAAAGTCAATGGATATTACGCATACAAGTATATGGTTGTATATATGCAACTTCATGACACAACAGTATGCGTATAGTCGG   1710
ACGTGACGACAAGCAACTACCTCGTGCAAGGATGCGAGGAGATACCAGATTAAAACCTGTAATACTTGAAGTTGACAAAATGCGATTCTT   1800
CAGACGATATAATAACAAGAACATTTCTGAATCTTCCTTTAAAAAATGTTGAATGCATAAAAGAATCTTAGCTGTGATGGCAACAACACG   1890
ACTTTCTGATAGTGACATTGGATCTTAGTTGAATCTGGCATCTTGCGTATGCGACCTTGCTTGGATCTGTCGGATACTCCCAACCCAGCA   1980
TAAATTACTGATGTCTGAAACTTTCTGAGCAAAGCGGGAACTCAGGCTAGTTGAGTCGCTCATCATCAAAAGTCAAGACAATACTTAAGT   2070
AAAAACAAAACAAATATCACTGTCGCAAAACCAGTGTAAACCTATTGGGATAATTTTAACAGTCTTACAACACCAGCGCCGAGACGTCTA   2160
GGTAACAAATTAAATCATCACTGACAATATTTGAAGATAAGTGAATCACATGTCTTTCTCAATGAACTTAAATTTATGAATGAACCAACC   2250
TATACATGCAACTAAATATGATATCAAGATCAGTTAAAAATCTTGTTTTGTGAAATTTCAAAACAAAAAAATAAAATTGCAGCGTACCTT   2340
TATTTCAAGAGAAATTTAATTCACTAAAAAAAAGTAATTGTATGTGCCAAATTTTACATTAAAAAATGGTATCTGCAATGTATTCTTAAG   2430
GATGATAAAATTATCTCAGTAATATTCAATCATTCATTAATAGAGGTGAACTGCTCGTTCTTTTACTCATCACACCTGTTGGATGAAAAG   2520
ATTGGTTGCTGCCACTAAAAAAATTAATCAACTCATTGCAGTTGGCAAAAAAGAAAAAAAAAGGTTTTCAGGCACTAGTATATGTGATGT   2610
TAGAAGGAATCCAAAACAGTATACATGCATCCACGCGCACCTCGGTTTTGCATTTCCCGCTGTCTGTGATCATGAATCATCCAATTAAAA   2700
ACAACCATCAACCATGGATTCCAATGTGTTCTGCCCCTATAAATAGTCCAGCATCTCATTCTCTCGTCTACTTCAAAGAATCACACACCA   2790
AAACCTCCATTAGTTCCTAAACCCTAGCAAGAAGCAGCACAAAACCTTGCCACACTTGGCTAGTGACACTGAGACACACCATGGCGACGT   2880
                                                                                 M  A  T         4
GCTTGGACAGCCTCGGGCCGCTTCTCGGCGGCGCGGCGAACTCCACCGACGCGGCCAACTACATCTGCAACAGGTTCACGGACACCTCCT   2970
 C  L  D  S  L  G  P  L  L  G  G  A  A  N  S  T  D  A  A  N  Y  I  C  N  R  F  T  D  T  S     34
CCGCGGTGGACGCGACGTACCTGCTCTTCTCGGCCTACCTCGTGTTCGCCATGCAGCTCGGGTTCGCCATGCTCTGCGCGGGCTCCGTCC   3060
 S  A  V  D  A  T  Y  L  L  F  S  A  Y  L  V  F  A  M  Q  L  G  F  A  M  L  C  A  G  S  V     64
GCGCCAAGAACTCAATGAACATCATGCTCACCAACGTGTTCGACGCCGCCGCCGGCGCGCTCTTCTACTACCTCTTCGGCTTCGCTTCGC   3150
 R  A  K  N  S  M  N  I  M  L  T  N  V  F  D  A  A  A  G  A  L  F  Y  Y  L  F  G  F  A  S     94
GTCGGACGCCGTCCAAGGGCTTCATCGGGAAGCAGTTCTTCGGGCTGAAGCACATGCCGCAGACAGGGTACGACTACGACTTCTTCCTCT   3240
 R  R  T  P  S  K  G  F  I  G  K  Q  F  F  G  L  K  H  M  P  Q  T  G  Y  D  Y  D  F  F  L    124
TCCAGTGGGCCTTCGCCATCGCCGCCGCCGGCATCACGTCCGGTTCCATCGCCGAACGGACGCGCTTCAGCGCGTATCTCATCTACTCCG   3330
 F  Q  W  A  F  A  I  A  A  A  G  I  T  S  G  S  I  A  E  R  T  R  F  S  A  Y  L  I  Y  S    154
CCTTCCTCACCGGGTTCGTGTACCCGGTGGTGTCGCACTGGTTCTGGTCCACCGACGGGTGGGCTTCGGCCGGCCGGCTGACGGGTCCGT   3420
 A  F  L  T  G  F  V  Y  P  V  V  S  H  W  F  W  S  T  D  G  W  A  S  A  G  R  L  T  G  P    184
TGCTGTTCAAGTCGGGCGTCATCGACTTCGCCGGCTCCGGCGTCGTCCATCTGGTCGGTGGCATTGCTGGCCTGTGGGGTGCCTTCATCG   3510
 L  L  F  K  S  G  V  I  D  F  A  G  S  G  V  V  H  L  V  G  G  I  A  G  L  W  G  A  F  I    214
AGGGCCCCCGCATCGGGCGCTTCGACGCCGCCGGCCGCACGGTGGCGATGAAGGGCACAGCGCCTCACTGGTCGTGCTCGGCACCTTCC   3600
 E  G  P  R  I  G  R  F  D  A  A  G  R  T  V  A  M  K  G  H  S  A  S  L  V  V  L  G  T  F    244
TGCTGTGGTTCGGGTGGTTCGGCTTCAACCCGGGGTCCTTCACCACCATCTCCAAGATCTACGGCGAGTCGGGCACGATCGACGGGCAGT   3690
 L  L  W  F  G  W  F  G  F  N  P  G  S  F  T  T  I  S  K  I  Y  G  E  S  G  T  I  D  G  Q    274
GGTCGGCGGTGGGCCGCACCGCCGTGACGACGTCGCTGGCGGGCAGCGTCGCCGCGCTTAACCACGCTGTACGGCAAGAGATGGCTGACG   3780
 W  S  A  V  G  R  T  A  V  T  T  S  L  A  G  S  V  A  A  L  N  H  A  V  R  Q  E  M  A  D    304
GGGCACTGGAACGTGACCGACGTCTGCAACGGTCTCCTCGGCGGGTTCGCGCGATCACCGCGGGCTGCTCCGTGGTCGACCCGTGGGCGT   3870
 W  A  L  E  R  D  R  R  L  Q  R  S  P  R  R  V  R  A  I  T  A  G  C  S  V  V  D  P  W  A    334
CGGTGATCTGCGGGTTCGTGTCGGCGTGGGTCCTCATCGGCTGCAACAAGCTGGCGCTGATGCTCAAGTTCGATGACCCGCTGGAGGCGA   3960
 S  V  I  C  G  F  V  S  A  W  V  L  I  G  C  N  K  L  A  L  M  L  K  F  D  D  P  L  E  A    364
CGCAGCTGCACGGCGGGTGCGGCGCGTGGGGGATCATCTTCACCGCGCTGTTCGCGCGCAAGGAGTACGTCGAGCTGATCTACGGGGTGC   4050
 T  Q  L  H  G  G  C  G  A  W  G  I  I  F  T  A  L  F  A  R  K  E  Y  V  E  L  I  Y  G  V    394
CGGGGAGGCCGTACGGGCTGTTCATGGGCGGCGGCGGGAGGCTTCTCGCGGCGCACATCGTGCAGATCCTGGTGATCGTCGGGTGGGTCA   4140
 P  G  R  P  Y  G  L  F  M  G  G  G  G  R  L  L  A  A  H  I  V  Q  I  L  V  I  V  G  W  V    424
GCGCCACCATGGGGACGCTCTTCTACGTGCTGCACAGGTTCGGGCTGCTCCGCGTCTCGACCTCGACAGAGATGGAAGGCATGGACCCGT   4230
 S  A  T  M  G  T  L  F  Y  V  L  H  R  F  G  L  L  R  V  S  T  S  T  E  M  E  G  M  D  P    454
CGTGCCACGGCGGGTTCGGGTACGTGGACGAGGACGAAGGCCAGCGCCGCGTCAGGGCCAAGTCGGCGGCGGAGACGGCTCGCGTGGAGC   4320
 S  C  H  G  G  F  G  Y  V  D  E  D  E  G  Q  R  R  V  R  A  K  S  A  A  E  T  A  R  V  E    484
CCAGAAAGTCGCCGGAGCAAGCCGCGGCGGGCCAGTTGGTGTAGTAGGATCATATCGATCGTGTCCGTTCGGGGAAAGTGTTTTGTGAAG   4410
 P  R  K  S  P  E  Q  A  A  A  G  Q  L  V  *                                                   498
TGTGCATATATAAGCTGAGGCAGTCAGTCGTGTGGGCGTGGTGGCACTTCAGCCCATGGTGGTTGTGGCTTTCTTTTGATATTTGCTTCC   4500
TTTCTTCTCTGCATTTGCATCTGTATGGATTTTTGTGGCTTTCAATCTTTTATGCTTTTCTTTAGGTATTCAGTCTTTTATGCTTTCTTG   4590
TACATGTTTAGACGTGTCCAGTTTGTATCAGTATTTAGGTCATTATGATGTTAACGTGGAGCTC   4654
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(C) *OsAMT1;3*

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ATTATCTCCAATGATTTCATAGCTAATCCATATGCTGGAAGGGTTAGGAATTCAAGCCATTTCAAATTCCAAAAAATTACCTATACTAAA   90
GTAAAAAAAAACCTATGACCTACCCTCAATGTTTGTTAACCAATTTAGGCCTTGTTTGATTCCACTTAGAATTATTATAATCCTGATTAT   180
TATTAGGAGTAAGCTGAAACAAACAGATAACNTATTATGATAGATTATTATAATCTATAAGCCAGATTACTATAATCTGGTAATCCACTC   270
TAAAGGTGCTTTTTTTAATTATTGGATAGCTAATAACTAGCAAACAGCTAATAATCCAGATAAACAAACAGCTAACAACTTATTCTATAT   360
CGGCTTATTATAATCTTATTATAATCCAATTTATAGTAATCTAGCTCAATAATATATATTATAATAATCTTAAACTGAAACAAACAGGGC   450
TTTAGAAATTCATATGTTTTGAAATGGAGATAGTACCACTCAGAAAGCTTGAAGGATTTCATGTGTTTTGGTTAACATATTCATGTGTGT   540
CTTTTCGTGCAACCAAAATTTTCTTTAGAAACATGGTGAACCAATTAGATTTAGAAATTATAAAATATTTCCAAGTGTTACAAGTGGAAA   630
TATAATAAAAATAATATTGTTAAAAAAGTAAAGAAAGTTTAAGTACAAACTGAGGAGGAAATATAACAAGTGCTTCACTATAGACAAATA   720
TAGAGGTGGACGAAATGTACAAACAGTCGTTTTTAAAAATACAAACCACCGTATTGCGACTCAGGCCTTGTTTAGATCCCAAAAAATTTT   810
AGCCAAAAATATCACATCAAATGTTTGGACACATGCATAGGATATTAAATATGGGGAAAAAAAATCAATTACACAGTTTGCAGGTAAATT   900
GCGAGACAAATCTTTTTAGTCTAATTACGTCATGATTTGACCATGTGATGCTATAGTAAACATTTACTAATGACAGATTGATTAGGCTTA   990
ATAAATTCATCTCGCAATTTACAAACAGAATCTATAATTTATTTTATTATTAGTCTATATTTAATATTTTAAATATATATCCGTGTAGTT   1080
CAAAAACTTTATATCAAAAGAACTAAACACAGCCTCCAGGCCGCAGCCTACAGTAGGCCTATAGAGAGATTCCACGGGATTCGATGAACT   1170
ACGACCACGAACAGGAGGGGGACAAATCAACAAGCAAATCATAGGGGTCGCACATTTCAGAGGTAGCCAAAGATTCACTGGCAGGTGGGC   1260
CCTTCACACTTTGAAGGAATCAACAACGACACCCCCCAAGTCATGGATTCCTTCTCGCTCCCTCTCCACGTCGCCTATAAATCCGACGCG   1350
GGCCGCTCCCCACTCCACCCACAGCCCACACTTCCATTGCTCCTCCCCTCTCCTCTACAGTCTGTGTTGAGCGCGCGTCGAGCGGCGAGG   1440
ATGGCAACGTGCGCGGATACCCTCGGCCCGCTGCTGGGCACGGCGGCGGCGAACGCGACGGACTACCTGTGCAACCAGTTCGCGGACACC   1530
 M   A   T   C   A   D   T   L   G   P   L   L   G   T   A   A   A   N   A   T   D   Y   L   C   N   Q   F   A   D   T    30
ACGTCGGCCGTGGACTCGACGTACCTGCTCTTCTCGGCGTACCTCGTGTTCGCCATGCAGCTCGGCTTCGCCATGCTCTGCGCCGGGTCC   1620
 T   S   A   V   D   S   T   Y   L   L   F   S   A   Y   L   V   F   A   M   Q   L   G   F   A   M   L   C   A   G   S    60
GTCCGCGCCAAGAACACCATGAACATCATGCTTACCAACGTGCTCGACGCCGCCGCCGGCGCGCTCTTCTACTACCTCTTCGGCTTCGCC   1710
 V   R   A   K   N   T   M   N   I   M   L   T   N   V   L   D   A   A   A   G   A   L   F   Y   Y   L   F   G   F   A    90
TTCGCCTTCGGGGCGCCGTCCAACGGCTTCATCGGGAAGCACTTCTTCGGCCTCAAGCAGGTCCCACAGGTCGGCTTCGACTACAGCTTC   1800
 F   A   F   G   A   P   S   N   G   F   I   G   K   H   F   F   G   L   K   Q   V   P   Q   V   G   F   D   Y   S   F   120
TTCCTCTTCCAGTGGGCCTTCGCCATCGCCGCCGCGGGCATCACGTCCGGCTCCATCGCCGAGCGGACCCAGTTCGTGGCGTACCTCATC   1890
 F   L   F   Q   W   A   F   A   I   A   A   A   G   I   T   S   G   S   I   A   E   R   T   Q   F   V   A   Y   L   I   150
TACTCCGCCTTCCTCACCGGCTTCGTCTACCCGGTGGTGTCCCACTGGATCTGGTCCGCCGACGGGTGGGCCTCGGCTTCCCGAACGTCG   1980
 Y   S   A   F   L   T   G   F   V   Y   P   V   V   S   H   W   I   W   S   A   D   G   W   A   S   A   S   R   T   S   180
GGGTCGCTGCTCTTCGGGTCCGGCGTCATCGACTTCGCCGGGTCAGGGGTTGTCCACATGGTGGCGGCGTGCCGGACTCTGGGGCGCCCT   2070
 G   S   L   L   F   G   S   G   V   I   D   F   A   G   S   G   V   V   H   M   V   A   A   C   R   T   L   G   R   P   210
CATCGAGGGCCCCCGCATTGGCGGTTCGACCACGCCGGCCGCTCGGTGGCGCTGCGCGGCCACAGCGCGTCGCTCGTCGTGCTCGGCAGC   2160
 H   R   G   P   P   H   W   R   F   D   H   A   G   R   S   V   A   L   R   G   H   S   A   S   L   V   V   L   G   S   240
TTCCTGCTGTGGTTCGGGTGGTACGGGTTTAACCCCGGCTCGTTCCTCACCATCCTCAAATCCTACGGCCCGCCCGGTAGCATCCACGGG   2250
 F   L   L   W   F   G   W   Y   G   F   N   P   G   S   F   L   T   I   L   K   S   Y   G   P   P   G   S   I   H   G   270
CAGTGGTCGGCGGTGGGACGCACCGCCGTGACCACCACCCTCGCCGGCAGCACGGCGGCGCTCACGACGCTCTTCGGGAAGAGGCTCCAG   2340
 Q   W   S   A   V   G   R   T   A   V   T   T   T   L   A   G   S   T   A   A   L   T   T   L   F   G   K   R   L   Q   300
ACGGGGCACTGGAACGTGATCGACGTCTGCAACGGCCTCCTCGGCGGCTTCGCGGCGATCACCGCCGGTTGCTCCGTCGTCGACCCGTGG   2430
 T   G   H   W   N   V   I   D   V   C   N   G   L   L   G   G   F   A   A   I   T   A   G   C   S   V   V   D   P   W   330
GCCGCGATCATCTGCGGGTTCGTCTCGGCGTGGGTGCTCATCGGCCTCAACGCGCTGGCGGCGAGGCTCAAGTTCGACGACCCGCTCGAG   2520
 A   A   I   I   C   G   F   V   S   A   W   V   L   I   G   L   N   A   L   A   A   R   L   K   F   D   D   P   L   E   360
GCGGCGCAGCTGCACGGCGGGTGCGGCGCGTGGGGGGTCATCTTCACGGCGCTGTTCGCGCGCAAGGAGTACGTGGACCAGATCTTCGGC   2610
 A   A   Q   L   H   G   G   C   G   A   W   G   V   I   F   T   A   L   F   A   R   K   E   Y   V   D   Q   I   F   G   390
CAGCCCGGGCGCCCGTATGGGCTGTTCATGGGCGGCGGCGGCCGGCTGCTCGGGGCGCACATAGTGGTAATCCTGGTCATCGCGGCGTGG   2700
 Q   P   G   R   P   Y   G   L   F   M   G   G   G   G   R   L   L   G   A   H   I   V   V   I   L   V   I   A   A   W   420
GTGAGCTTCACCATGGCGCCGCTGTTCCTGGTGCTCAACAAGCTGGGATTGCTGCGCATCTCGGCCGAGGACGAGATGGCCGGCATGGAC   2790
 V   S   F   T   M   A   P   L   F   L   V   L   N   K   L   G   L   L   R   I   S   A   E   D   E   M   A   G   M   D   450
CAGACGCGCCACGGCGGGTTCGCGTACGCGTACCACGACGACGACGCGAGCGGCAAGCCGGACCGCAGCTTCGGCGGGTTCATGCTCAAG   2880
 Q   T   R   H   G   G   F   A   Y   A   Y   H   D   D   D   A   S   G   K   P   D   R   S   F   G   G   F   M   L   K   480
TCGGCGCACGGCACGCAGGTCGCCGCCGAGATGGGAGGCCATGTCTAGTGGAACCGGAGGAGCTGAGCTAGTAGTACATACATGCAGCAT   2970
 S   A   H   G   T   Q   V   A   A   E   M   G   G   H   V   *                                                            496
CATCGATCCTCGAGCTC   298
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Table 5.1 Percent identity of neucleotide and peptide among three members of *OsAMT1* family and peptide percent identity between *OsAMT1;1* cDNA and three genomic members of *OsAMT1*.

		OsAMT1;1	*OsAMT1;2*	*OsAMT1;3*
OsAMT1;1	nt		80%	85%
	pep		76%	81%
OsAMT1;2	nt			79%
	pep			77%
OsAMT1;1 (cDNA)	pep	95.65%	73.00%	80.77%

CHAPTER 6

GENERAL DISCUSSIONS

6.1 Diversity and Evolution of the *AMT*

The uptake and assimilation of inorganic nitrogen on to carbon skeletons have marked effects on plant productivity, biomass and crop yield (Lawlor *et al.*, 1989). There are various sources of NH_4^+ for plants, including absorbtion from soil, NO_3^- reduction, and as a by product of photorespiration, protein degradation or amino acid catabolism (Mack, 1995). The existence of multiple ammonium transporters in plants presumably allows efficient use of different NH_4^+ sources. Recent studies have thrown some light on the molecular identities of different ammonium transporters. There are at least two gene families encoding for NH_4^+ uptake proteins in *Arabidopsis*, including *AMT1* and *AMT2* (Sohlenkamp *et al.*, 2000). Three *AMT1* family members have been isolated in *Arabidopsis* and in tomato (Gazzarrini *et al.*, 1999; Lauter *et al.*, 1996; Ninnemann *et al.*, 1994; Von Wiren *et al.*, 2000). Yeast has three NH_4^+ transporters, encoded by the *Mep1*, *Mep2* and *Mep3* genes of the same family (Marini *et al.*, 1997; Marini *et al.*, 1994). During the course of this study genomic clones of three putative rice ammonium transporters were isolated, including the one identical to the published *OsAMT1;1* cDNA (Von Wiren *et al.*, 1997). The completion of *Arabidopsis* genome sequencing has revealed at least five *AtAMT1* family members and one *AtAMT2*. As rice genome sequencing progresses it is likely that other ammonium transporters will be identified. The first bacterial NH_4^+ transporter gene, *Amt* was isolated from *Corynebacterium glutamicum* (Siewe *et al.*, 1996). The AMT protein of *C. glutamicum* shows significant identity to the MEP1 and MEP2 of *S. cerevisiae* (Marini *et al.*, 1994) and *AtAMT1;1* (Ninnemann *et al.*,, 1994). It has now been recognized that ammonium transporters are encoded by multigene families in plants, reflecting the fact that different transporters are utilized under different physiological conditions. However, it is still not clear about the functional redundancy among different transporters. If there is significant redundancy then mutation or knockout at the DNA level in a single transporter gene may have little or no obvious phenotype.

6.2 Function of *AMT*

Initial data regarding the possible physiological roles of specific plant ammonium transporters came from gene expression studies. Most of the plant ammonium transporter genes so far described including *OsAMT1;1*, are expressed both in roots and shoots. Exceptions are *AtAMT1;2* and *AtAMTT1;3* which are expressed only in roots and *LeAMT1;3* which is expressed only in shoots (Gazzarrini *et al.*, 1999; Lauter *et al.*, 1996; Ninnemann *et al.*, 1994; Sohlenkamp *et al.*, 2000; Von Wiren *et al.*, 2000). In most cases transcript levels are higher in roots than in shoots, suggesting a possible role of the cognate proteins in acquisition of NH_4^+ from growth medium (Ninnemann et al., 1994; Von Wiren *et al.*, 2000). All three tomato NH_4^+ transporters and the *Arabidopsis AtAMT1;1-3* complemented a yeast mutant defective in NH_4^+ uptake confirming their role in ammonium uptake (Gazzarrini *et al.*, 1999; Lauter *et al.*, 1996; Ninnemann *et al.*, 1994; Von Wiren *et al.*, 2000). It has been shown in bean and rice that NH_4^+ is partially assimilated at root hairs (Cullimore, 1991; Ishiyama *et al.*, 1998). Two related NH_4^+ transporter genes *LeAMT1;1* and *LeAMt1;2* are preferentially expressed in root hairs. Promoter–reporter gene expression and protein immunolocalization studies showed high expression of GS1 and NADH-GOGAT in root hairs as well as in epidermal and exodermal root cells, respectively (Watson and Cullimore, 1996). Finally, transcriptional upregulation of *AtAMT1;1* and *LeAMT1;1* were closely correlated with enhanced $^{15}NH_4^+$ influx after plant transfer to nitrogen free nutrient solution (Gazzarrini *et al.*, 1999; Von Wiren *et al.*, 2000). It appears that plant N nutritional status and substrate availability differentially control transcription of some *AMT* genes. Thus transcript levels of *AtAMT1;1* and *LeAMT1;1* increased rapidly during nitrogen deprivation and decreased rapidly in response to high nitrogen supply (Gazzarrini *et al.*, 1999; Von Wiren *et al.*, 2000). On the other hand, transcript levels of *AtAMT1;2* & *AtAMT1;3* did not increase significantly following nitrogen starvation (Gazzarrini *et al.*, 1999). Similarly, *OsAMT1;1* showed constitutive expression under all nitrogen conditions (this thesis). Rather than decreasing or remaining the same, transcription of *LeAMT1;2* increased after NH_4^+ or NO_3^- supply (Von Wiren *et al.*, 2000). It seems likely that *AMT* genes which are up-regulated in response to N limitation encoded transporters that enable NH_4^+ uptake at low concentration. This hypothesis is supported by the high affinity for ammonium that some of these proteins show when expressed in yeast (Gazzarrini *et al.*, 1999). In contrast, NH_4^+-inducible *LeAMT1;2* expression suggests a function for the cognate protein in NH_4^+ uptake at higher external concentrations, that could serve in retrieval of NH_4^+, thus compensating constant NH_4^+ efflux from roots derived from amino acid catabolism (Feng, Volk, and Jackson, 1998). Constitutive expression of *OsAMT1;1* in roots as observed in this study, suggests that *OsAMT1;1* may be involved in ammonium uptake under N limiting conditions and/or recovery of ammonium lost from roots during metabolism. Many species that normally use NO_3^- also have an efficient system(s) for absorbing NH_4^+ which is constitutively expressed at high N levels (Forde and Clarkson, 1999). Where such species are presented with a mixed NO_3^-/NH_4^+ source, NH_4^+ is absorbed more rapidly, for example in perennial rye grass and barley (Clarkson *et al.*, 1986; Macduff and Jackson, 1991). Such a role requires a plasma membrane location for these transporters which has not yet been demonstrated.

The expression of *AMT*s in leaves suggests that they may be involved in photorespiratory ammonium transport (Gazzarrini *et al.*, 1999; Sohlenkamp *et al.*, 2000). Low atmospheric CO_2 concentrations stimulate photorespiration leading to release of NH_3 from glycine in mitochondria (Ogren, 1984). As the reassimilation of photorespiratory NH_3 is catalysed by glutamine synthetase localized in the chloroplast (Wallsgrove *et al.*, 1987), it must be transported across the membranes of both organelles. However, nothing is known about ammonium transport in these organelles. One recent study supported the idea of involvement of members of *AMTs* in uptake and/or retrieval during photorespiration (Von Wiren *et al.*, 2000). It has been shown that at elevated CO_2 levels, transcripts of *LeAMT1;2* and *LeAMT1;3* slightly decreased in leaves with simultaneous strong decrease in chloroplast glutamine synthetase (GS2) and photorespiratory serine hydroxymethyl transferase (Von Wiren *et al.*, 2000). The authors proposed that elevated CO_2 levels decrease photorespiratory NH_3 evolution, which in turn, repressed *Le*GS2 transcription as well as *LeAMT1;2* and *LeAMT1;3.* It was suggested that these transporters, particularly *LeAMT1;2* may be involved in the retrieval and import of photorespiratory NH_3 escaping from mitochondria. This is because accumulation of *LeAMT1;2* transcripts is higher during daylight when photorespiration peaks and when a considerable amount of NH_4^+ is translocated in the xylem (Husted and Schjoerring, 1995). Although the diurnal variation in *OsAMT1;1* expression or its expression patterns at different CO_2 levels is not known, its expression in leaves indicates that it may play a similar role as *LeAMT1;2.* Expression studies of *AMT*s under different conditions suggest that the transporters are involved in NH_4^+ uptake from soil and ammonium recycling in shoots. To fulfill such roles, ammonium transporters must be located in the plasmamembrane and possibly other membranes, such as the inner membrane of the chloroplast. Unfortunately, no information is yet available on the location of *AMT* proteins in plant cells. Another serious gap in our understanding about the function of *AMT*s is that there are no reports of any *AMT* mutants or transgenic lines with altered *AMT* expression. The data presented in this study is the first report of alteration of expression of an *AMT* in plants.

6.3 Transgenic Approach to Study the Function of *OsAMT1;1*

Expression of the *OsAMT1;1* gene in an antisense orientation (to inhibit gene expression) or in a sense orientation (to enhance gene expression) in transgenic plants was an obvious approach to understand its physiological function. Due to the progress of foreign gene delivery and expression, transgenic rice is useful model to investigate gene functions (McElroy and Brettell, 1994; Upadhyaya *et al.*, 2000). The sense and antisense *OsAMT1;1* constructs (pPIMP161 and pPIMP145) produced in this work were introduced into the two rice cultivars Taipei 309 and Jarrah by using a well established *Agrobacterium*-mediated transformation system (Upadhyaya *et al.*, 2000; Wang *et al.*, 1997). Transformation efficiencies were higher in Taipei 309 compared to Jarrah. Differences in transformation efficiencies among rice cultivars has been reported previously (Upadhyaya *et al.*, 2000). A small number of transgenic plants were regenerated from calli transformed with antisense *OsAMT1;1* construct (pPIMP145) for cultivar Taipei 309. No antisense transgenic plants were recovered for cultivar Jarrah. The number of sterile plants was higher in antisense transgenic lines compared to sense transgenic lines. Therefore, it appears that there could be some lethal effect of the antisense transgene in rice. Overall, results suggest *OsAMT1;1* may be essential for plant survival. This is curious results given the number of *AMT1* genes present in rice and other species like *Arabidopsis*. It is assumed that most, if not all *AMT1* genes in plants have overlapping physiological roles. The results presented suggest that not all *AMT* proteins are redundant or dispensable. It is also possible that during callus induction and regeneration, expression of antisense RNA decreased the endogenous target message and thereby prevented the synthesis of ammonium transporters. The small number of antisense transgenic lines are probably the ones having incomplete repression of endogenous ammonium transporters. This could have resulted from transgene position effect such as in or near repetitive DNA or heterochromatin (Prols and Meyer, 1992). If this were not to be the case, more lines with effective antisense co-suppression need to be generated, preferably using recently reported inverted repeat technology (Wang and Waterhouse, 2000).

The introduction of the *OsAMT1;1* cDNA in sense orientation into the rice cultivars Taipei 309 and Jarrah under the control of the maize *Ubi-1* promoter led to an increased accumulation of the mRNA. Transgenic plants with multiple copies of the transgene showed higher transcript levels. The positive correlation between *OsAMT1;1* mRNA levels and transgene copy number suggested an additive effect of transgene copies, as observed previously using a reporter gene (Hobbs *et al.*, 1993). Multiple T-DNA integrations are a common event with *Agrobacterium*-mediated transformation and there is conflicting evidence as to how such an increased copy number affects the expression of the introduced genes. The correlation between copy number and gene expression in transformants has been reported to be positive (Gendloff *et al.*, 1990), indeterminate (Shirsat *et al.*, 1989) or negative (Hobbs *et al.*, 1990). Differences in expression levels in lines with a single transgene copy may be due to transgene positional effects. For example a single transgene when inserted into the transcriptionally active region of euchromatin will produce higher expression due to the influence of the regulatory sequences of nearby host genes (Herman *et al.*, 1990; Kerbundit *et al.*, 1991; Koncz *et al.*, 1989). On the other hand, if the transgene inserts in or near repetitive DNA or heterochromatin, transgene expression can be inactivated (Prols and Meyer, 1992).

6.3.1 *OsAMT1;1* Transgenic Plants Showed Higher NH_4^+-Dependent Membrane Depolarisation

The membrane electric potential (E_m) of plant root cells is the driving force for ammonium uptake (Smith and Waker, 1978; Ullrich *et al.*, 1984; Wang *et al.*, 1994). Ammonium uptake by plant cells generally leads to depolarisation of the plasma membrane, the magnitude of which depends on the rate of NH_4^+ uptake. Rice seedlings (21-27 d old) acclimatised in nitrogen free solution for 1 or 2 d exhibited a rapid depolarisation of root cell membrane electrical potential upon addition of ammonium. Transgenic lines (e.g. line 46) that over-expressed *OsAMT1;1* showed mean depolarisation values nearly twice that of control plants. This presumably reflected the involvement of more transporters in the transgenic plants. Estimated half-saturation values for net depolarisation (K_m) were similar in N acclimatised wild type and transgenic plants despite the differences in maximum depolarisation values (V_{max}). This indicated that ammonium uptake in the wild type is achieved by *OsAMT1;1* or another *AMT1* family member with a similar affinity for ammonium.

6.3.2 Does High NH_4^+ Accumulation in *OsAMT1;1* Overexpressing Lines Lead to Ammonium Toxicity in Transgenic Plants ?

In this study, under adequate N nutrition, *OsAMT1;1* overexpressing plants had (i) higher NH_4^+ uptake rates; (ii) higher NH_4^+ concentration in the roots; and (iii) lower biomass in comparison to control plants. However, biomass and root ammonium contents remained the same in both transgenic and wild type plants when plants were grown for 4 weeks at a very low concentration of ammonium (10 μM). The higher NH_4^+ uptake rate, increase in root NH_4^+ pool and reduction in biomass, in over-expressing plants positively-correlated with transgene copy number and *OsAMT1;1* mRNA expression levels. Segregants (T_2) from multiple copy transgenic lines (Line 46 & line 38) were highly variable in terms of plant phenotype. Some of the dwarf type plants from line 46 did not survive. It is conceivable that the excess accumulation of free NH_4^+ in overexpressing plants was toxic. Presumably ammonium uptake by these plants exceeded their capacity to assimilate the ammonium. Interestingly, NH_4^+ efflux from root cells appeared to be insufficient to remove the excess ammonium. The biochemical bases for ammonium toxicity or tolerance in plant species is not yet fully understood. High ammonium can affect both morphology and physiology of plants. At the biochemical level NH_4^+ toxicity blocks ATP production and reduces CO_2 fixation in the chloroplast (Ikeda and Yamada, 1981; Puritch and Baker, 1967), and reduces starch synthesis (Marwaham and Juliano, 1976). High ammonium uptake may also prevent water movement from root to shoot, and as a result plants may wilt and die (Anderson *et al.*, 1991) as was the case for some T2 segregants from line 46. Plants may avoid the toxic effects of ammonium by storing excess ammonium in 'safe' intracellular locations and by rapid consumption of ammonium in the cytoplasm or plastids. Data obtained by different techniques show that NH_4^+ concentrations may range between 6-18 mM in ear components of wheat (Maheswari *et al.*, 1988), 3-8 and 10-20 mM, respectively in cytoplasm and vacuoles of maize root cells (Lee and Ratcliffe, 1991), up to 40 mM in the cytoplasm of rice and spruce roots (Kronzucker *et al.*, 1995; Wang *et al.*, 1993), up to 2 mM in xylem of barley, wheat and maize plants (Cramer and Lewis, 1993; Mattsson and Schjoerring, 1996) and up to about 2 mM in the apoplast of *B. napus* leaves (Husted and Schjoerring, 1995). The NH_4^+ concentration in plant tissues can thus in many cases be quite substantial. The mechanism by which plants avoid the accumulation of exceedingly high NH_4^+ concentrations is still not yet fully understood.

The enzymes involved in the assimilation of NH_4^+ into the nitrogen transporting amino acids (glutamine, glutamate, aspartate, and asparagine) are cytosolic and chloroplastic glutamine synthetase (GS1 and GS2), NADH, ferredoxin dependent glutamate synthase (NADH-GOGAT and Fd-GOGAT), aspartate aminotransferase (AspAT), and asparagine synthetase (AS) (Lam *et al.*, 1995). Effects of variations in external N supply on expression of GS isoforms have been investigated in several studies with contradictory results. Kozaki *et al.*, (1992) concluded that NH_4^+ activated the GS2 promoter of rice. GS2 polypeptide and mRNA did not change in *Phaseolus vulgaris* following NH_4^+ addition (Cock *et al.*, 1990). Induction of GS by NO_3^- has been proposed (De la Haba *et al.*, 1992). GS isoforms from barley leaves and roots were fully active not only in NH_4^+ and NO_3^- grown seedlings but also in N free grown plants (Mack, 1995), thus indicating that external nitrogen was not necessary for the induction of the barley GS isoforms. The conflicting reports concerning effects of nitrogen supply on GS activity may be due to the fact that carbon and nitrogen metabolism are regulated by dynamic changes in C/N ratios and by changes in metabolic status (Lam *et al.*, 1995). Decreases in C or

N resources upregulate genes involved in their acquisition while abundance of these resources induces genes associated with use and storage (Lam *et al.*, 1995). Nitrogen assimilation in *Arabidopsis* is regulated by changes in metabolic status. Light and sugars, both of which increase C/N balance, up-regulate the expression of genes involved in ammonia assimilation into glutamine and glutamate by chloroplastic GS and Fd-GOGAT, while they repress asparagine synthetase (AS) expression (Lam *et al.*, 1996). Developmental events also appear to be important for regulation of the different GS isoforms. In barley while GS1a was already active in germinating seed, GS1b and GS2 became active several days later in the young seedlings (Mack, 1995).

There are differences between different plant genera in the inherent capacity to assimilate exogenously supplied ammonium. It has been shown in the past that older mustard seedlings (*Sinapis alba* L.) can assimilate excessive supplied nitrogen (5-30 mM NH_4^+) by avoiding excessive levels of internal ammonium. In contrast, seedlings of Scots pine (*Pinus sylvestris* L.) accumulated NH_4^+ in cotyledons and roots and showed no stimulation of GS activity after the application of NH_4^+. In addition, root growth was drastically reduced in Scot pine (Vollbrecht *et al.*, 1989). Ammonium is often the preferred source for conifers like *Pinus sylvestris*, but is normally only present in low concentrations in soil (Adams and Attiwill, 1982; Cole, 1981). Conifers that are well adapted to such conditions may have intrinsically low levels of ammonium assimilation and insufficient capacity to assimilate high concentrations of exogenously supplied ammonium. This could explain the deleterious effects of high ammonium on young seedlings. The two diverse cultivars used in this study also showed inherent differences in ammonium uptake and assimilation, as manifest by their differential response to N nutrition.

The results obtained from this study indicate that the manipulation of the source, ammonium transport, in rice plants is not sufficient to increase ammonium uptake and utilisation. Simultaneous manipulation of N assimilatory pathways including manipulation of the metabolism in the sink tissues themselves, such as shoots and seeds may facilitate improved NH_4^+ utilization and growth in plants. It has been shown that tobacco plants overexpressing pea cytosolic GS1 had considerable growth advantage over wild type plants. In contrast, plants co-suppressed for both chloroplastic and cytosolic GS had decreased GS activity (T. Brears and G.Coruzzi, unpublished results, cited by Lam *et al.*, 1995) and caused plants to grow poorly. Therefore, this indicates that GS could be a rate limiting enzyme in plant growth and nitrogen use. Transgenic tobacco plants overexpressing the asparagine synthetase (AS) gene showed increased levels of free asparagine in leaves (Brears *et al.*, 1993). By increasing the sink for ammonium, via increases in GS and/or other N assimilating enzymes, it may be possible to avoid the deleterious effects of *AMT1* overexpression in rice. In that case, it may well be possible to enhance N use efficiency and growth in rice in the future.

6.4 Future Work

From the above discussions it is clear that expression of different isoforms of rice GS under different nitrogen conditions need to be studied in *OsAMT1;1* overexpressing lines to understand more about ammonium transporters and ammonium assimilation pathways. It will be very interesting to study the effect of overexpression *OsAMT1;1* on GS and other downstream metabolic enzymes involved in ammonium assimilation and storage. This may be possibely by creating lines that overexpress both *AMT* and GS. It will also be interesting to know the effect of *OsAMT1;1* overexpression on the carbon skeleton and the C/N ratio of the plants grown under different nitrogen conditions. Another important question that needs to be addressed is; "Does an increase in accumulation of NH_4^+ in transgenic plants inhibit CO_2 fixation due to inhibition of photosynthesis?" (Platt and Anthon, 1981). There are some reports (Ikeda and Yamada, 1981; Walker *et al*, 1984; Johansson and Larsson, 1986) showing no correlation between high accumulation of NH_4^+ and inhibition of photosynthesis. To understand the environmental and developmental regulation of ammonium transport activity and the tissue specific expression of this gene, a promoter–*gus* fusion analysis also needs to be carried out. Detailed analysis of lines with *OsAMt1;1* promoter-*gus* transgenes will unravel environmental, spatial and developmental regulation of these genes.

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