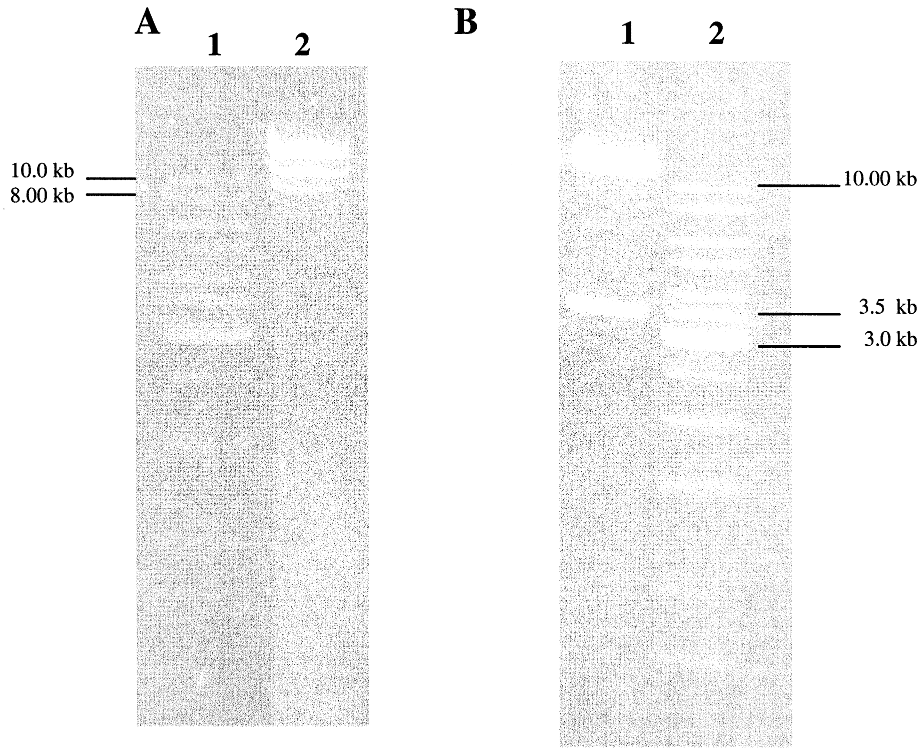


Figure 5.3 (A) Genomic λclone E2 digested with *Sac*I (lane 2) and Marker (lane 1); (B) clone pPIMP 770 digested with *Sac*I liberating the 12 kb insert (lane 1).

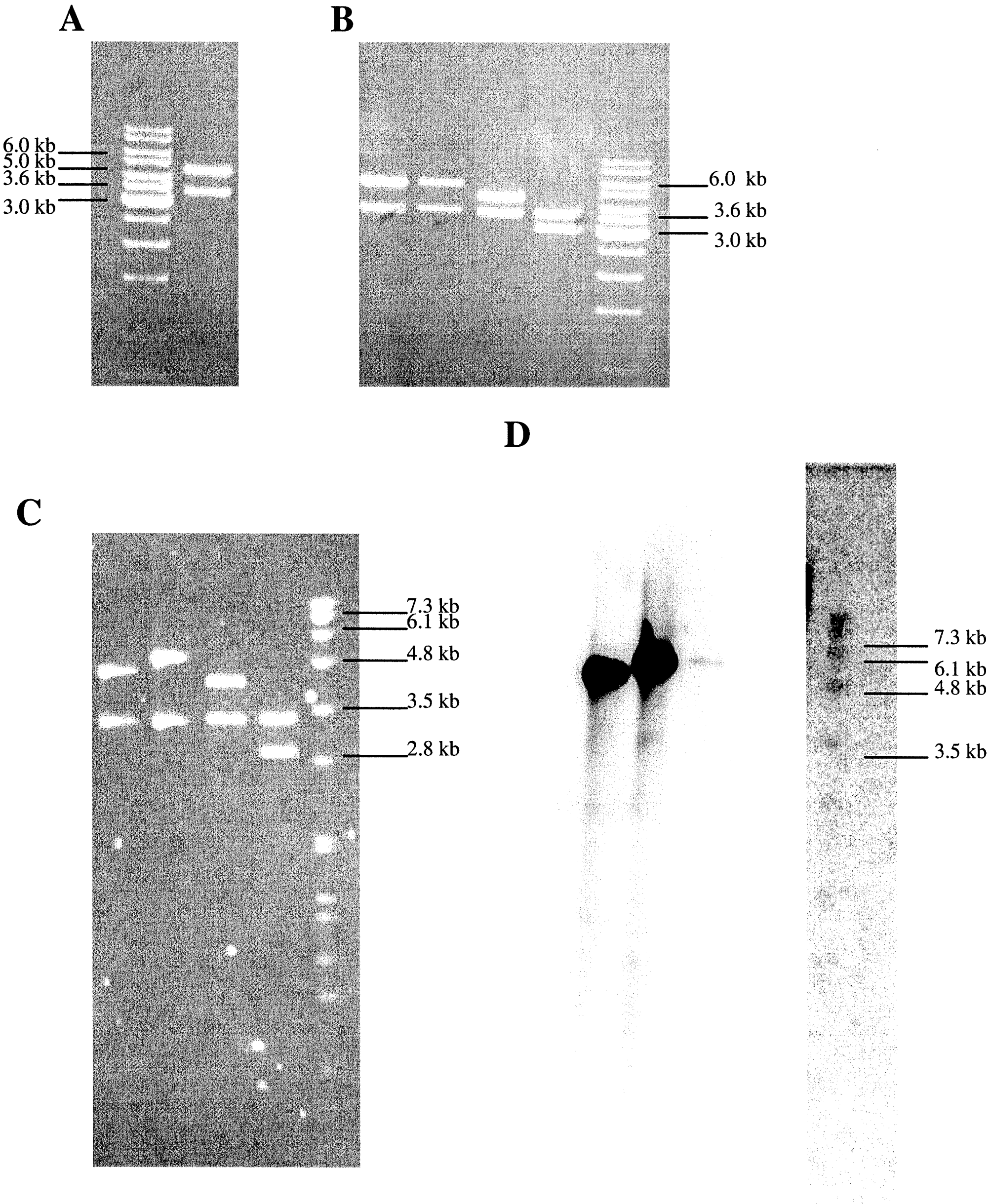


Figure 5.4 (A) pPIMP 827 digested with *Sac*I showing 4.8 kb insert (lane 2), and marker (lane 1); (B) pPIMP 833 (lane 1), pPIMP 834 (lane 2), pPIMP 936 (lane 3) and pPIMP 937 (lane 4) digested with *Sac*I showing different sized inserts and marker (lane 5); (C) pPIMP 827 (lane 1) pPIMP 834 (lane 2), 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).

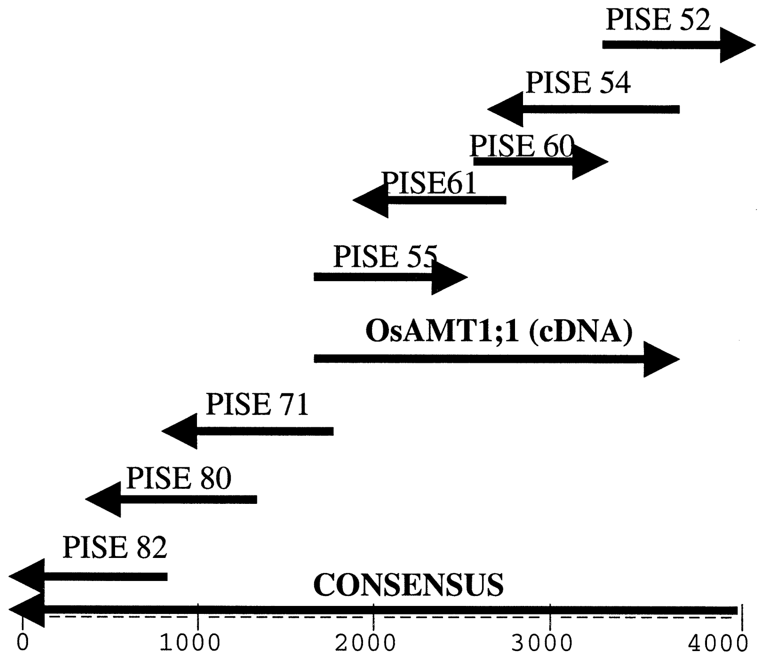


Figure 5.5. Sequencing strategy for *OsAMT1;1* (λclone E_2) from clone pPIMP770. Sequence PISE 52 with internal primer OsAMT1; 1 -1698$^+$, PISE 54 with internal primer OsAMT1; 1 -2058$^-$, PISE 60 with internal primer OsAMT1;1-864$^+$, PISE 61 with internal primer OsAMT1;1-1143$^-$, PISE 55 with internal 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$^+$.

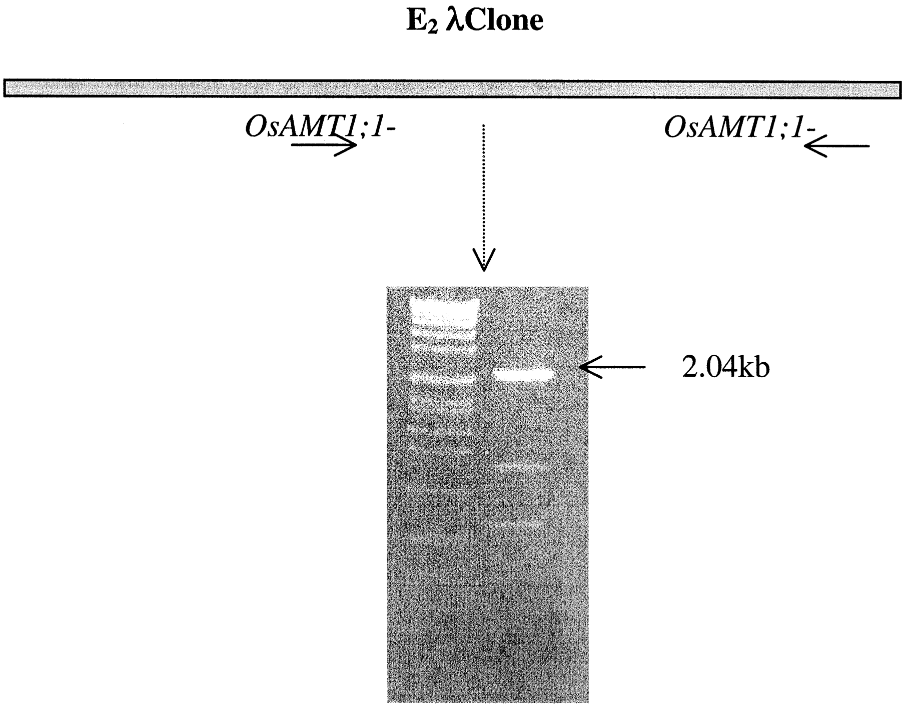


Figure 5.6 PCR amplification of an *AMT1* gene product from clone E2. A PCR product of 2.04 kb was amplified from λ E2, using OsAMT1;1-44$^+$ forward primer and OsAMT1;1-2058$^-$ reverse primer.

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 gcacgccgtccaacggcttcatcgggaagcagttcttcggcctcaagcacatgccg--ca 459
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||   |
OsAMT1;1   378 gcacgccgtccaacggcttcatcgggaagcagttcttcggcctcaagcacatgccggcaa 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     aagccaaagtttgatagagaggtagagcactgcaggacaaaaggacccacgcaaagtct 161
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsAMT1;1 2046    aagccaaagtttgatagagaggtagagcactgcaggacaaaaggacccacgcaaagtct1987

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
```

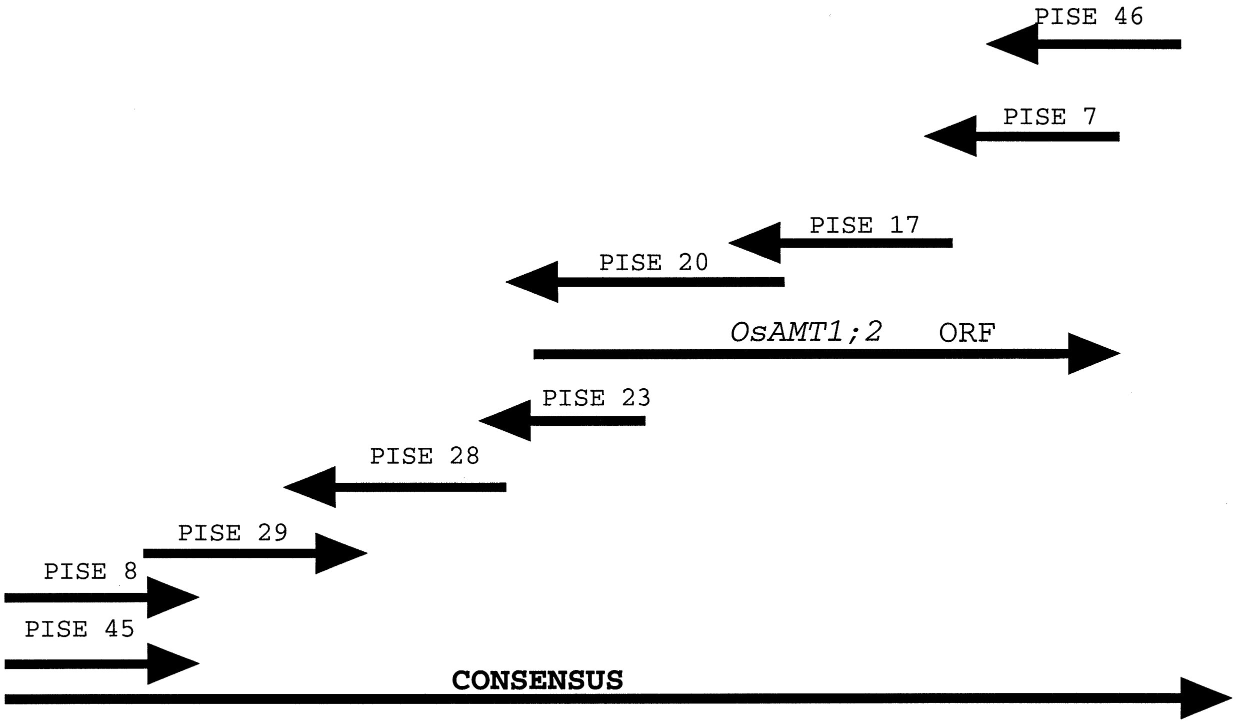


Figure 5.8 Sequencing strategy for *OsAMT1;2* (λclone A3 & J) from the subclones pPIMP321 and pPIMP827. Sequence PISE 46 with universal reverse primer, PISE7 with M13 universal forward primer, PISE17 with internal primer SE7-696$^+$, PISE 20 with internal primer SE17-658$^+$, PISE23 with internal primer 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    ****_*_*** _***_*____ *_******** ********** *__*******
```

```
            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 tctGgggttg gtgggttcat
{OsAMT1_3}  GcgtACcAcG ACGAcGaCgc GagCggCaag ccgGaccgca gcttcggcgg
{OsAMT1_2}  GtggACgAgG ACGAaGgCca GcgCcgCgtc aggGccaagt cggcggcgga
Consensus   *___**_*_* ****_*_*__ *__*__*___ ___*______ __________
```

```
             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    ---------- ---------- ---------- ---------- ----------
```

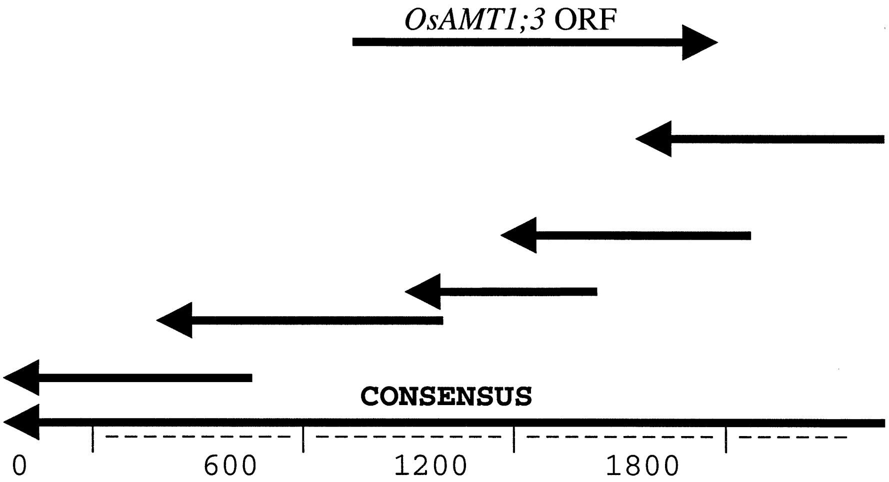
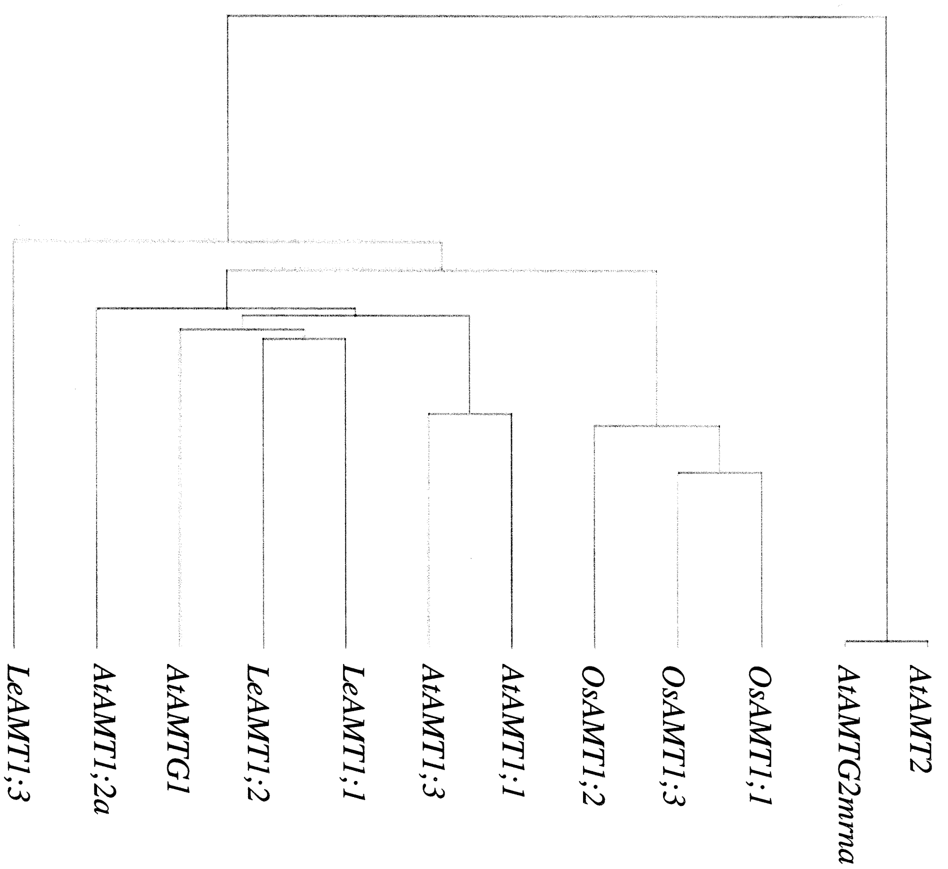


Figure 5.10 Sequencing strategy for *OsAMT1;3.* (λclone K) from the sub clone pPIMP834. Sequence PISE47 with M13 universal reverse primer, PISE 57 with clone K internal primer SE47- 538$^{+,}$ PISE 62 with internal primer SE57-565^{+}, PISE 69 with internal primer SE62- 624^{+} and PISE 75 with internal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

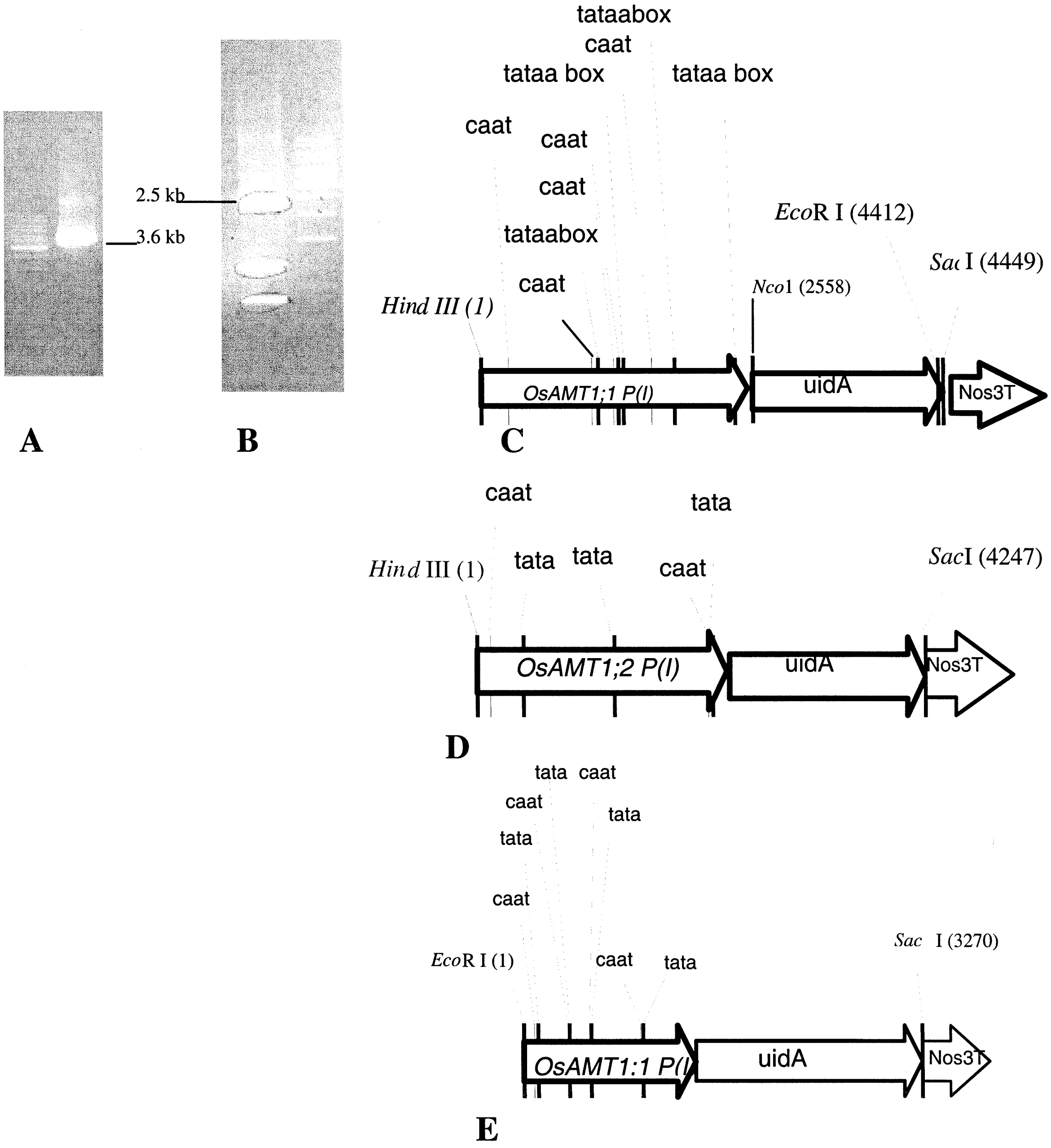


Figure 5.12 Construction of promoter-*gus* fusions. (A) A 3.6 kb PCR product amplified with the forward primer SE74-668$^+$ and the reverse primer *OsAMT1;1*-90$^-$; (B) PCR product was digested with *Hind*III and *Nco*I to produce three fragments including a 2.5 kb *Hind*III/*Nco*I fragment; (C) construct 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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 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
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 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
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 S  R  H  K  F  D  P  H  I  *                                                                  533
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(B). *OsAMT1;2*

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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
```

(C) *OsAMT1;3*

ATTATCTCCAATGATTTCATAGCTAATCCATATGCTGGAAGGGTTAGGAATTCAAGCCATTTCAAATTCCAAAAAATTACCTATACTAAA 90

GTAAAAAAAAACCTATGACCTACCCTCAATGTTTGTTAACCAATTTAGGCCTTGTTTGATTCCACTTAGAATTATTATAATCCTGATTAT 180

TATTAGGAGTAAGCTGAAACAAACAGATAACNTATTATGATAGATTATTATAATCTATAAGCCAGATTACTATAATCTGGTAATCCACTC 270

TAAAGGTGCTTTTTTTAATTATTGGATAGCTAATAACTAGCAAACAGCTAATAATCCAGATAAACAAACAGCTAACAACTTATTCTATAT 360

CGGCTTATTATAATCTTATTATAATCCAATTTATAGTAATCTAGCTCAATAATATATATTATAATAATCTTAAACTGAAACAAACAGGGC 450

TTTAGAAATTCATATGTTTTGAAATGGAGATAGTACCACTCAGAAAGCTTGAAGGATTTCATGTGTTTTGGTTAACATATTCATGTGTGT 540

CTTTTCGTGCAACCAAAATTTTCTTTAGAAACATGGTGAACCAATTAGATTTAGAAATTATAAAATATTTCCAAGTGTTACAAGTGGAAA 630

TATAATAAAAATAATATTGTTAAAAAAGTAAAGAAAGTTTAAGTACAAACTGAGGAGGAAATATAACAAGTGCTTCACTATAGACAAATA 720

TAGAGGTGGACGAAATGTACAAACAGTCGTTTTTAAAAATACAAACCACCGTATTGCGACTCAGGCCTTGTTTAGATCCCAAAAAATTTT 810

AGCCAAAAATATCACATCAAATGTTTGGACACATGCATAGGATATTAAATATGGGAAAAAAAATCAATTACACAGTTTGCAGGTAAATT 900

GCGAGACAAATCTTTTTAGTCTAATTACGTCATGATTTGACCATGTGATGCTATAGTAAACATTTACTAATGACAGATTGATTAGGCTTA 990

ATAAATTCATCTCGCAATTTACAAACAGAATCTATAATTTATTTTATTATTAGTCTATATTTAATATTTTAAATATATATCCGTGTAGTT 1080

CAAAAACTTTATATCAAAAGAACTAAACACAGCCTCCAGGCCGCAGCCTACAGTAGGCCTATAGAGAGATTCCACGGGATTCGATGAACT 1170

ACGACCACGAACAGGAGGGGGACAAATCAACAAG**CAAAT**CATAGGGGTCGCACATTTCAGAGGTAGCCAAAGATTCACTGGCAGGTGGGC 1260

CCTTCACACTTTGAAGGAATCAACAACGACACCCCCCAAGTCATGGATTCCTTCTCGCTCCCTCTCCACGTCGCC**TATAAAT**CCGACGCG 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

TCGGCGCACGGCACGCAGGTCGCCGCCGAGATGGGAGGCCATGTC***TAG***TGGAACCGGAGGAGCTGAGCTAGTAGTACATACATGCAGCAT 2970
S A H G T Q V A A E M G G H V * 496

CATCGATCCTCGAGCTC 298

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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