

Oligomerization of Ammonium Transporter LeAMT1;1 and Its Interactions with Other Proteins

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Summary

Ammonium is one of the important nitrogen sources for plant growth and development. Ammonium uptake and retrieval mediated by high-affinity ammonium transporters at the plasma membrane is the initial step in ammonium assimilation and nitrogen metabolism. Molecular and genetic studies have revealed a large protein family of ammonium transporters functionally diverse but evolutionarily conserved in all domains of organisms. Despite the available information regarding the physiological aspects, biochemical characteristics and gene regulations of ammonium transporters, the function of the transporter proteins is still poorly understood, and the sensory/regulatory mechanism coupling ammonium transport in plants remains elusive.

To test the hypothesis that plant ammonium transporters form multimeric complexes at the plasma membrane, the well-characterized plant ammonium transporters LeAMT1;1 and LeAMT1;2 from tomato were used as a model system. Protein-protein interactions between these transporters were monitored in two *in vivo* systems: the split ubiquitin system and the *Xenopus* oocyte expression system. Results firmly demonstrate that plant ammonium transporters, at least in the case of the LeAMT1;1 and LeAMT1;2, exist in homo-oligomeric forms.

A dominant-negative point mutation at the conserved cytoplasmic C-termini of the LeAMT proteins, corresponding to the reported inhibitory mep1-1 mutation in the yeast ammonium transporter Mep1, was characterized. It is non-functional in both yeast and oocytes and specifically reduces the activity of co-expressed wild-type LeAMT proteins in oocytes in a dominant negative manner. These results demonstrate that LeAMT1;1 and LeAMT1;2 form not only homo- but also hetero-oligomeric structures. GFP-fusion of LeAMT1;1 indicates that this mutation does not impair the subcellular localization of the transporter. Taken together, these results suggest that oligomerization of the transporter proteins is necessary for maintaining an efficient transport of the substrate across the plasma membrane. Stable transgenic lines of tomato were generated to over-express the wild type and the mutant genes under the 35S promoter. These transgenic lines facilitate further analysis of the physiological effects of the dominant-negative mutant of LeAMT1;1.

By screening yeast two-hybrid libraries with the C-terminal cytoplasmic fragment of LeAMT1;1 as a bait, putative interacting proteins were identified. One cDNA was isolated three-times independently and encodes a polypeptide that maps to the C2 domain of a plant phosphatidylinositol-specific phospholipase C (PI-PLC). Plant PI-PLC has been found participating in a number of cellular signal transduction pathways in response to environmental stimulus. The possible roles underlying the putative interaction between LeAMT1;1 and the tomato PI-PLC cDNA, in terms of the ammonium sensory and signaling network in plants, is discussed. The identification of a plant PI-PLC as a putative interaction partner of LeAMT1;1 will initiate further investigations in this area.

Zusammenfassung

Ammonium ist eine der wichtigen Stickstoffquellen und spielt eine zentrale Rolle für das Wachstum und Entwicklung der Pflanzen. Die Aufnahme von Ammonium durch hoch-affine Transporter in der zellulären Plasmamembran stellt den ersten Schritt der Stickstoffassimilation sowie des Stickstoffmetabolismus dar. Molekulare und genetische Studien zeigen eine Superfamilie von Ammoniumtransportern und Ammoniumtransporter-ähnlichen Proteinen aus allen bekannten phylogenetischen Domänen des Lebens. Trotz der vielen Informationen über die physiologischen und biochemischen Charakteristika, sowie die Expressionsregulation der Transporter, bleibt der genaue Funktionsmechanismus der pflanzlichen Transporterproteine weitestgehend unerforscht. Ebenfalls unbekannt ist wie Ammoniumtransporter in Pflanzen durch das Stickstoffangebot reguliert werden.

Ob pflanzliche Ammoniumtransporter in der Plasmamembran als multimere Komplexe vorliegen können, war die erste Fragestellung die es zu untersuchen galt. Hierzu wurden die bisher am weitesten charakterisierten LeAMT1;1 und LeAMT1;2, aus der Tomate stammend, als Modellsystem gewählt. Die Protein-Protein Interaktionen zwischen diesen Transportern wurden mit Hilfe von zwei in vivo Systemen untersucht: dem Split Ubiquitin System und durch Expressionsstudien in *Xenopus* Oocyten. Die Ergebnisse zeigen, dass zumindest im Falle von LeAMT1;1 und LeAMT1;2, pflanzliche Ammoniumtransporter Homooligomere bilden können.

Als nächstes wurde der Einfluss einer Punktmutation im konservierten C-terminalen Bereich von LeAMT Proteinen auf den Transport untersucht. Es ist bekannt, dass eine entsprechende Punktmutation "mep1-1" des Hefetransporters Mep1 einen dominant-negativen Effekt auf den Ammoniumtransport ausübt. Die Punktmutationen von LeAMT1;1 und LeAMT1;2 führten zur Inhibition ihrer Transporteraktivität, wie durch Hefekomplementation und Expression in *Xenopus* Oocyten gezeigt werden konnte. Die Co-Expression von Punktmutanten mit den Wildtyp LeAMTs in *Xenopus* Oocyten führte zu einer spezifischen Reduktion des Ammoniumtransports. Diese Ergebnisse zeigen, dass LeAMT1;1 und LeAMT1;2 nicht nur homo- sondern auch heteromere Komplexe bilden können. Versuche mit GFP Fusionen sprechen dafür, dass die Punktmutation die subzelluläre Lokalisierung der LeAMTs nicht beeinträchtigt. Zusammenfassend legen diese Ergebnisse die Vermutung nahe, dass die Oligomerisierung von LeAMT Transportern für einen effizienten Transport des Ammoniums durch die Plasmamembran wichtig sein könnte. Stabile, transgene Tomatenlinien, welche den Wildtyp und die Punktmutante von LeAMT1;1 unter Kontrolle des konstitutiven 35S Promotors exprimieren, wurden hergestellt. Diese Linien ermöglichen die physiologischen Effekte der dominant-negativen Punktmutation von LeAMT1;1 bezüglich der Pflanze zu charakterisieren.

Durch "Screening" von "Yeast-two-hybrid" cDNA Bibliotheken mit dem cytoplasmatischen C-terminus von LeAMT1;1 wurden mögliche interagierende Partner von LeAMT1;1 gesucht und identifiziert. Mehrere unabhängig isolierte cDNA Klone kodierten für die C2 Domäne der Phosphatidylinositol-spezifischen Phospholipase C (PI-PLC) aus Pflanzen. Pflanzliche PI-PLCs sind an der Signalübertragung verschiedener extra- und intrazellulärer Stimuli beteiligt. Mögliche Bedeutung der Interaktion von LeAMT1;1 und PI-PLC bezüglich der Funktion von LeAMT1;1 als Stickstoffsensoren, sowie die damit verbundenen Signalübertragungswege werden diskutiert. Die Identifizierung von PI-PLC als möglichen Interaktionspartner von LeAMT1;1 eröffnet die Möglichkeit zu weiterführenden Studien auf diesem Gebiet.

1. Introduction

1.1. A conserved family of ammonium transporters from all domains of life

Ammonium represents an important source of nitrogen for bacteria, fungi and plants. In plants, both NH_4^+ and NO_3^- are the predominant sources of nitrogen taken up by the root system. Although many plants adaptive for aerobic soils do not grow well with NH_4^+ as a sole source of nitrogen, most plants preferentially take up NH_4^+ when both forms are present – even when NO_3^- exceeds NH_4^+ by as much as 10-fold (Gessler *et al.* 1998). In addition, optimal plant growth is usually observed when nitrogen is supplied in both forms (Bloom *et al.* 1993), despite the differences among plant species in the extent of their utilization of the two nitrogen sources. Uptake and assimilation of NH_4^+ consumes less energy than NO_3^- , but an excessive supply of NH_4^+ is harmful to plants and the accumulation of high NH_4^+ concentration in the cytoplasm is toxic. Therefore, intracellular NH_4^+ concentration must be tightly controlled. Apart from root acquisition of NH_4^+ , it is also important for plants to retrieve $\text{NH}_3/\text{NH}_4^+$ from shoot xylem or leaf apoplast, where long-distance translocated or photorespiratory-released $\text{NH}_3/\text{NH}_4^+$ is accumulated (von Wiren *et al.* 2000).

Earlier experiments on the physiological and biochemical characterization of root influx of NH_4^+ have distinguished two components of the uptake kinetics: NH_4^+ uptake at <1 mM external concentration displays typical Michaelis-Menten kinetics, while, at higher concentration, the uptake has non-saturable linear kinetics (Ullrich *et al.* 1984; Wang *et al.* 1993 and Kronzucker *et al.* 1996). The first component at low concentration of NH_4^+ is a high affinity transport system (low K_m , ranging from 20-360 μM , von Wiren *et al.* 2001) and is always linked to low capacity (low V_{max}), whereas the second component is characterized as a low affinity high capacity transport system.

Although the low affinity high capacity transport system is believed to be mediated by channels or transport proteins that do not discriminate NH_4^+ from other cations, the underlying biophysical mechanism has not been fully dissected, nor have any genes yet been cloned that encode these transport systems. On the contrary, physiological evidences regarding the induction and regulation of the high affinity low capacity transport system in a number of plant species are well documented using whole plant models (Glass *et al.* 2002).

Genetic studies in the budding yeast *Saccharomyces cerevisiae* led to the identification and cloning of three ammonium transporter genes: MEP1, MEP2 and MEP3 (Marini *et al.* 1994; Marini *et al.* 1997). The three encoded proteins comprise a small family of ammonium transporters in yeast and each has different affinity for NH_4^+ , ordered from highest to the lowest, Mep2p ($K_m = 1\text{-}2 \mu\text{M}$), Mep1p ($K_m = 5\text{-}10 \mu\text{M}$) and Mep3p ($K_m = 1.4\text{-}2.1 \text{mM}$) (Marini *et al.* 1997).

The use of yeast double mutant 26972c (*mep1-1 mep2-1*) and later triple mutant 31019b (*mep1Δ mep2Δ mep3Δ*) has greatly assisted the cloning of plant ammonium transporter genes. The yeast strain 31019b is a null mutant of all three endogenous Mep genes, while in 26972c Mep2 is deleted in the genome and Mep1 carries a point mutation at its C-terminus resulting in loss of its activity as well as in a specific inhibition of the activity of Mep3, which is present in the genome as a wild type gene. Both mutant strains lack ammonium transport activity and thus cannot grow on medium with ammonium concentration lower than 5 mM as a sole nitrogen source. Hetero-expression of a cDNA coding for a functional ammonium transporter in these strains will allow the complementation of the growth defect, facilitating the identification and isolation of functional ammonium transporters from plants and other organisms. AtAMT1;1 is the first plant ammonium transporter isolated by heterologous complementation of the yeast double mutant strain 26972c (*mep1-1 mep2-1*) with cDNAs from an *Arabidopsis thaliana* library (Ninnemann *et al.* 1994). Since then, five members of the AMT1 family, namely AtAMT1;1, AtAMT1;2, AtAMT1;3, AtAMT1;4 and AtAMT1;5 have been identified in *Arabidopsis thaliana*. Sequences of these proteins show 70% - 90% similarity at the amino acid level (Gazzarrini *et al.* 1999; von Wiren *et al.* 2002). An additional gene AtAMT2;1 was also cloned from *Arabidopsis thaliana*, which encodes a high affinity ammonium transporter more closely related to the Mep proteins than to the plant AMT1 transporters (Sohlenkamp *et al.* 2000).

In tomato (*Lycopersicon esculentum*), three homologs, LeAMT1;1, LeAMT1;2 and LeAMT1;3 were isolated and functionally expressed in yeast (von Wiren *et al.* 2000). LeAMT1;1 and LeAMT1;2 share 76% amino acid sequence similarity and are preferentially expressed in root hairs, while LeAMT1;3 is more distantly related (62.8% identity) and mainly expressed in leaves.

Subsequently, a number of ammonium transporters from eubacteria, archaeobacteria and fungi have been identified and isolated (Siewe *et al.* 1996; Soupene *et al.* 1998; Javelle *et al.* 2003). Heterologously expressed human Rhesus-associated RhAG protein and a kidney homologue RhGK were shown to function as ammonium transporters (Marini *et al.* 2000; Westhoff *et al.* 2002). In fact, phylogenetic analysis indicates a definite sequence relationship (20-27% identity) between the Mep/Amt transporters and the Rh family proteins, especially RhAG proteins (Matassi *et al.* 1998; Marini *et al.* 2000). Since RhAG homologues are also found in slime mold, sponge, nematode and fruit fly, these proteins probably derived from Mep/Amt proteins and originated later in evolution from a RhAG-like ancestor (Matassi *et al.* 1999; Kitano and Saitou, 2000).

A non-redundant homology search for Amt/Mep/Rh proteins in the GeneBank (RefSeq) has yielded 258 entries and the number of genes identified is increasing, especially thanks to the genome sequencing projects on several species. Thus the Amt/Mep/Rh protein family is functionally diverse but evolutionarily conserved in all domains of life (Fig. 1.1).

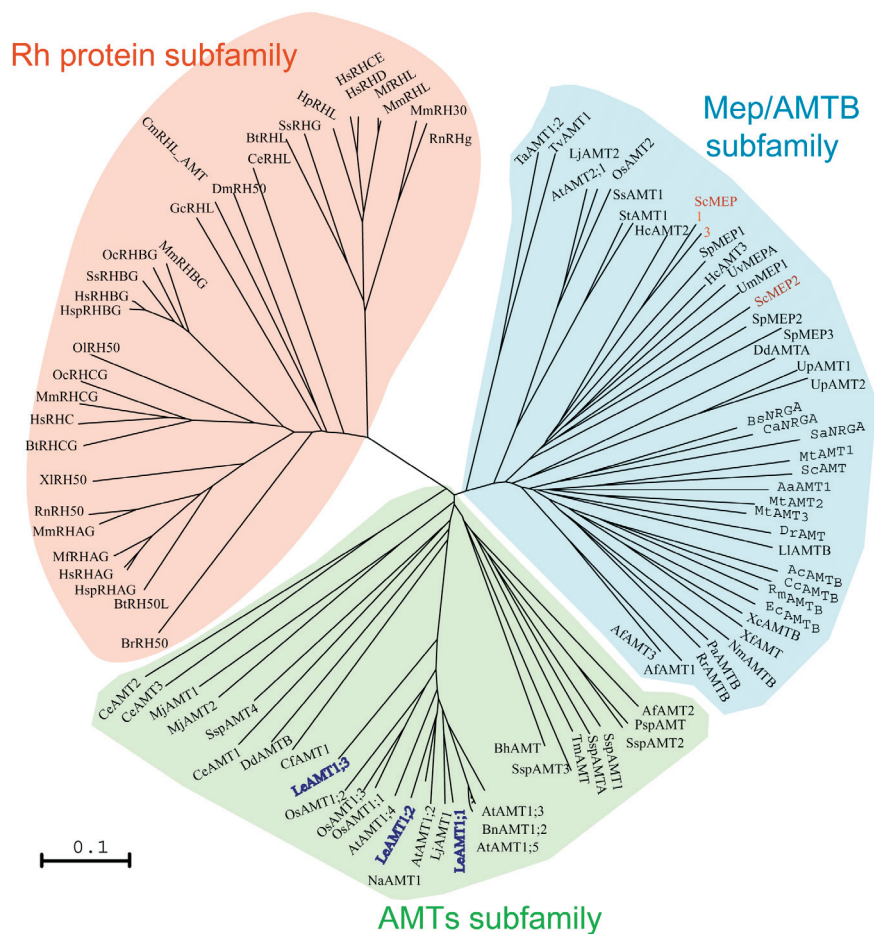


Fig. 1.1 Phylogenetic tree of selected members of the Amt/Mep/Rh family, the family of ammonium transporters and related proteins. The selected protein sequences were obtained from Swissprot Database and abbreviated protein names were taken from the annotations associated with each sequence. The tree is based on Maximum Parsimony Analysis from sequences of aligned proteins. It was drawn using the DRAWTREE program from the PHYLIP package (Baum, 1989)

1.2. Characterization of the ammonium transport kinetics and mechanism

Despite the large number of ammonium transporters being identified and isolated, the mechanism regarding ammonium movement across the plasma membrane mediated by these transporters remains less clear (Ludewig *et al.* 2002; Westhoff *et al.* 2002).

Because the charged NH_4^+ and uncharged NH_3 are in equilibrium with a pKa of 9.25 in aqueous solutions, NH_4^+ is the predominant form (c.a. 99%) presented at typical cytosolic pH. Although in some cases bidirectional NH_3 diffusion was observed to be facilitated by Mep/AmtB (Soupene *et al.* 2002), the real substrate of ammonium transporters is the

charged form. This is supported by most studies using radiotracer direct-uptake experiments with the yeast knock-out mutant defect in ammonium uptake (Marini *et al.* 1994; Ninnemann *et al.* 1994; Lauter *et al.* 1996; Gazzarrini *et al.* 1999; Meier-Wagner *et al.* 2001). Kinetic properties of individual transporter expressed separately in the yeast mutant have also been examined in these studies. Collected data show that different substrate affinities and regulation exist among various ammonium transporters from any particular species tested.

Electrophysiological studies based on the *Xenopus* oocyte heterologous expression system demonstrated a uniport mechanism for the tomato high-affinity ammonium transporter LeAMT1;1. The NH_4^+ -induced inward currents in oocytes injected with LeAMT1;1 cRNA were concentration-dependent and membrane potential-driven but external pH-independent (over a range of pH 5.5 – pH 8.5). The NH_4^+ -induced currents are highly specific since no inhibition by Na^+ or K^+ is observed (Ludewig *et al.* 2002; Westhoff *et al.* 2002).

1.3. Structural considerations of ammonium transporters

Most of the members in the ammonium transporter family consist of 400-500 amino acids in length though in some cases the C-terminal region is considerably extended, increasing their length to well over 510 residues. Based on computer-aided hydrophathy analysis, most of the plant and fungal ammonium transporters have predicted 11 transmembrane helices with the C-terminus always being intracellular and the N-terminus extracellular. This membrane topology was experimentally confirmed for the yeast Mep2 protein (Marini *et al.* 2000). On the other hand, some members of the family, especially the Rhesus proteins and the bacterial AmtB proteins, contain an additional transmembrane helix at the N-terminus resulting in both C- and N-termini being intracellular (Avent *et al.* 1996; Thomas *et al.* 2000).

Tertiary and quaternary structures of a transporter protein can provide extensive information on the mechanism of the transport and on its functions. It is now commonly believed that proteins in general most often do not function as monomeric entities but rather interact with each other forming stable or transient complexes. For membrane transporters such as the Amt/Mep proteins, a solved molecular structure may give hints to fundamental questions such as how the substrate specificity can be achieved and how the activity of substrate translocation across the lipid barrier is fulfilled. Over the last few years the three-dimensional structure of a number of membrane transporters was resolved, including several differently-gated tetrameric potassium channels (review in MacKinnon 2003), the dimeric prokaryotic chloride channels (Dutzler *et al.* 2002), the dimeric lactose permease from *E. coli* (Abramson *et al.* 2003), the dimeric bacterial multidrug transporter EmrE (Ubarretxena-Belandia *et al.* 2003) and the symmetry glycerol-3-phosphate transporter from *E. coli* (Huang *et al.* 2003).

The lactose permease of *E. coli* (LacY) is a member of the oligosaccharide/proton symport subfamily of the major facilitator superfamily (MFS) transporters. MFS transporters transduce free energy stored in electrochemical proton gradients into substrate concentration gradients and LacY is a well-studied representative of membrane transporter of this kind. LacY transports lactose against its concentration gradient by coupling the free energy released from downhill translocation of protons in response to an electrochemical proton gradient (Kaback *et al.* 2001). Structural information from a combination of site-directed mutagenesis, cross-linking and x-ray crystallography studies reveal that each of the two identical monomers of LacY acts as a functional unit of the transporter. The monomer contains 12 transmembrane helices, divided into N-terminal and C-terminal domains of six transmembrane helices each. Between the N- and C-terminal domains, a centrally-located interior hydrophilic cavity containing one substrate-binding site with similar distance from both sides of the membrane is formed and packed by the two-fold symmetrically distributed alpha-helices. Residues in the N-domain determine the substrate binding specificity, whereas those in the C-domain contribute to the affinity of the binding. The interactions between residues in both domains are critical for the proton translocation coupling with the substrate binding. Conformational switches between outward- and inward-facing states are thought to be the mechanism of proton-coupled lactose symport (Abramson *et al.* 2003).

Although x-ray crystallography is a powerful tool for deciphering structure and function of protein complexes, the difficulties in obtaining a large quantity of homogeneously purified membrane proteins for well-ordered crystal growth, especially eukaryotic membrane proteins, remain a challenge (Loll, 2003). Nevertheless, a variety of biochemical, molecular and genetic approaches can also be chosen to monitor the membrane protein complexes of interest.

As for the ammonium transporters, accumulating evidences do point out that they might actually form a multimeric protein complex at the plasma membrane. Rh antigen polypeptides have already been described to form hetero-tetramers consisting of two subunits of Rh and of RhAG in the erythrocyte membrane (Eyers *et al.* 1994). A molecular mass estimation of native AmtB proteins from *Escherichia coli*, by density ultracentrifugation and a polypeptide mass determination in denaturing SDS-PAGE, suggested that bacterial AmtB forms trimers (Blakey *et al.* 2002). In yeast, which encodes in its genome three high-affinity ammonium transporters (Mep1, Mep2 and Mep3), genetic evidence for the interaction between different Mep proteins has been coincidentally observed (Marini *et al.* 2000). Indications of such an interaction came from a detailed analysis of the yeast mutant 26972c (mep1-1 mep2-1) initially used to isolate ammonium transporters (Dubois and Grenson, 1979). The mutant carries a deletion in the Mep2 gene, whereas the Mep3 genomic sequence is present and unchanged compared with wild type. A single point mutation (G413D) in the Mep1 gene inactivated both Mep1 and Mep3 simultaneously (Marini *et*

al. 2000), which supports the idea of direct interaction of Mep proteins. However, because the inhibitory effect of the Mep1-G413D mutant was studied in *S. cerevisiae*, endogenous interacting factors and indirect effects could not be ruled out. Highly similar results were obtained for ammonium transporters from *Aspergillus*, where the corresponding mutation in the endogenous ammonium transporter inhibited wild type ammonium transporters (Monahan *et al.* 2002).

1.4. Regulatory aspects of ammonium transport activity

The multiplicity of ammonium transporters, with different but complementary substrate affinities in any one organism, obviously implicates the existence of a complex and sophisticated regulatory network, which regulates the transport process in accordance to the external and internal nitrogen status and coordinates ammonium assimilation with the cellular availability of carbon and energy.

In plants, Amt genes are differentially expressed among various tissues and organs and the transcript levels are subjected to down-regulation or induction upon addition of external nitrogen sources (Gazzarrini *et al.* 1999; von Wiren *et al.* 2000). For example, in roots of tomato plants, transcript levels of LeAMT1;2 increases after NH_4^+ or NO_3^- supply, while LeAMT1;1 is induced by nitrogen deficiency and its expression increases with a decline of cellular glutamine and NH_4^+ concentrations. In addition, expression of LeAMT1;1 and LeAMT1;2 is higher in root hairs than in the remaining root fraction. Growth of tomato plants at elevated CO_2 slightly decreases expression of LeAMT1;2 and LeAMT1;3 in leaves, but strongly represses transcript levels of chloroplast glutamine synthetase and photorespiratory serine hydroxymethyl-transferase, two important enzymes for ammonium metabolism in the chloroplast (von Wiren *et al.* 2000).

Upon import into the cytoplasm, ammonium has to be incorporated into amino acids or redistributed in other subcellular compartments rapidly since high concentration of NH_4^+ in the cytosol is toxic. The major assimilation pathway for ammonium in both bacteria and plants is the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, which is highly regulated at both transcriptional and posttranslational levels. In enteric bacteria, GS represents a well-studied nitrogen metabolism enzyme, biochemically and genetically. Both, the enzyme activity as well as the expression of the corresponding gene GlnA is essentially controlled by the activity of a small trimeric signal transmitter protein called PII (encoded by GlnB) in response to nitrogen limitation. This regulation is in fact part of a so-called “global nitrogen regulatory system” (Ntr) (Kamberov *et al.* 1994). Ntr is an intricate network involving Urase/UR, GlnB and the proteins of the two-component histidine protein kinase system, NtrB and NtrC, which in enteric are cotranscribed in the *glnA-ntrBC* operon. Urase/UR, uridylyltransferase/uridylyl-removing enzyme, catalyzes the uridylylation state of the PII protein GlnB, which in turn controls a number of nitrogen metabolism enzymes including GS. The later discovery of GlnK, a GlnB homolog and a new member of the PII

family in *E. coli* and in many other bacteria, increased the complexity of the system (van Heeswijk *et al.* 1995; Arcondeguy *et al.* 2001).

In *E. coli*, the PII proteins encoded by GlnB and GlnK undergo covalent modification via uridylylation of residue Tyr51. This uridylylation takes place in response to nitrogen deprivation, and the process is reversed in nitrogen sufficiency (Atkinson and Ninfa, 1999). The ubiquitous linkage of genes encoding AmtB and the PII protein GlnK in the same operon has led to the proposal that these proteins are functionally related (Thomas *et al.* 2000). Recent results suggest that both in *E. coli* and *Azotobacter vinelandii*, GlnK binds to the membrane in an AmtB-dependent manner and that GlnK acts as a negative regulator of the transport activity of AmtB. The interaction between GlnK and the C-terminal cytoplasmic domains of the AmtB depends on the uridylylation state of GlnK and is modulated in accordance to the cellular nitrogen status such that it is maximal in nitrogen-sufficient situations (Cou tts *et al.* 2002). A further experiment from the same laboratory using mutated forms of AmtB expressed in the *E. coli* strain GT1000 (Δ glnK Δ amtB) reveals that the GlnK-AmtB association requires ammonium transport activity, suggesting a role of AmtB in ammonium sensing (Javelle *et al.* 2003).

Although a single-copy nuclear gene encoding PII homolog is found in the genome of plants including Arabidopsis, castor bean (*Ricinus communis*), rice (*Oryza sativa*), alfalfa (*Medicago sativa*) and tomato (*Lycopersicon esculentum*), the plant PII-like protein is localized in chloroplast. Thus the function of the PII homologs in plant may be limited to chloroplast and there is no cytoplasmic GlnK-like protein in plants (Hsieh *et al.* 1998; Moorhead and Smith, 2003). In addition, no PII homolog has been identified in the completely sequenced yeast genome. Therefore, in eukaryotes the functional analog of GlnK protein remains to be sought.

In mammalian, insect, and yeast cells, the rapamycin-sensitive Tor signaling pathway is thought to be essential for mediating cell growth in response to nutrient availability (Crespo *et al.* 2002; Rohde and Cardenas, 2003) and nitrogen is a particularly important nutrient in Tor signaling (Beck and Hall, 1999; Cooper, 2002). However, a direct link between the ammonium transporters and the Tor kinase proteins has not been revealed.

1.5. Ammonium sensing

In addition to the feedback control by downstream components, substrate sensing is crucial for cells to regulate the expression and the activity of membrane transporters in accordance to the changing environment and the availability of nutrient resources. In order to be able to sense the substrate and generate the signal, sensor proteins must possess substrate-binding domains and signal transmit domains. In addition, sensor proteins often form oligomeric functional complexes and interact with downstream signal transduction proteins.

Sensor proteins can be categorized into receptor-like kinase sensors or transporter-like sensors.

Receptor-like kinase sensors

The well-studied receptor-like kinase sensors are members of the bacterial two-component systems, which are also present in plants. A two-component system consists of a receptor protein and a response regulator protein. The former functions as a homodimer in which each monomer can phosphorylate the other, whereas the latter can be activated upon receiving a phosphate group at its conserved aspartate from the histidine residue of the receptor kinase. The receptor contains an extracellular domain that binds the substrate, a transmembrane domain of variable-length and a cytoplasmic C-terminal extension. When activated by substrate binding, a kinase activity located in the C-terminus autophosphorylates each subunit at a specific histidine residue, which in turn pass the phosphate group to an aspartate residue of the response regulator. In its phosphorylated form, the regulator controls a wide range of cellular processes. Removal of the phosphate group inactivates the regulator (Parkinson, 1993).

Other examples of transmembrane receptor-like kinase sensors include histidine-kinase receptors involving in ethylene sensing (Bleecker and Kende, 2000) and in cytokinin sensing (Estelle, 2001), and serine-threonine kinases such as the brassinosteroid-receptor BRI1 (Wang *et al.* 2001).

Transporter-like sensors

Transporter-like sensor proteins also exist in many organisms from bacteria and fungi to plants and animals. The best characterized system is the yeast Ssy1p-Ptr3p-Ssy5p (SPS) amino acid sensor complex in which Ssy1p, a unique member of the amino acid permease (AAP) family, physically interacts with two membrane-associated cytoplasmic proteins Ptr3p and Ssy5p (Forsberg and Ljungdahl, 2001). The Ssy1p shows 22-28% identity at amino acid level with other members of the AAP family, but contains a largely extended N-terminus (140aa) distinct to other members. This unique N-terminal extension is oriented towards the cytoplasm and is essential for sensor function (Klasson *et al.* 1999). The SPS system is capable of sensing external amino acids and transmitting signal to the downstream components, leading to the transcriptional control of a number of amino acid transporters and peptide transporters (Forsberg *et al.* 2001). Two members of the yeast glucose transporter family, Snf3p and Rgt2p, are shown to function as glucose sensors that induce expression of other hexose transporter genes depending on extracellular glucose concentration (Ozcan *et al.* 1996). While Rgt2p functions as a low-affinity glucose sensor, Snf3p is shown to be a high-affinity sensor mediating glucose uptake at low external glucose concentrations (Ozcan *et al.* 1998).

In plants, LeSUT2 and AtSUT2 belong to the sucrose transporter family but have distinct

structural features similar to the yeast glucose sensor Snf3p and Rgt2p. They are suggested to function as sucrose sensors in plants (Barker *et al.* 2000; Schulze *et al.* 2000).

The ammonium sensory mechanism, however, is still not fully understood.

In *E. coli*, as suggested by Javelle *et al.* (2004), AmtB act as an ammonium sensor under normal physiological conditions. But direct evidence is still missing.

The high affinity low capacity ammonium transporter Mep2p, one of the three members of the yeast ammonium transporter family, has been suggested to have sensor function that generates signals for the induction of pseudohyphal growth of diploid cells on limited ammonium source (Lorenz and Heitman, 1998). The other two Mep proteins are unable to induce the pseudohyphal growth, however, insertion of the first intracellular loop of Mep2p into Mep1p enables the chimera Mep1 protein to partially restore the pseudohyphal growth of a mep2 null mutant. This implies the importance of this loop in signal transduction (Lorenz and Heitman, 1998). Although it remains to be elucidated how the Mep2p senses the extracellular substrates and what are the downstream signal components of the transduction pathways, Gpa2p has been suggested to be a candidate downstream of Mep2p, functioning in the regulation of filament formations under nitrogen-limited conditions (Kübler *et al.* 1997). Gpa2p is one of the two α -subunits (Gpa2p and Gpa1p) of the GTP-binding protein found in yeast and has been shown to induce pseudohyphal differentiation upon Mep2 activation, via mediation of cAMP levels (Kübler *et al.* 1997). Interestingly, Gpr1p, a GTP binding protein-coupled receptor, is also required for yeast filament growth under nitrogen depletion (Ansari *et al.* 1999; Tamaki *et al.* 2000; Lorenz *et al.* 2000). Moreover, both Gpr1p and Gpa2p are known to function in a cAMP-mediated glucose sensing system which is independent of Snf3p and Rgt2p (Rolland *et al.* 2000). Therefore, Gpr1p seems to be a possible checkpoint of cellular C/N coordination in yeast.

The Gpr1p is a typical seven-transmembrane receptor consisting of 961 amino acids with an extracellular-oriented N-terminus and a long (290 amino acids) cytoplasmic C-terminus and an even longer (381 aa) cytoplasmic central loop between the V and the VI transmembrane helices. The Gpa2p contains a conserved GTP-binding site and a hydrolysis site but is a cytoplasmic protein that interacts with the cytoplasmic C-terminus (residues 839-961) of the Gpr1p (Xue *et al.* 1998). Another protein Plc1p, the phosphatidylinositol-specific phospholipase C (PI-PLC) is also found to interact with the Gpr1p at its cytoplasmic C-terminal region (Ansari *et al.* 1998). The PI-PLC, represented by a single gene in the yeast genome, belongs to the δ subgroup of the large PLC family, which only exist in eukaryotes. PI-PLCs are known key players in phosphoinositide/calcium signaling pathways (review in Rebecchi and Pentylala, 2000). The involvement of Gpr1p, Gpa2p and PI-PLC in the control of yeast filament growth under nitrogen limitation might suggest a new pathway of ammonium sensing and signaling, but clearly it needs further dissections.

In plants, no ammonium sensor has been proposed so far. With Mep2p as an example of ammonium sensor, however, it is possible that some Amt protein may also play a role in sensing of the substrate.

1.6. Purpose of the present study

The main aim of this study was to determine the oligomeric interactions of the well-characterized plant ammonium transporters LeAMT1;1 and LeAMT1;2 from *Lycopersicon esculentum* *M in vivo*, and to search for putative cytoplasmic proteins interacting with LeAMT1;1.

To test the hypothesis that ammonium transporters form a multimeric complex at the plasma membrane, interactions between these transporters were monitored in a split ubiquitin system.

Heterologous expression in *Xenopus* oocyte allows electrophysiological characterization of the ammonium transport activity of individual transporters. Thus co-expression of LeAMTs might reveal the biophysical information underlying their interactions. Generation of point mutants of the LeAMT proteins at their conserved cytoplasmic C-termini, which correspond to the inhibitory mutant mep1-1 in yeast, and co-expression of the mutant and wild type LeAMT proteins in oocytes should help the elucidation at the molecular level of the genetic interaction observed in yeast.

Since mutated plasma-membrane proteins often fail in their subcellular targeting, GFP-fusions of wild type and mutant LeAMT1;1 were constructed and the subcellular localization of these GFP-fusion was examined via transient expression in plant protoplasts and stable expression in yeast triple null mutant. To facilitate the analysis of the physiological effects of the point mutation of the LeAMT1;1 protein in plants, stable transgenic lines of tomato were generated to over-express the wild type and the mutant genes under the 35S promoter.

Finally, the absence of cytoplasmic PII-like proteins in plants raises the question whether any other proteins might interact with the plant ammonium transporter and thus participate in ammonium sensing and signaling in plants. In order to search for such proteins, a yeast two-hybrid system was employed. Using the last 45 amino acid sequence at the cytoplasmic C-terminus of LeAMT1;1 as a bait, a cDNA library from Arabidopsis and from tomato was screened.

2. Materials and methods

2.1. Basic molecular and cellular biology protocols

All of the procedures for DNA and RNA handling, DNA cloning and subcloning, DNA amplification and sequencing; bacteria culture, maintaining and transformation were based on standard protocols as in Ausubel *et al.* (2002) and Sambrook *et al.* (2001). If modifications to the protocols were necessary, details were given in the following sections.

The following *E. coli* strains were used for cloning and maintaining of plasmid vectors:

DH5 α , genotype: F⁻ *gyrA96* (*Nal*^r) *recA1* *recA1* *endA1* *thi-1* *hsdR17* (*r_k⁻m_k⁺*) *glnV44* *deoR* Δ (*lacZYA-argF*)U169 [ϕ 80d Δ (*lacZ*)*M15*];

XL1-Blue, genotype: F[']::Tn10 (*Tet*^r) *proA*⁺*B*⁺ *lacI^q* Δ (*lacZ*)*M15* / *recA1* *endA1* *gyrA96*(*Nal*^r) *thi-1* *hsdR17* (*r_k⁻m_k⁺*) *glnV44* *relA1* *lac*

Transformation of *Saccharomyces cerevisiae* was performed according to protocols of Gietz and Woods (2002) (<http://www.umanitoba.ca/faculties/medicine/biochem/gietz/method.html>).

2.1.1. Cloning of LeAMT1;1, LeAMT1;2 and LeAMT1;3

The primary clones of LeAMT1;1/pBS, LeAMT1;2/pFL61 and LeAMT1;3/pFL61 were described in Lauter *et al.* (1996) and von Wiren *et al.* (2000), respectively. These cDNAs were used as templates for subcloning in this study.

Table 2.1 Conditions for generating LeAMT1;1G458D-pOO2 and LeAMT1;2G465D-pOO2

Mutant	Amino acid changed	Primers	PCR cycles used
LeAMT1;1G458D-pOO2	Glycine ⁴⁵⁸ to aspartate	FW ¹ : 5'GACGAAATGGCGG _a TATGGATCTGACC3' RV: 5'GGTCAGATCCATA _t CCGCCATTTTCGTC3'	1 cycle of 1 minute at 95°C 10 cycles of 30 seconds at 95°C, 1 minute at 55°C, 8 minutes at 68°C 1 cycle of 15 minute at 68°C
LeAMT1;2G465D-pOO2	Glycine ⁴⁶⁵ to aspartate	FW: 5'GACGATGAAACAGCAG _{at} ATGGATTTAACAAGAC3' RV: 5'GTCTTGTTAAATCCAT _{at} CTGCTGTTTCATCGTC3'	

Note: The mutated sequences incorporated in the primers are in lower case letters and underlined

2.1.2. Site-specific mutagenesis of LeAMT1;1 and LeAMT1;2

LeAMT1;1 and LeAMT1;2 cDNAs were PCR amplified and ligated subsequently into

¹ The abbreviations used are: FW, forward primer; RV, reverse primer

the oocyte expression vector pOO2 (Ludewig *et al.* 2002). To generate the mutants LeAMT1;1G458D-pOO2 and LeAMT1;2G465D-pOO2, PCR site-specific mutagenesis was conducted based on the instructions of Quickchange© kit from Stratagene using the condition shown in Table 2.1. The mutant cDNA clones were verified by sequencing.

2.1.3 Functional complementation of ammonium transport in yeast mutant 31019b

Functional complementation of the yeast mutant 31019b defective in ammonium transport activity was carried out based on Marini *et al.* (1997). After transformation with appropriate plasmids, single colonies were streaked out on fresh plates of nitrogen-free SD (1.7% YNB plus 2% glucose) medium supplemented with 1 mM $(\text{NH}_4)_2\text{SO}_4$ and grown for 3 days at 28°C. In some cases, 0.1% glutamine or arginine was used in place of $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source to unnesessitate the presence of an ammonium transporter.

2.1.4. Fluorescence microscopy bio-imaging

Images were obtained using a four-channel Leica TCS laser scanning confocal microscope system.

2.2. Split ubiquitin system

2.2.1. Plasmid constructs used in the split ubiquitin system

Table 2.2 List of plasmids used in the split ubiquitin system

Plasmid	Function	Promoter for yeast expression	Yeast Marker and replicon	Reference
pY-CubPLV	Vector for bait fusion with CubPLV	WBP1	LEU2, chromosome integration	Stagljar <i>et al.</i> 1998
pX-NubG	Vector for prey fusion with the NubG	ADH1	TRP1, 2 μ	Stagljar <i>et al.</i> 1998
pNubG-AtKAT1	For expression of AtKAT1 gene fused to the C-terminus of NubG	ADH1	TRP1, 2 μ	Reinders <i>et al.</i> 2001
LeSUT2-CubPLV	For expression of LeSUT2 gene fused to the CubPLV	WBP1	LEU2, chromosome integration	Reinders <i>et al.</i> 2001
pNubG-LeSUT2	For expression of LeSUT2 gene fused to the C-terminus of NubG	ADH1	TRP1, 2 μ	Reinders <i>et al.</i> 2001
pNubG-LeSUT4	For expression of LeSUT4 gene fused to the C-terminus of NubG	ADH1	TRP1, 2 μ	Reinders <i>et al.</i> 2001
metYCgate	Vector for bait fusion with CubPLV	MET25	LEU2, CEN/ARS	Obrdlik <i>et al.</i> to be publish
XNgate	Vector for prey fusion with the NubG	ADH1	TRP1, 2 μ	Obrdlik <i>et al.</i> to be publish
pX-NubWT	Positive control vector for expressing the WT Nub alone or fused with prey	ADH1	TRP1, 2 μ	This study

pX-NubA	For expressing the NubA alone or fused with prey	ADH1	TRP1, 2 μ	This study
LeAMT1;1-pCubPLV	LeAMT1;1 fused with CubPLV	WBP1	LEU2, chromosome integration	This study
LeAMT1;2-pCubPLV	LeAMT1;2 fused with CubPLV	WBP1	LEU2, chromosome integration	This study
LeAMT1;3-pCubPLV	LeAMT1;3 fused with CubPLV	WBP1	LEU2, chromosome integration	This study
LeAMT1;1-merYCgate	LeAMT1;1 fused with CubPLV	MET25	LEU2, CEN/ARS	This study
LeAMT1;1G458D-merYCgate	LeAMT1;1G458D fused with CubPLV	MET25	LEU2, CEN/ARS	This study
LeAMT1;1-pNubG	LeAMT1;1 fused with NubG	ADH1	TRP1, 2 μ	This study
LeAMT1;2-pNubG	LeAMT1;2 fused with NubG	ADH1	TRP1, 2 μ	This study
LeAMT1;3-pNubG	LeAMT1;3 fused with NubG	ADH1	TRP1, 2 μ	This study
LeAMT1;1G458D-pNubG	LeAMT1;1G458D fused with NubG	ADH1	TRP1, 2 μ	This study
LeAMT1;1 Δ C45-pNubG	LeAMT1;1 Δ C45 fused with NubG	ADH1	TRP1, 2 μ	This study
LeAMT1;1-pNubWT	Expression of LeAMT1;1 fused at the C-terminus with the NubWT	ADH1	TRP1, 2 μ	This study

pX-NubWT and **pX-NubA** were constructed using the protocol of Quickchange© kit (see section 2.1.2.) based on the plasmid pX-NubG with the following primers:

pX-NubWT

FW, 5'CTTTGACCGGTAAAACCAACATTGGAAGTTGAATC3'

RV, 5'GATTCAACTTCCAATGTTatGGTTTTACCGGTCAAAG3'

pX-NubA

FW, 5'CTTTGACCGGTAAAACCGcAACATTGGAAGTTGAATC3'

RV, 5'GATTCAACTTCCAATGTTgCGGTTTTACCGGTCAAAG3'

LeAMT1;1-pCubPLV, **LeAMT1;2-pCubPLV** and **LeAMT1;3-pCubPLV** were constructed by ligating the respective PCR products into the NdeI-XbaI cleaved pX-CubPLV vector with the compatible coherent ends AseI-XbaI generated by the PCR reaction using the following primers:

LeAMT1;1

FW 5'gagagaATTAATATGGCTTGTTCGTCGATACTC3'

RV 5'gagagaTCTAGAAGTTGAGGTTGTTGGTTCAATTCTTC3'

LeAMT1;2

FW 5'gagagaATTAATATGGCCTCCGCCATGACTTG3'

RV 5'gagagaTCTAGATACAACCACATTAACAGATCTATTTTGATGATC3'

*LeAMT1;3*FW 5'gagagaATTAATATGGATTCTTCATGGGAAGCTAG3'RV 5'gagagaTCTAGATTGTTGTTGCATCCTTAAATACTCG3'

This cloning strategy generated a peptide linker SRLESGGSTMSG between the AMTs and the CubPLV.

LeAMT1;1-pNubG, **LeAMT1;2-pNubG** and **LeAMT1;3-pNubG** were constructed by *in vivo* cloning in yeast (Ma *et al.* 1987; Oldenburg *et al.* 1997). A general strategy of the recombination-mediated *in vivo* cloning is outlined in Fig 2.1. The oligos used to produce PCR products for *in vivo* cloning were:

*LeAMT1;1*FW 5'tttctgcacaatatttcaagctataccaagcatacaatcaactcaATGGCTTGTTCCGTCGATACTC3'RV 5'caaagtcttgacgaaaatctgcatggctgcaggtcgacggatcccAGTTGAGGTTGTTGGTTCAATTCTTC3'*LeAMT1;2*FW 5'tttctgcacaatatttcaagctataccaagcatacaatcaactcaATGGCCTCCGCCATGACTTG3'RV 5'caaagtcttgacgaaaatctgcatggctgcaggtcgacggatcccTACAACCACATTAACAGATCTATTTTGATG3'*LeAMT1;3*FW 5'tttctgcacaatatttcaagctataccaagcatacaatcaactcaATGGATTCTTCATGGGAAGCTAG3'RV 5'caaagtcttgacgaaaatctgcatggctgcaggtcgacggatcccTTGTTGTTGCATCCTTAAATACTCG3'

The underlined sequences in the above oligonucleotides show the 45 bps regions homologous to the vector pX-NubG (Fig. 2.1). All the final constructs contained a peptide linker

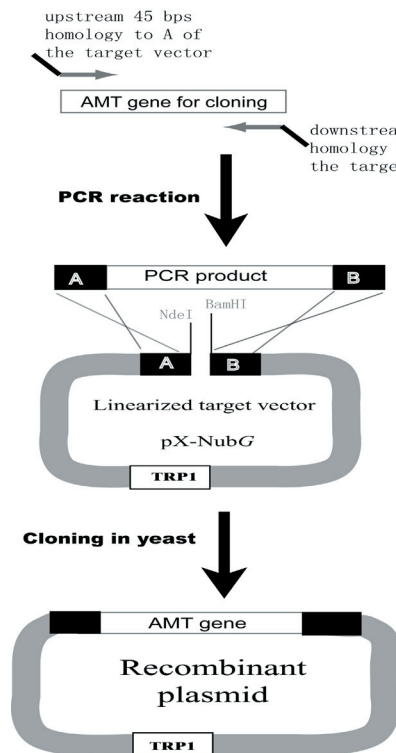


Fig. 2.1 Recombination-mediated *in vivo* cloning in yeast. The primers are designed in such a way that the resulting PCR product contains, in this case, 45 nt of homology at each end to the region of the plasmid at which crossing-over is to occur, which in this case are the A and B regions flanking the NdeI and the BamHI of the vector pX-NubG. Recombination taking place in yeast cells results in the re-circulation of the plasmid containing the PCR product fused at the exact sites. [Adapted from Oldenburg *et al.* 1997]

GIRRPAA between the Amt proteins and the NubG. After cloning in yeast, plasmids were recovered, amplified in *E. coli*, isolated from *E. coli* and sequenced.

Plasmids **LeAMT1;1-metYCgate** and **LeAMT1;1G458D-metYCgate** were also constructed using the *in vivo* cloning strategies (Fig. 2.1). PCR amplifications were performed using LeAMT1;1-pOO2 and LeAMT1;1G458D-pOO2 as the template respectively, with the following primers:

LeAMT1;1-sub-F,

5'acaagtttgtaaaaaagcaggctctccaaccaccATGGCTTGTTCGTCGATACTC3'

LeAMT1;1-sub-R,

5'tccgccaccaccaaccactttgtacaagaagctgggtaAGTTGAGGTTGTTGGTTCAATTCTTC3'

The lengths of the regions homologous to the vector in this case were 36 and 39 bps, respectively. A peptide YPAFLYKVVGGGGSGGGGS was engineered to link the Amts and CubPLV in these two constructs.

To generate the plasmid **LeAMT1;1G458D-pNubG**, a 819bps fragment containing the mutated region of LeAMT1;1G458D was excised from plasmid LeAMT1;1G458D-pOO2 by NdeI-BsaMI cleavage and ligated into the same restriction cleaved plasmid LeAMT1;1-pNubG, replacing the wild type region and thus yielding the plasmid LeAMT1;1G458D-pNubG.

LeAMT1;1ΔC45-pNubG was constructed by the same strategy as LeAMT1;1-pNubG, with the following primers:

FW, 5'tttctgcacaatatttcaagctataccaagcacaacaactcaATGGCTTGTTCGTCGATACTC3'

RV, 5'caagctctgacgaaaatctgcatggctgcaggctgcagatcccGAATTTATGAAGTATATAAAAAAGC3'

The resulting construct contains truncated LeAMT1;1 without the last 45aa fused to NubG.

LeAMT1;1-pNubWT was constructed by excising the NubWT fragment from the plasmid pNubWT with NdeI and inserting it into the NdeI-site of LeAMT1;1-pNubG.

2.2.2. Yeast strains, transformation and growth selection

The following yeast reporter strains were used:

L40: MAT α his3- Δ 200 trp1-901 leu2-3,112 ade2 lys2-801 LYS2::(*lexAop*)₄-HIS3 URA3::(*lexAop*)₈-lacZ GAL4

THY.AP4: MAT α leu2-3,112 ura3-52 trp1-289 *lex*::HIS3 *lexA*::ADE2 *lexA*::lacZ

THY.AP5: MAT α URA3 leu2-3,112 trp1-289 his3- Δ 1 ade2 Δ ::loxP

For maintenance yeast cells were grown in full medium (YPD: yeast extract 10 g/L, peptone 20 g/L, glucose 20 g/L). For selection, yeast cells were grown on minimal medium (SD: yeast nitrogen base 1.7 g/L, ammonium sulfate 5 g/L, pH 5.6, glucose 20 g/L) supplement-

tal with necessary amino acids as indicated in the result section. Solid medium contained an additional 2% Bacto-Agar or 1% agarose. If not stated otherwise, yeast growth was incubated at 28°C.

2.2.3. β -galactosidase assay

The activity of β -galactosidase was determined using a filter assay (Ramer *et al.* 1992). Cells were streaked out on reinforced nitrocellulose membrane (Schleicher & Schuell), placed on top of SD agar plates containing adenine and histidine and grown for two days. Filters were then removed from the plates, frozen in liquid nitrogen for 10 seconds (repeated twice), and subsequently placed on filter paper saturated with Z-buffer (60 mM Na_2PO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4) containing 35 mM β -mercaptoethanol and 1.5 mg/mL 5-bromo-4-chloro-3-inolyl-3-D-galactoside (X-Gal). Filters were then incubated at 37°C for 1h or prolonged to over night. In some cases, the X-Gal agarose overlay assay was used (Dumay *et al.* 1999).

2.3. Oocyte expression system

2.3.1. Plasmid constructs and *in vitro* transcription

For electrophysiological analysis, the following plasmid constructs were used: LeAMT1;1-pOO2, LeAMT1;2-pOO2 (Ludewig *et al.* 2002), LeAMT1;1G458D-pOO2 and LeAMT1;2G465D-pOO2 (see section 2.1.2). Mutant LeAMT1;2G421R-pOO2 was previously shown to yield identical current to the wild type and served as a control. For unrelated control, the amino acid transporter AtAAP6 cloned in the pOO2 vector (Fischer *et al.* 2002) was used.

For GFP fusion studies, the plasmids LeAMT1;1-GFPs65t-pOO2 and LeAMT1;1G458D-GFPs65t-pOO2 were generated. First, a NcoI-PstI DNA fragment containing the GFPs65t gene from the plasmid GFP-pDR195 (kindly provided by Sakiko Okumoto) was excised and ligated into the NcoI and PstI sites of the plasmid LeAMT1;1-pOO2 to generate a plasmid called LeAMT1;1-stop-GFPs65t-pOO2. This plasmid was then cleaved with NheI and EcoRV, which removed the stop codon and a part of LeAMT1;1. This linear DNA was subsequently ligated with corresponding NheI/blunt end fragments without stop codon from plasmids LeAMT1;1-pNubG, LeAMT1;1G458D-pNubG and LeAMT1;1 Δ C45-pNubG (see section 2.2.1); thus yielding plasmids LeAMT1;1-GFPs65t-pOO2, LeAMT1;1G458D-GFPs65t-pOO2 and LeAMT1;1 Δ C45-GFPs65t-pOO2, respectively. In these constructs the GFP is fused to the C-terminus of LeAMT1;1, LeAMT1;1G458D and LeAMT1;1 Δ C45 by a short peptide linker GIRRPS. All the constructs used had been sequenced to verify.

The cRNA for injection into oocytes was prepared as follows: The *in vitro* transcription reaction was performed using Ambion's mMessage mMachine™ kit. Capped cRNA was

transcribed by SP6 RNA polymerase after linearization of plasmids with MluI. Approximately 500ng of each linearized plasmid were used in a 10 μ l reaction for 2h. The cRNA was recovered at the end of the reaction by LiCl precipitation. An aliquot of each cRNA was resuspended in Nuclease-free dH₂O and analysed on a 1% agarose gel for quantification. At least two independent cRNA preparations were used for each construct.

2.3.2. Preparation, Injection, and Electrophysiology of Oocytes

Xenopus oocytes were removed from adult female frogs by surgery and manually dissected. Oocytes (Dumont stage V or VI) were defolliculated using collagenase 10 mg/ml (Roche Applied Science) and trypsin inhibitor (Sigma) for 1 h and injected with 50 nl of cRNA diluted in diethyl pyrocarbonate-treated water (6-50 ng/oocyte). For co-expression experiments, oocytes were injected with low (6-12 ng/nl) concentrations of cRNA to avoid expression saturation effects due to limiting translation and processing of heterologously expressed AMT proteins in oocytes. These cRNA concentrations allowed a linear increase of transporter activity with cRNA amount injected. Each coinjection experiment was repeated multiple times, and 4-15 oocytes were measured for each construct. After injection oocytes were kept for 2-5 days at 16 °C in ND96 (in mM: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 Hepes (pH 7.4), and gentamycin (20 μ g/ml) with added Na-pyruvate (2.5 mM). Total data were collected from more than 10 batches of oocytes from different frogs. Electrophysiological measurements were done as described in Ludewig *et al.* (2002). Standard bath solutions contained (in mM): 100 NaCl, 2 CaCl₂, 2 MgCl₂, and 4 Tris, pH adjusted to 7.5 with MES.

2.3.3. Data Analysis

All data were given as means \pm S.D. The concentration dependence of ammonium-induced current at each voltage was fitted using Equation 2.1,

$$I = I_{\max}(1 + K_m/c) \quad (\text{Eq. 2.1})$$

where Imax is maximal current at saturating ammonium concentration, Km is substrate concentration permitting half-maximal currents, and c is experimentally used concentration. For the co-injections, Equation 2.2 was used.

$$I = I_{\max} \times [1/(1 + K_{m1}/c) + 1/(1 + K_{m2}/c)] \quad (\text{Eq. 2.2})$$

Voltage dependence of Km was fitted with Equation 2.3,

$$K_m(\delta) = K_m^{(0mV)} \times \exp(\delta \times e \times V/k \times T) \quad (\text{Eq. 2.3})$$

where δ is fractional electrical distance, e is elementary charge, V is membrane potential, k is Boltzmann's constant, and T is absolute temperature. Statistical significance was evaluated by using a paired t test. A p value < 0.05 was considered significant.

2.4. Plant transformation

2.4.1. Protoplast transformation for transient expression of GFP-fusions

Plasmid constructs

Plasmids pJIT60 and pGreen0229 (Hellens *et al.* 2000) were kindly provided by Gabriel Schaaf. pG60 was constructed by excising the 35S cassette from pJIT60 with SacI-XhoI and ligated into the same sites of pGreen0229. The pG60 binary vector then possessed a bar selection mark gene (resistant to herbicide BASTA™, Rathore *et al.* 1993) close to the left border (LB), and to the right border (RB) a double-35S-driven expression cassette from pJIT60.

Starting from pG60, a number of plasmids for expressing GFP-fusions were constructed. First, the gene of mGFP5 was excised from plasmid CF203 (kindly provided by Sakiko Okumoto) with the BspH I and PstI restriction enzymes and ligated into NcoI and PstI restricted plasmid LeAMT1;1-pOO2 (section 2.3.1), yielding plasmid LeAMT1;1-stop-mGFP5-pOO2. This plasmid was then cleaved with NheI and EcoRV removing the stop codon and a part of the LeAMT1;1 sequence, yielding a linear DNA for ligation, respectively, with the corresponding fragments without stop codon from plasmid LeAMT1;1-pNubG, LeAMT1;1G458D-pNubG and LeAMT1;1ΔC45-pNubG (section 2.2.1). The resulting plasmids were named LeAMT1;1-mGFP5-pOO2, LeAMT1;1G458D-mGFP5-pOO2 and LeAMT1;1ΔC45-mGFP5-pOO2, respectively. From these three plasmids, fragments containing the respective AMT 1;1 and mGFP5 fusions were then excised with HindIII and PstI, and ligated into the same HindIII-PstI restricted pG60 vector, yielding plasmids **pG60-LeAMT1;1-mGFP5**, **pG60-LeAMT1;1G458D-mGFP5** and **pG60-LeAMT1;1ΔC45-mGFP5**, respectively. These final plasmids contains a peptide sequence *GIRRRPS* linking the mGFP5 to the C-terminus of the respective LeAMT1;1.

The plasmid **pG60-C45-mGFP5** for expression of the cytosolic C-terminal fragment of the LeAMT1;1 fused to mGFP5 was constructed by cleaved the plasmid LeAMT1;1-mGFP5-pOO2 with HindIII-EcoRV and subsequently re-ligation using the synthesized oligo adapters,

5' agcttatgAAGTTGCTTCGGAT3'
3' atacTTCAACGAAGCCTA5'

The resulting plasmid was named C45-mGFP5-pOO2. The HindIII-PstI fragment of this plasmid containing C45-mGFP5 sequence was then ligated into the HindIII-PstI restriction sites of pG60 to give the final plasmid pG60-C45-mGFP5.

The plasmid **pG60-mGFP5** for the expression of soluble mGFP5 was constructed by excising the mGFP5 cDNA from LeAMT1;1-mGFP5-pOO2 with EcoRV and PstI and subsequently fusing the fragment into HindII/blunt end and PstI restricted pG60.

Cell cultures and protoplast transformations

Tobacco BY2 cells were cultured and maintained in MSB medium (4.3g/l MS salts, 100mg/l myo-Inositol, 1mg/l Thiamin, 0.2mg/l 2,4-D, 255mg/l KH_2PO_4 , 30g/l sucrose, pH5.0), whereas *Arabidopsis thaliana* (var. Columbia) mesophyll cells were cultured and maintained in MSCol medium (4.3g/l MS salts, 100mg/l myo-Inositol, 1mg/l B5-Vitamins mix, 1mg/l 2,4-D, 30g/l sucrose, pH5.8). Both were incubated in a shaker of 120rpm at 26°C. Sub-culturing was done by inoculation in fresh medium every week. Tobacco BY2 protoplasts were prepared from the cell cultures by digesting cell walls at 26°C with enzyme solutions containing cellulose, macerozyme and pectinase in the dark for 12 hours without shaking. Protoplasts of *Arabidopsis* were obtained by digestion of cell walls at 26°C with enzyme mixture of cellulose and macerozyme for 6 hours shaking at 50 rpm in the dark. PEG-mediated protoplast transformations (Abel and Theologis, 1998) were conducted using 15-30 µg plasmid DNA/transformation. The transformed protoplasts were resuspended in culture medium and inoculated at 26°C for 20-24 hours in order to allow gene expression.

2.4.2. Stable transformation of tomato plants

Plasmid constructs

To construct the plasmids **pG60-LeAMT1;1** and **pG60-LeAMT1;1G458D** with the plant selectable marker bar gene, pG60 was cleaved with HindIII and PstI and ligated with the fragments of LeAMT1;1 and LeAMT1;1G458D, which were excised from LeAMT1;1-pOO2 and LeAMT1;1G458D-pOO2 via HindIII and PstI restriction.

To construct the plasmids **pJH212gck-LeAMT1;1** and **pJH212gck-LeAMT1;1G458D**, which bear the plant selectable marker neomycin phosphotransferase gene (NPTII), the binary vector pJH212gck (kindly provided by Dr. Karin Schumacher) was cleaved with KpnI, and ligated with fragments from pG60-LeAMT1;1 and pG60-LeAMT1;1G458D as shown in Fig. 2.2.

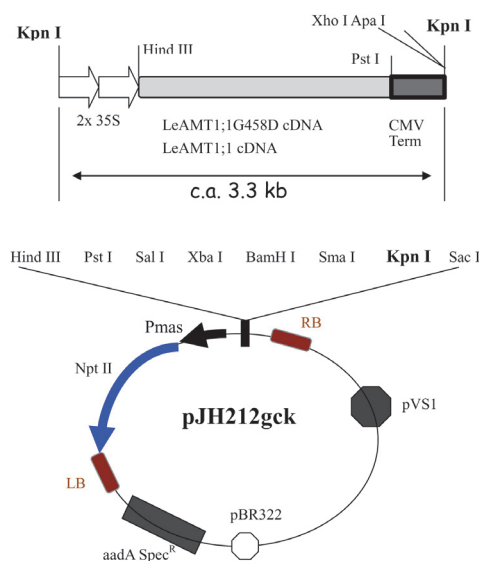


Fig. 2.2 Plasmid constructs for tomato transformation to obtain transgenic plants over-expressing the wild type and the point mutant of LeAMT1;1. The constructs in which the expression of Amt genes are under the control of the CaMV35S promoter with a duplicated enhancer are cloned into the KpnI site of the binary vector pJH212gck, which confers spectinomycin resistance in bacteria and kanamycin resistance in plants.

Agrobacterium-mediated tomato cotyledon transformation

Agrobacterium tumefaciens strain GV2260 (Rifampicin and Ampicillin resistance) was transformed with plasmids pJH212gck-LeAMT1;1 and pJH212gck-LeAMT1;1G458D via electroporation respectively. Transformants were selected on YEB+ Rifampicin (100µg/ml) + Ampicillin (100µg/ml) + Sectinomycin (100µg/ml) medium.

Tomato (*Lycopersicon esculentum* L. Mill. var. Moneymaker) cotyledons were used as explant sources for the *Agrobacterium*-mediated transformation as described in Frary and Earle (1996).

PCR screening of transgenic plants

PCR detection of the neomycin phosphotransferase gene (NptII) was performed with the specific forward and reverse primers 5'AAGATGGATTGCACGCAGGTTC and 5'GAAGAAGCTCGTCAAGAAGGCGA, respectively, using genomic DNA prepared from leaves of the regenerated transgenic plants (R₀) as templates.

2.5. Yeast two hybrid system

The GAL4-based two-hybrid system was adopted for testing the self-interaction of the cytoplasmic C-terminal fragment of LeAMT1;1, C45 (containing the last 45 amino acids: KLLRISSEDEMAGMDLTRHGGFAYYHEEDPKLGMQMRRIEPTTST) and for screening cDNA libraries for putative interaction partners that might associate with this cytoplasmic region.

2.5.1. Plasmid constructs, yeast strains, reporter assay and plasmid rescue

Plasmid constructs

The bait was constructed as follows: C45 fragment was PCR amplified, cleaved with EcoRI and PstI and ligated into a EcoRI-PstI linearized vector pGBKT7 (Clontech, Inc. USA). The primers for PCR were:

FW, 5' gagagagaattcAAGTTGCTTCGGATATCGTCCGAAGAC3';

RV, 5' gagagactgcagCTAAGTTGAGGTTGTTGGTTCAATTCTTC3'

This bait plasmid pGBKT7-C45 enables the expression of the GAL4 DNA-binding domain (BD domain) fused with the C45 fragment under the control of ADH1 promoter.

The same C45-fragment was ligated into the EcoRI and PstI sites of pGAD424 vector(Clontech, Inc. USA). The resulting prey plasmid pGAD424-C45 allows the expression of the GAL4 DNA activation domain (AD domain) fused with the C45 fragment. The bait plasmid pGBKT7 bears a bacterial selection marker for kanamycine resistance, which differs from that of prey plasmids (ampicillin resistance), making it easier to isolate the prey plasmids after screening cDNA AD-fusion libraries.

Yeast strains

Two reporter strains were used:

AH109 (MAT α , trp1-901, leu2-3, 112, ura3-52, his3-200, gal4 Δ , gal80 Δ , LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ, MEL1); **Y187** (MAT α , ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4 Δ , met⁻, gal80 Δ , URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ, MEL1).

Reporter assay

To assay the activities of α -galactosidase, encoded by the reporter gene MEL1 (in both AH109 and Y187), 200 μ l of X- α -Gal (5-Bromo-4-Chloro-3-indolyl α -D-galactopyranoside, Glycosynth Limited, England) stock solution (4 mg/ml in dimethylformamide) were spread evenly onto a 150 mm SD selection plate (or 100 μ l for a 90 mm plate) and dried at room temperature for 20 minutes. Cells were spread or streaked on these plates and incubated at 28°C overnight or until blue colonies formed. Determination of β -galactosidase activities encoded by the reporter gene LacZ (in both AH109 and Y187) was described in Section 2.2.3.

Plasmid rescue

Plasmids from the positive clones, which were isolated by the library screening, were recovered by shuttling them back to *E.coli* strain XL1-Blue. Reconfirmation of interactions was done by co-transformation of isolated plasmids and the bait plasmid pGBKT7-C45 into the yeast strain AH109. cDNA of the re-confirmed clones were sequenced.

2.5.2. Testing the self-interaction of the LeAMT1;1 C-terminal fragment

The yeast strain AH109 was co-transformed with plasmids pGBKT7-C45 and pGAD424-C45 using the protocol of Gietz and Woods (2002). The transformants were selected on SD plates without leucine and tryptophan. Single colonies were streaked out on fresh SD-leu-trp plates and grown for 4 days at 28°C. The activity of β -galactosidase was determined as in Section 2.2.3.

The plasmids pGBKT7-53 and pGADT7-T (Clontech, Inc. USA) encoded fusions between the GAL4 DNA-BD and AD and murine p53 and SV40 large T-antigen, respectively. p53 and large T-antigen interacted in a yeast two-hybrid assay (Li and Fields, 1993) and served as a positive control.

2.5.3. Screening of the *Arabidopsis thaliana* cDNA AD-fusion library

The AD-fusion cDNA library from 7-week-old leaves of *Arabidopsis thaliana* was kindly provided by Drs. Ulrike Zentgraf and Ying Miao. It was constructed in the AD vector pGADT7-Rec using the Matchmaker™ Library Construction & Screening Kit from Clon-

tech Inc. This library was made directly in the yeast reporter strain AH109 by homologous recombination-mediated *in vivo* cloning, according to the User Manual, which was provided with the kit. The cDNA was made by oligo d(T) priming and the range of insert length was 200-800 bps. The estimated transformation efficiency was 1×10^5 transformants/ μg and the library titer 7.52×10^7 cfu/ml.

Screening was performed by mating the strain Y187 harboring the bait plasmid pGBKT7-C45 with the strain AH109 bearing the cDNA AD-fusion library. The protocol is described in the User Manual. Briefly, a single colony of Y187 containing pGBKT7-C45 was picked and grown in 50 ml of SD-trp+kan (50 $\mu\text{g}/\text{ml}$) liquid medium overnight at 28°C until the cell optical density at 600nm (OD600) reached 0.8. The cells were then collected and resuspended in about 5 ml SD-trp liquid medium. This was combined with a thawed 1-ml aliquot of the AH109 library strain (7.5×10^7 cells) and allowed for mating in 50 ml of 2 x YPDA+kan (50 $\mu\text{g}/\text{ml}$) medium at 28°C for 2 days with gentle swirling at 40 rpm. The mating mixture was collected, rinsed and resuspended in 10 ml of 0.5 x YPDA+kan (50 $\mu\text{g}/\text{ml}$) and subsequently plated on 42 x 150mm plates of selection medium SD-trp-leu-ade-his. After 7 through 14 days, positive (Trp+ Leu+ Ade+ His+) colonies were picked and re-streaked out on fresh plates maintaining the same selection.

2.5.4. Screening of the *Lycopersicon esculentum* cDNA AD-fusion library

The *Lycopersicon esculentum* cDNA AD-fusion library, kindly provided by Rama Panford-Walsh, was constructed in the AD-fusion vector pACT2 (Clontech, Inc. USA) by ligating the cDNA (random priming) unidirectionally between the EcoRI and XhoI sites of pACT2. The average length of the cDNA inserts was 1 kb and the primary transformants had an estimated cell titer of 10^{10} cfu/ml in *E.coli*. The library was amplified only once in *E.coli*.

To perform a large scale screening with this tomato cDNA library, a total of 40 μg library plasmid DNA were used. These library cDNA were combined with 40 μg of the bait plasmid pGBKT7-C45 and used in the transformation of the yeast strain AH109. The transformation was conducted in 40 separate eppendorf tubes. After transformation, yeast cells in each tube were resuspended in 600 μl sterile double distilled H₂O and every 300 μl of these transformed cells were spread on a 150 mm selection plate with SD-trp-leu-ade-his medium, totaling to 80 plates. From 7 through 14 days, growing colonies were picked and re-streaked on fresh plates with the same selection medium. Transformation efficiency was determined in 90 mm plates with SD-trp, SD-leu or SD-trp-leu medium using serial dilution of transformant cells. Plasmid rescue, isolation and re-transformation and sequencing were done as described in above sections.

2.6. Invertase fusion and expression in yeast

2.6.1. Plasmid constructs

Table 2.2 List of plasmids constructed in invertase fusion experiments

Plasmid	Function	Promoter for yeast expression	Yeast marker and replicon
pMET25	Yeast expression vector under the control of MET25 promoter, methionine repressible	Met25	LEU2, 2 μ
pMET25-Suc2	Expressing the yeast Suc2 gene coding for the secreted invertase (invertase with its native transmit signal sequence)	Met25	LEU2, 2 μ
pMI	Yeast expression vector for fusion proteins, 2 MCS linked by a sequence coding for 3x GGGGS polypeptide.	Met25	LEU2, 2 μ
pMInv-Y	Expressing the yeast non-secreted invertase (The N-fusion)	Met25	LEU2, 2 μ
pMN377-Y	Expressing the yeast non-secreted invertase N-terminal fragment 377 aa (The N-fusion)	Met25	LEU2, 2 μ
pMInv-LeAMT1;1	Expressing the yeast non-secreted invertase fused at the N-terminus of LeAMT1;1	Met25	LEU2, 2 μ
pMInv-LeAMT1;2	Expressing the yeast non-secreted invertase fused at the N-terminus of LeAMT1;2	Met25	LEU2, 2 μ
pMInv-ScSte2	Expressing the yeast non-secreted invertase fused at the N-terminus of ScSte2, the yeast α -factor receptor	Met25	LEU2, 2 μ
pMSin-LeAMT1;1	Expressing the yeast non-secreted invertase N-terminal fragment 377 aa fused at the N-terminus of LeAMT1;1	Met25	LEU2, 2 μ
pMN377-LeAMT1;2	Expressing the yeast non-secreted invertase N-terminal fragment 377 aa fused at the N-terminus of LeAMT1;2	Met25	LEU2, 2 μ
pMN377-ScSte2	Expressing the yeast non-secreted invertase N-terminal fragment 377 aa fused at the N-terminus of ScSte2	Met25	LEU2, 2 μ
pMY-Inv	Expressing the yeast non-secreted invertase (The C-fusion)	Met25	LEU2, 2 μ
pMY-N377	Expressing the yeast non-secreted invertase N-terminal fragment 377 aa (The C-fusion)	Met25	LEU2, 2 μ
pMhTfR-Inv	Expressing the yeast non-secreted invertase fused at the C-terminus of hTfR, a human transferrine receptor	Met25	LEU2, 2 μ

pMhTfR-N377	Expressing the yeast non-secreted invertase N-terminal fragment 377 aa fused at the C-terminus of hTfR, a human transferrine receptor	Met25	LEU2, 2 μ
pC135-X	Expressing the C-terminal fragment (135 aa) of yeast non-secreted invertase (The N-fusion)	ADH1	TRP1, 2 μ
pC211-X	Expressing the C-terminal fragment (211 aa) of yeast non-secreted invertase (The N-fusion)	ADH1	TRP1, 2 μ
pC135-ScSte2	Expressing the C-terminal fragment (135 aa) of yeast non-secreted invertase fused at the N-terminus of ScSte2	ADH1	TRP1, 2 μ
pC211-ScSte2	Expressing the C-terminal fragment (211 aa) of yeast non-secreted invertase fused at the N-terminus of ScSte2	ADH1	TRP1, 2 μ
pC135-LeAMT1;1	Expressing the C-terminal fragment (135 aa) of yeast non-secreted invertase fused at the N-terminus of LeAMT1;1	ADH1	TRP1, 2 μ
pC211-LeAMT1;1	Expressing the C-terminal fragment (211 aa) of yeast non-secreted invertase fused at the N-terminus of LeAMT1;1	ADH1	TRP1, 2 μ
pX-C135	Expressing the C-terminal fragment (135 aa) of yeast non-secreted invertase (The C-fusion)	ADH1	TRP1, 2 μ
pX-C211	Expressing the C-terminal fragment (211 aa) of yeast non-secreted invertase (The C-fusion)	ADH1	TRP1, 2 μ
phTfR-C135	Expressing the C-terminal fragment (135 aa) of yeast non-secreted invertase fused at the C-terminus of hTfR	ADH1	TRP1, 2 μ
phTfR-C211	Expressing the C-terminal fragment (211 aa) of yeast non-secreted invertase fused at the C-terminus of hTfR	ADH1	TRP1, 2 μ
pSS-C135	Expressing the secreted C-terminal fragment (135 aa) of yeast invertase (Containing the native transmit signal sequence)	ADH1	TRP1, 2 μ
pSS-C211	Expressing the secreted C-terminal fragment (211 aa) of yeast invertase (Containing the native transmit signal sequence)	ADH1	TRP1, 2 μ

The plasmid **pMET25** was a derivate of pGAD424 (Clontech Inc) by first restriction digesting with SphI to remove the GAL4-AD sequence together with the ADH1 promoter and terminator sequence and blunting with T4 polymerase and then ligated with an 820 bp fragment containing the MET25 promoter followed by a polylinker and the CYC1 terminator, obtained by blunting after excision from pRS426Met25 (Mumberg *et al.* 1994) with SacI and KpnI restriction.

pMET25-Suc2 was constructed via blunt-end ligation of the PCR product of Suc2 gene from yeast strain 31019b genomic DNA into the pMET25 at the site XbaI blunted by T4 polymerase. The primer sequences were:

FW, 5'gagagactcgagATGCTTTTGCAAGCTTTCCTTTTC

RV, 5'gagagactcgagCTATTTTACTTCCCTTACTTGGAACTTG.

pMI was constructed by restriction digestion of pMET25 with XbaI and PstI to destroy its MCS sites and ligated again with the following synthetic oligo adaptors.

Adaptor 1 (XbaI-NdeI)	5' CTAGAGAGAGACA3' 3' TCTCTCTGTAT5'
Adaptor 2 (NdeI-XhoI)	5' TATGGTCGACACCACGCGTAGATCCGGTGGTGGTTCCTGGTGGTGGTTCCTGGTGGTGGTGGTCC3' 3' ACCAGCTGTGGTGCATCTAGGCCACCACCACCAAGACCACCACCAAGACCACCACCAGGAGAGT5'
Adaptor 3 (XhoI-PstI)	5' TCGAGGCTAGCCCCGGGGCGGCCGCACTAGTGCATGCAGGCCTATGCATGTAATAACTGCA3' 3' CCGATCGGGGCCCGCCGCGTGATCACGTACGTCCGGATACGTACATTATTG5'

pMY-Inv, **pMY-N377**, **pMInv-Y** and **pMN377-Y** were constructed by PCR amplification of either full-length yeast invertase or its N-terminal 377aa fragment from yeast genomic DNA of the strain 31019b. Subsequently the fragments were ligated into appropriate sites of the vector pMI (see Fig. 2.3 and Fig. 2.4).

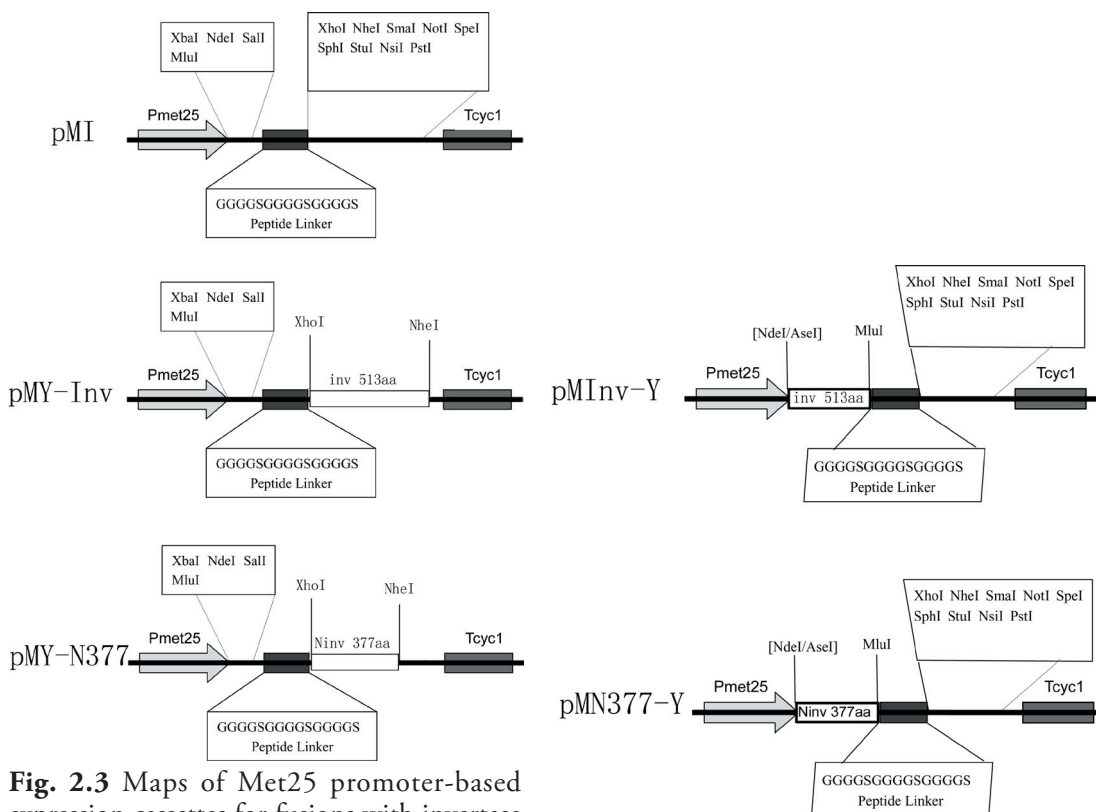
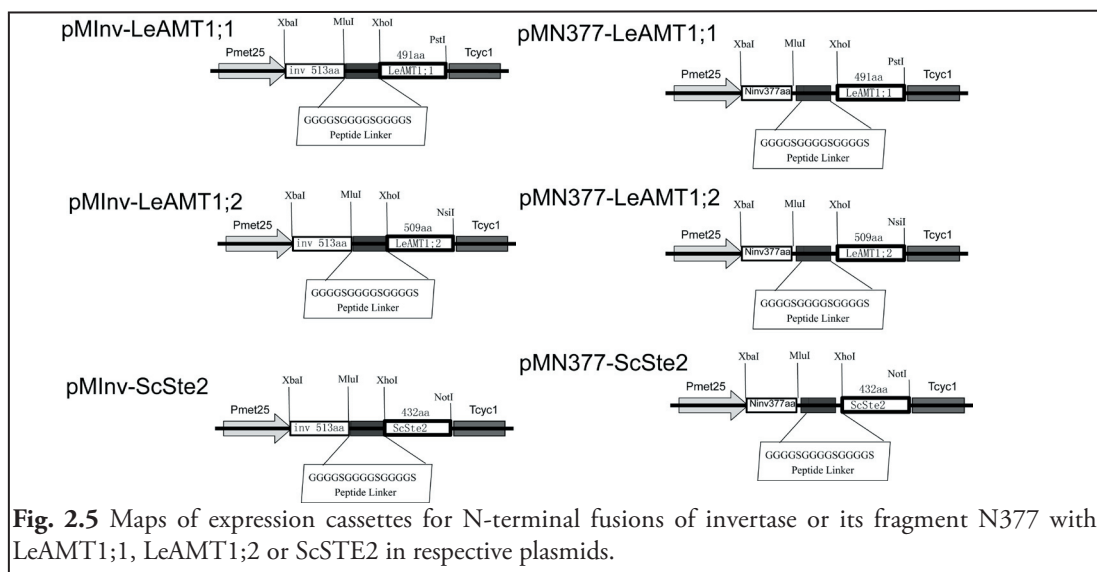


Fig. 2.3 Maps of Met25 promoter-based expression cassettes for fusions with invertase and its fragment N377 in vectors pMY-Inv and pMY-N377, which are derivatives of vector pMI. pMY-Inv and pMY-N377 are designed for C-terminal fusions of invertase and its fragment N377, respectively.

Fig. 2.4 Maps of Met25 promoter-based expression cassettes for N-terminal fusions with invertase and its fragment N377 in vectors pMInv-Y and pMN377-Y.

pMInv-LeAMT1;1, **pMN377-LeAMT1;1**, **pMInv-LeAMT1;2**, **pMN377-LeAMT1;2**, **pMInv-ScSte2** and **pMN377-ScSte2** were constructed by amplifying the specific genes via PCR and ligating them into the vectors pMInv-Y and pMN377-Y at appropriate sites (Fig. 2.5). Genomic DNA isolated from the yeast strain 31019b was used as template for PCR cloning the gene ScSte2. The primer sequences for ScSte2 were:
 Ste2-Xho-F, 5'gagagactcgagATGTCTGATGCGGCTCCTTC3'
 Ste2-Not-R, 5'tgatgagcggccgcTAAATTAT'TAT'TATCTTCAGTCCAG3'

pMhTfR-Inv and **pMhTfR-N377** were constructed by sticky-end PCR (Zeng, 1998) amplifying the human transferrin receptor gene hTfR (Terng *et al.* 1998) from plasmid



pGEM-hTfR (kindly provided by Dr. C. Langs) and subsequent ligation into the NdeI sites of vectors pMY-Inv and pMY-N377 (Fig. 2.6). The primers used in the sticky-end PCR were,

Fa, 5'TATGATGGATCAAGCTAGATCAG3'

Fb, 5'TGATGGATCAAGCTAGATCAG3'

Ra 5'AACTCATTGTCAATGTCCCAAAC3'

Rb 5'TAAACTCATTGTCAATGTCCCAAAC3'.

The vector pX-NubG was used to construct a series of vectors for expressing different C-terminal fragments of the invertase fused with membrane proteins. pX-NubG was first cleaved by NdeI-PstI and then re-ligated with the synthetic adapter:

5' tatgtaccatcacgatgttccagattactaccatcacgatgttccagattaccatggtaataataagctagcaggcctcggcggcagatctttaataactgca3'
 3' acatgggtatgctacaaggctcaatgatgggtatgctacaaggctcaatgggtaccattattatctgatcgctcggacgcccggcgtctagaaattattg5'

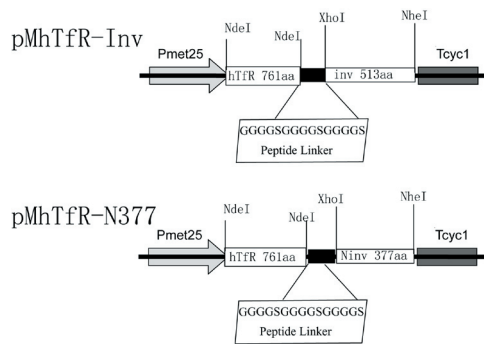


Fig. 2.6 Maps of expression cassettes for C-terminal fusions of invertase or its fragment N377 with hTfR in plasmids pMhTfR-Inv or pMhTfR-N377.

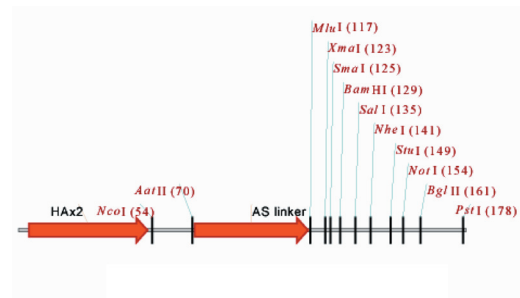


Fig. 2.7 Maps of the multi-cloning sites showing the duplicated HA tag and the AS linker (amino acids: ASASNGASASNGASA) in between the AatII and MluI sites.

The resulting vector pXIIHA was then cleaved by NcoI-NheI and re-ligated with the synthetic adapter:

```
5' catgGTATACAGACGTCGCTTCTGCTTCTAAACGGTGCTTCTGCTTCTAACGGTGCTTCTGCTACGCGTCCC GGGGGATCCGTCGACG3'
3' CATATGCTCTGCAGCGAAGACGAAGATTGCCACGAAGACGAAGATTGCCACGAAGACGATGCGCAGGGCCCCCTAGGCAGCTGcgatc5'
```

The final vector was called pYXII, containing two repeats of HA tag followed by an AS peptide linker in between the multicloning sites (Fig. 2.7).

pC135-X, **pC211-X**, **pX-C135** and **pX-C211** were constructed by ligating the PCR products of the C-terminal 135aa or 211aa fragments of the yeast invertase into the appropriate sites of the vector pYXII (Fig. 2.8).

pC135-LeAMT1;1, **pC211-LeAMT1;1**, **pC135-ScSte2**, **pC211-ScSte2**, **phTfR-C135** and **phTfR-C211** were constructed by PCR amplification of the specific gene sequences and ligation into pC135-X, pC211-X, pX-C135 or pX-C211 (Fig. 2.9).

pSS-C135 and **pSS-C211** were constructed by cleavage of the vectors pX-C135 and pX-C211 with NdeI and AatII and subsequent ligation with the synthetic adapter:

```
5' tATGCTTTTGCAGCTTTTCCTTTTTCCTTTTGGCTGGTTTTGCAGCCAAAATATCTGCATCAATGgacgt3'
3' ACGAAAACGTTTCGAAAGGAAAAGGAAAACCGACCAAAAACGTCGGTTTTATAGCGTAGTTACc5'
```

The adapter contains the native secretory signal sequence of the Suc2 gene.

2.6.2. Yeast strain and growth conditions

Yeast strain **YSH2.64** (MAT α ura3-52 leu2, 3-112 trp1 suc2 Δ ::URA3 mal0) (Riesmeier *et al.* 1992) was used as the host strain for expression of various invertase-fusions. The endogenous Suc2 gene of this strain was completely deleted so that no background invertase activity could be detected. After transformation with appropriate plasmids, yeast colonies were selected and maintained on minimal selection SD medium with appropriate amino acids at 28°C. For invertase dependent growth assay, sucrose at final concentration of 2%

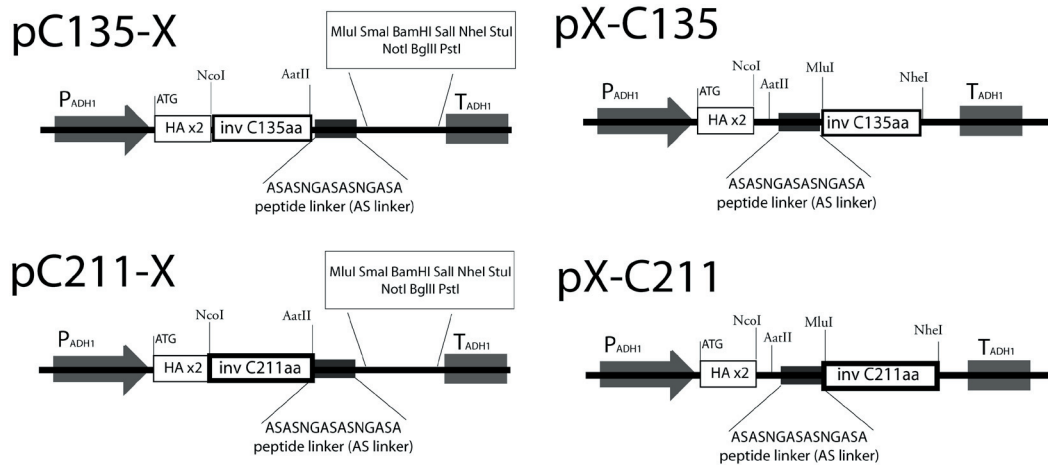


Fig. 2.8 Maps of ADH1 promoter-based expression cassettes for fusions with the invertase C-terminal fragments C135 or C211 in respective vectors. pC135-X and pC211-X are for N-terminal fusions with the invertase fragments, whereas pX-C135 and pX-C211 are for C-terminal fusions.

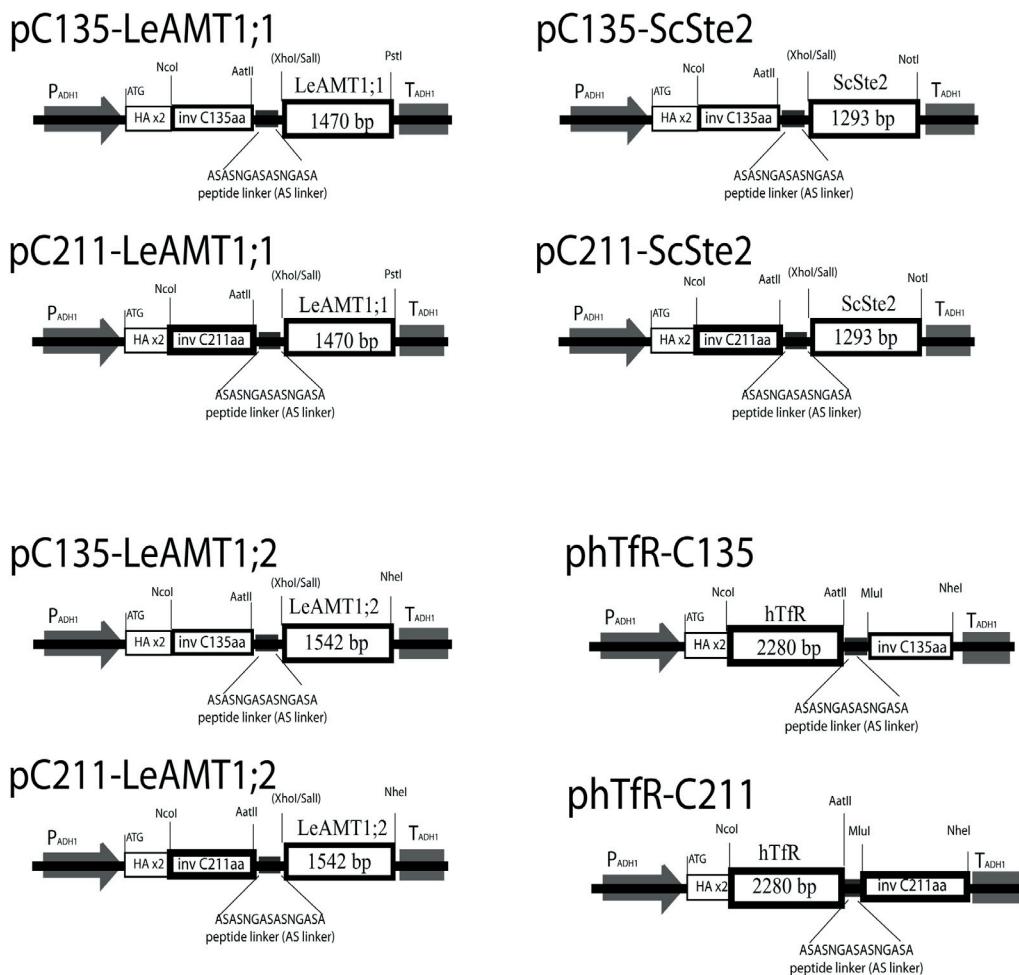


Fig. 2.9 Maps of ADH1 promoter-based expression constructs for fusions of the invertase C-terminal fragments C135 or C211 with LeAMT1;1, LeAMT1;2, ScSTE2 and hTfR in respective plasmids.

was used instead of glucose as the sole carbohydrate source, and 0.7% agarose was used for solidification of the medium. Antimycin A was added to the medium at a final concentration of 2 mg/L for better scoring of invertase-dependent growth (Robinson *et al.* 1988).

2.6.3. Invertase activity assays and data analysis

Invertase activity assays were performed as outlined in Fig. 2.10 and modified from Westphal *et al.* (1996). Glucose contents were determined using the GOD/POD Perid kit from Roche Diagnostics GmbH and the enzyme reaction products were quantified at 420 nm in 96-well microtiter plates using SAFIRE microplate reader (TECAN GmbH Austria). The amounts of yeast cells were estimated as optical density at 600nm and expressed as OD600. Invertase activity was then calculated as g glucose released/OD600/10min. The assays were repeated three times in different days, with 3 replications each. The final data were shown as mean \pm s.d. Statistical significance was evaluated using a paired t test. A p value < 0.05 was considered significant.

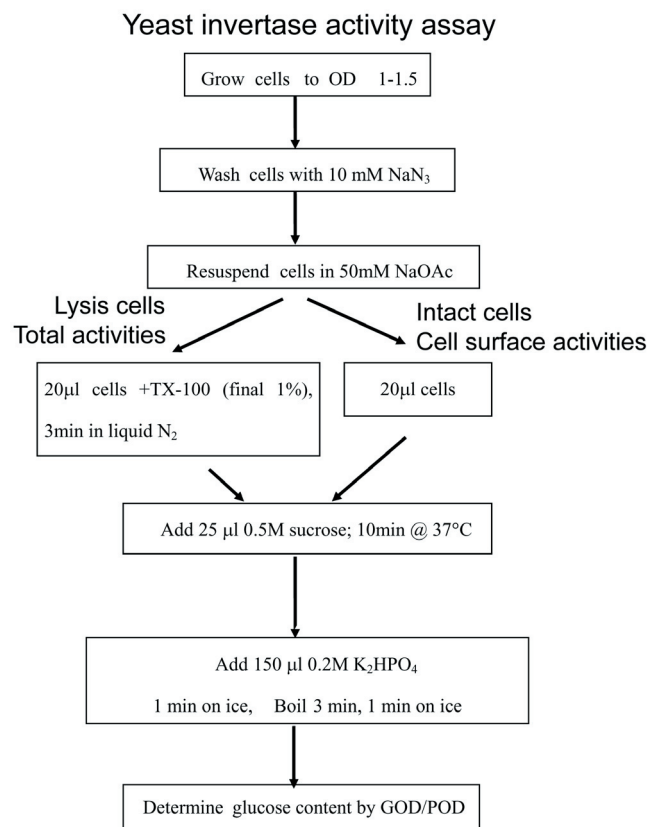


Fig. 2.10 Flow-chart outlining yeast cell invertase activity assay. The protocol is modified from Westphal *et al.* (1996).

3. Results

3.1 LeAMT1;1 self-interactions detected by an improved split ubiquitin system

Protein-protein interactions between two membrane proteins can be monitored in yeast by the membrane protein split ubiquitin system (Stagljar *et al.* 1998). It is based on the *in vivo* reconstitution of the ubiquitin, a cellular protein of 76 amino acids, which has been split into two fragments, the Nub (1-36 amino acids at N-terminus) and the Cub (35-76 amino acids at C-terminus) and co-expressed in the same cells. The discovery that a mutated form of the Nub fragment (designated as NubG, Ile at position 13 changed to Gly), in contrast to the wild-type Nub (designated as NubWT, original sequence without any mutation), was unable to interact with the Cub and reconstitute the functional ubiquitin, unless both were brought in close contact by two interacting proteins, led to the proposal of the split ubiquitin as a *in vivo* sensor of protein interaction or proximity (Johnsson and Varshavsky, 1994). The principle of the membrane protein split ubiquitin system was illustrated in Fig. 3.1. This system had been successfully used to demonstrate the interactions between sucrose transporters (Reinders, A. *et al.* 2002a, 2002b; Schulze *et al.* 2003).

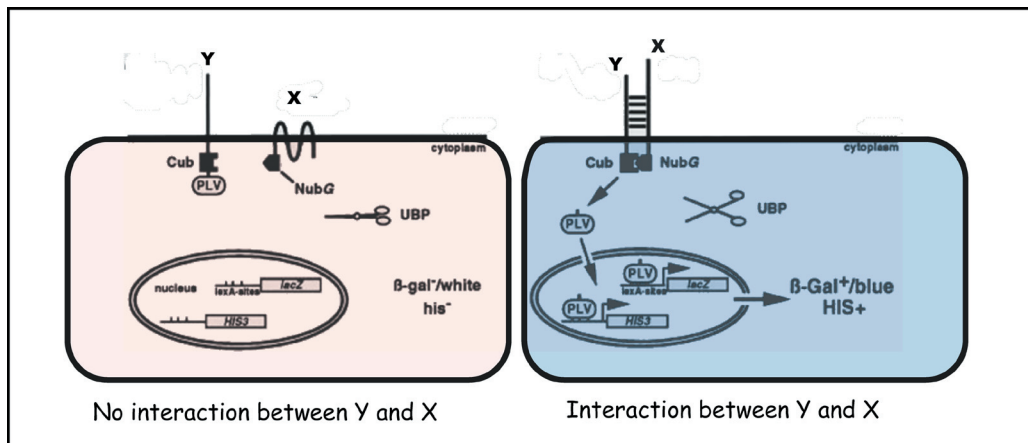


Fig. 3.1 Illustrated principle of the membrane protein split ubiquitin system. The split fragments of the ubiquitin Cub and NubG are fused to two membrane proteins Y and X, respectively. The fusions Y-Cub-PLV and X-NubG are co-expressed in the same cells. **Left panel:** when there is no interaction between Y and X, the Cub and NubG can not reconstitute a functional ubiquitin and the artificial transcription factor (PLV) fused at the C-terminus of the Cub will not be cleaved by ubiquitin-specific protease (UBP). No activation of the reporter genes (His3 and LacZ) occurs. **Right panel:** if Y and X interact with each other, the interaction will bring the linked Cub and NubG together, enabling them to reconstitute a functional ubiquitin. The reconstituted ubiquitin can be correctly recognized by the UBP thus the PLV is released from the fusion by UBP cleavage. The released PLV will enter the nucleus and activate reporter genes LacZ and His3. [Adapted from Stagljjar, et al. 1998]

In a primary experiment, LeAMT1;1, LeAMT1;2 and LeAMT1;3 were cloned into the original bait vector pY-CubPLV, a single copy integration vector where the CubPLV fusions were driven by the weak promoter Wbp1p. When co-expressed with designated NubG-fusions, LeAMT1;1-Cub and LeAMT1;2-Cub (Fig.3.2A and B) did not reveal any interactions with the co-expressed prey fusions LeAMT1;1, 1;2 and 1;3 and other control membrane proteins, while LeAMT1;3-Cub (Fig. 3.2C) generated high level of background signals. A NubWT fusion with the glucose transporter (pNubWT-GLU) serving as a positive control did not show the expected interaction with the bait Cub fusions, indicating that the expression of LeAMT1;1-Cub and LeAMT1;2 might be too low (Fig. 3.2). A similar result was also encountered when using the same system to test the interaction between ScMEP proteins, the yeast homologs of the AMTs (Andre, B. personal communication).

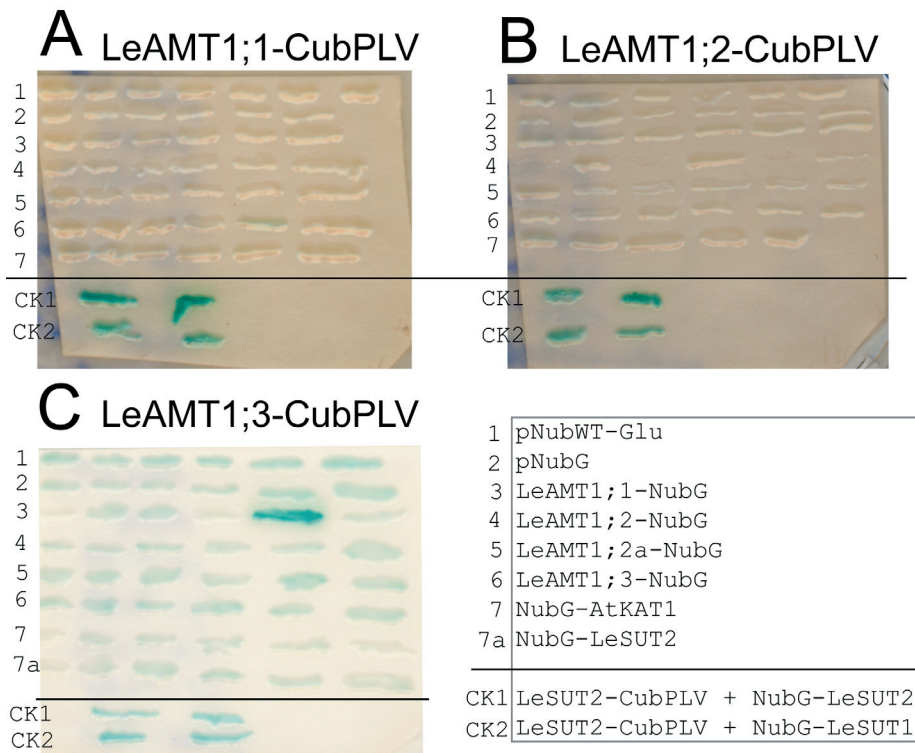


Fig. 3.2 Measuring protein-protein interactions between LeAMTs with the original ubiquitin system. The CubPLV fusions of LeAMT1;1 (**A**), LeAMT1;2 (**B**) or LeAMT1;3 (**C**) under the control of Wbp1 promoter in the vector pY-CubPLV were co-expressed in the L40 yeast strain with different NubG fusions as numbered and shown in the lower-right panel. Among the prey-proteins are glucose transporter (Glu), ammonium transporters (LeAMT1;1, LeAMT1;2 and LeAMT1;3), potassium channel (AtKAT1) and sucrose transporters (LeSUT1 and LeSUT2). LeSUT2 forms oligomer and interacts with LeSUT1, thus cells expressing LeSUT2-CubPLV together with NubG-LeSUT2 (CK1) or with NubG-LeSUT1 (CK2) served as positive controls. Yeast cells harboring different combinations of bait and prey were streaked on filter papers placed on top of agar medium with SD-leu-trp and grown for 4 days.

In order to tackle the problem of bait expression, a newly developed mating-based split ubiquitin system (Obrdlík, *et al.* to be published) was adopted. In this improved system, bait fusions are expressed in a CEN/ARS vector and driven by a strong MET25 promoter that can be repressed by adding various amount of methionine to the growth medium, thus allowing fine-tuning of the expression levels of bait fusions. The reporter strain THY.AP5 contains one more reporter gene Ade2, in addition to His3 and lacZ in the original strain L40, which allows counter-selection based on the red colour of yeast colony lacking the activity of the Ade2 gene.

With this improved system, a weak but significant self-interaction of LeAMT1;1 was detected. In a growth assay using both reporters His3 and Ade2, cells coexpressing LeAMT1;1-Cub and LeAMT1;1-NubG showed significant growth over those coexpressing LeAMT1;1-Cub and the NubG, indicating the activations of His3 and Ade2 reporters by self-interaction of LeAMT1;1 (Fig. 3.3). LeAMT1;1 did not interact with other plasma membrane proteins, such as the potassium channel AtKAT1 or the sucrose transporter AtSUC2. As expected, self-interactions of AtKAT1, a shaker-like K⁺ channel known to form tetramers (Daram *et al.* 1997; Pilot *et al.* 2001) was also observed. Since the NubWT does not harbor a mutation, it interacts strongly with the Cub. Thus, soluble NubWT and LeAMT1;1 fused to NubWT served as positive controls.

Moreover, NubG fusion with a mutant form of the LeAMT1;1 (designated as

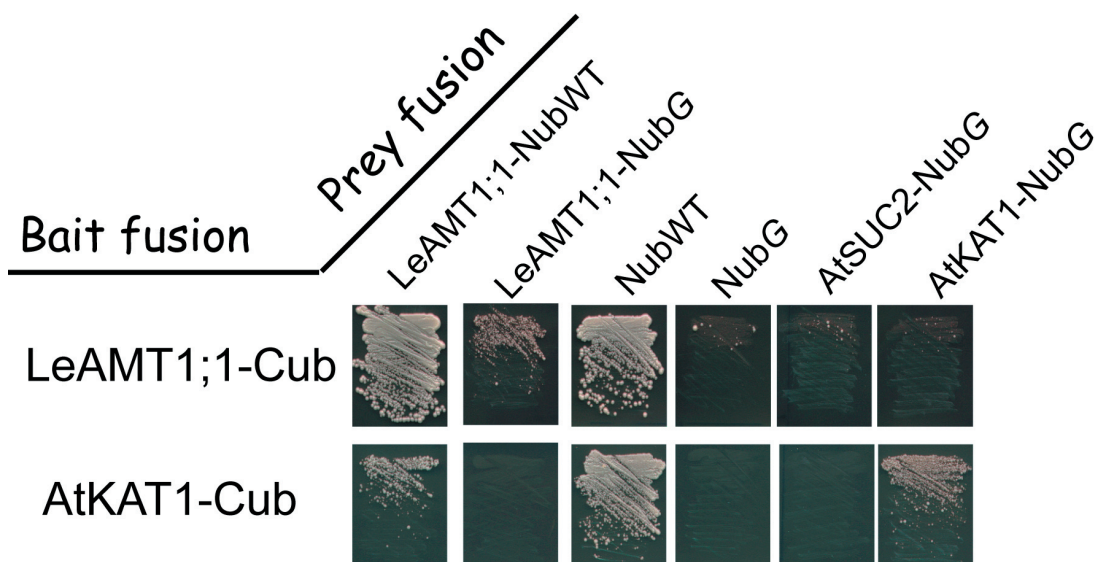


Fig. 3.3 Self-interaction of LeAMT1;1 detected in the improved split ubiquitin system. The bait fusions were expressed under the control of MET25 promoter in the vector pmetYCGate. Interactions among different NubG, NubWT, and Cub fusions were monitored via growth assay on a synthetic minimal medium lacking adenine and histidine but supplemented with 0.075 mM methionine. The empty Cub vector was used as a negative control. The potassium channel (AtKAT1) forms tetramer thus used as a positive control here.

The inability of the NubG fusion of the C-terminus truncated LeAMT1;1 (LeAMT1;1 Δ C45-NubG) to interact with the LeAMT1;1-Cub fusion gave the impression that the cytoplasmic C-terminal tail might be crucial for self-interaction of the LeAMT1;1. To test this the cytoplasmic C-terminal fragment containing the last 45 amino acids (C45) of LeAMT1;1 was used as both bait and prey in a yeast two-hybrid assay. No self-interaction was observed after coexpression of the C45 fusions with both the GAL4 DNA binding domain (bait fusion) and DNA activation domain (prey fusion) in the reporter strain AH109 (Fig. 3.5), indicating that the cytoplasmic C-terminus does not contain an interaction domain.

Using LeAMT1;1 as a bait protein, no interactions were observed between LeAMT1;1 and LeAMT1;2 or LeAMT1;3 (Fig. 3.4).

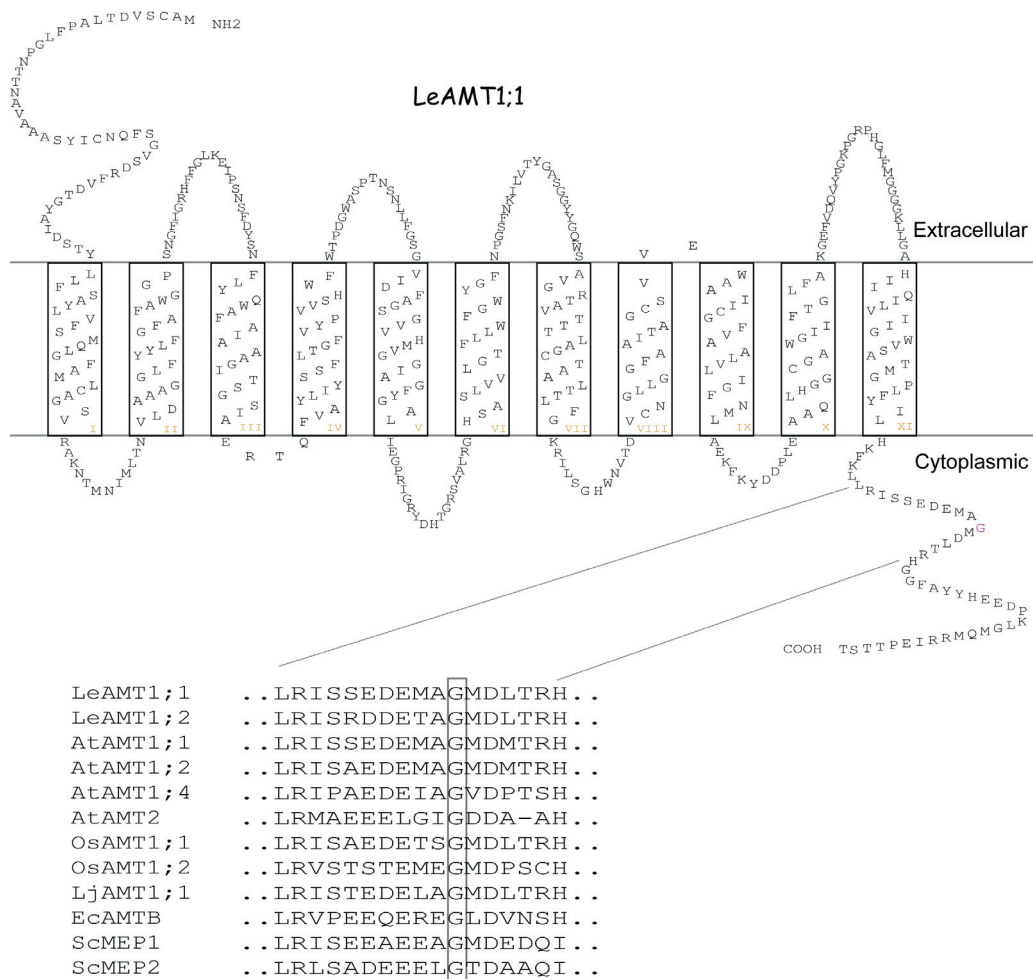


Fig. 3.6 LeAMT1;1 membrane topology and structure based on hydrophathy analysis and prediction by the TMHMM algorithm (<http://www.cbs.dtu.dk/services/TMHMM/>). The C-terminal region with the conserved motif and the glycine residue is shown below.

3.2. Despite functional defect, plasma membrane localization of the point mutant LeAMT1;1G458D

The amino acid sequence EDEMAGMDMTR in the cytoplasmic C-terminus of LeAMT1;1 represents a highly conserved motif among ammonium transporters from bacteria, fungi, and plants (Fig. 3.6). The function of this conserved motif remains unclear, despite the known dominant-negative effects of a single amino acid substitution from the conserved glycine to aspartate, which results in a strong reduction of ammonium transport activities (Marini *et al.* 2000; Monahan *et al.* 2002). To test whether the corresponding mutation in the plant ammonium transporter LeAMT1;1 has a similar effect, wild type and mutant (G458D) genes were expressed in the ammonium uptake-defective yeast strain 31019b. Although wild type LeAMT1;1 restored the growth defect of 31019b on medium containing ammonium as the sole nitrogen source, the point mutant (LeAMT1;1G458D) was unable to mediate efficient uptake, nor was the C-terminus-truncated mutant (LeAMT1;1 Δ C45) (Fig. 3.7).

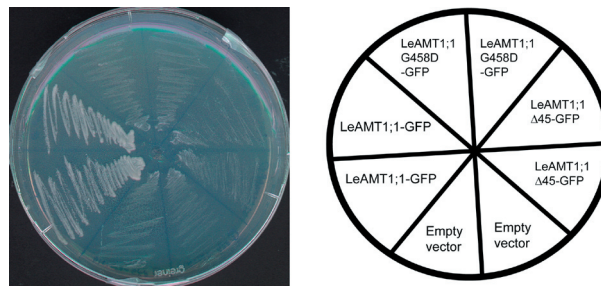


Fig. 3.7 Complementation of yeast null strain 31019b defective in ammonium-dependent growth with the expression of LeAMT1;1 and its point mutant or truncation mutant. Yeast strain 31019b (MATa ura3 mep1D mep2D::LEU2 mep3D::kanMX2) was transformed with 2 μ plasmid pDR196 harboring different constructs: LeAMT1;1-GFPs65t, LeAMT1;1G458D-GFPs65t and LeAMT1;1 Δ C45-GFPs65t, respectively. Isolated colonies were re-streaked out on fresh plate of YNB-Glc medium containing 1mM ammonium as the sole N source and grown for 4 days.

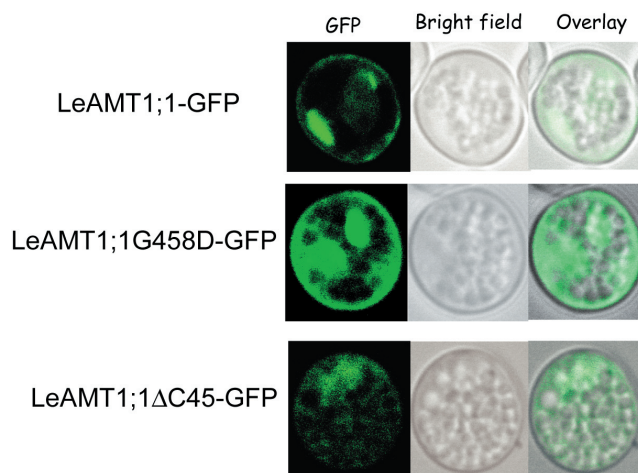


Fig. 3.8 Subcellular localization of GFP-fusions of LeAMT1;1 and its point mutant G458D and truncation mutant Δ C45 in yeast strain 31019b. Cells expressing GFP-fusions of LeAMT1;1, LeAMT1;1G458D and LeAMT1;1 Δ C45 were examined using a Leica confocal microscope system. Representative images are shown.

In order to see if the uptake deficiency of the G458D and the $\Delta C45$ mutants may be caused by improper targeting of the respective proteins to the plasma membrane, both mutants, together with the wild-type, were fused at the C-terminus with GFP and expressed in the yeast strain 31019b. GFP fusions of wild type and the point mutant G458D showed similar localization, while GFP fusion of the truncated mutant yielded very weak fluorescence all over the cytoplasm of the cell, indicating inappropriate expressions or mistargeting of the truncated forms (Fig. 3.8).

To further examine the localization in plant cells, the GFP fusion constructs were transiently expressed in tobacco BY2 and *Arabidopsis* protoplasts. Analysis of 10 randomly selected cells of BY2 and 70 cells of *Arabidopsis* protoplasts from over 10 different samples for each construct revealed that both wild type and point mutant G458D GFP fusions localized predominantly as a peripheral ring consistent with plasma membrane localization (Fig. 3.9). Although the highest fluorescent expression with both wild type and point mutant G458D GFP fusions was observed in 16-18 hours after transformation, fluorescent cells expressing the truncation mutant LeAMT1; $\Delta C45$ -GFP were rarely found even in a prolonged sampling time from 12-20 hours after transformation and only a few weak fluorescent cells were observed (Fig. 3.9).

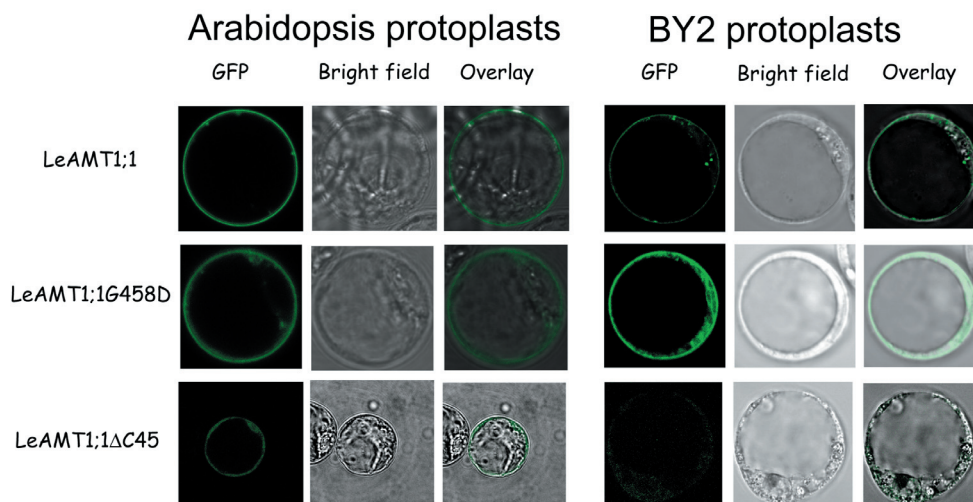


Fig. 3.9 Subcellular localization of GFP-fusions of LeAMT1;1, LeAMT1;1G458D and LeAMT1; $\Delta C45$ in plant protoplasts. Protoplasts of *Arabidopsis* and tobacco BY2 were transformed with plasmids harboring different constructs for transient expression of the respective GFP-fusions. Fluorescent cells were examined 12 hours after transformation and the representative images are shown here.

3.3. Cross-inhibitions of transport activities by mutant LeAMT1;1G458D and LeAMT1;2G465D in oocytes

3.3.1. Both mutant LeAMT1;1G458D and LeAMT1;2G465D are not functional in oocytes

Using the *Xenopus* oocyte heterologous expression system, it had been demonstrated that LeAMT1;1 mediated potential-driven NH_4^+ uptake and retrieval depend on membrane potential and NH_4^+ concentration gradient (Ludewig *et al.* 2002). Similarly, LeAMT1;2 is also characterized as a voltage-dependent, pH-independent NH_4^+ specific uniporter with a 6-fold lower affinity than LeAMT1;1 in oocytes (Ludewig *et al.* 2003).

To analyze the effect of the point mutation in LeAMTs on the protein functionality in more detail, mutant forms of LeAMT1;1 and LeAMT1;2 were generated and subcloned into the oocyte expression vector. The corresponding point mutant in LeAMT1;2 is G465D, while in LeAMT1;1 it is G458D.

Both LeAMT1;1 and LeAMT1;2 and their respective mutants G458D and G465D were expressed in parallel in oocytes. NH_4^+ induced significant currents in both LeAMT1;1- and LeAMT1;2 expressing oocytes, whereas the mutants permitted no detectable ammonium-induced currents above background when expressed at different cRNA concentrations (n=3 batches of oocytes). Thus, both mutants of the LeAMT1;1G458D and LeAMT1;2G465D were fully inactive in oocytes (Fig. 3.10).

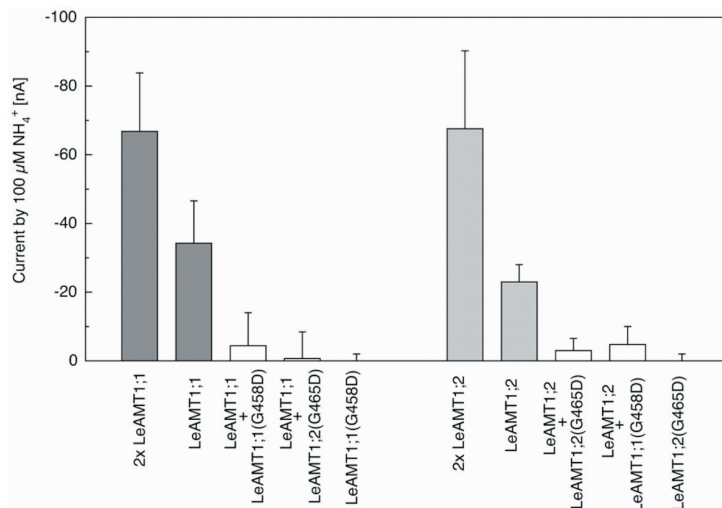


Fig. 3.10 NH_4^+ -induced currents in oocytes expressing LeAMT1;1, LeAMT1;2 or their point mutants, or co-expressing wild type and mutant form of the LeAMTs. Each individual bar represents currents recorded from a set of more than 5 oocytes from the same frog to minimize variability due to different expression levels. Experiments were repeated on at least six different batches of oocytes.

3.3.2. Specific inhibition of LeAMT1;1 and LeAMT1;2 activities by their point mutants

The mutated plant transporter was then used as a tool to study whether AMT^T transporters exist as oligomers in a heterologous system, which might lack other proteins potentially required to mediate interaction between ammonium transporters. To be able to evaluate possible interactions between mutant and wild type ammonium transporters, it was necessary to measure transport under conditions in which expression levels of the transporters correlate with activity. Increasing amounts of cRNA injected per oocyte led to larger ammonium currents, but the maximum current obtained saturated with ≈ 50 ng cRNA. Such saturation is a typical feature of oocyte expression of a variety of channels and transporters (e.g. Steinmeyer *et al.* 1994).

At low amounts of cRNA injected per oocyte (≈ 6 – 12 ng), ammonium-induced currents increased linearly with the amount of injected cRNA (Fig. 3.10). The linear correlation between functional LeAMT1;1 and LeAMT1;2-mediated ammonium transport and cRNA concentration (Fig. 3.10) allowed direct quantitative analysis of co-expressed mutant and wild type proteins in oocytes. Co-expression of equal amounts of wild type and mutant cRNA led to a dramatic decrease of ammonium transport to $\approx 12\%$ for LeAMT1;1 and $\approx 10\%$ for LeAMT1;2 (Fig. 3.10). Protein biosynthesis was not saturated in oocytes, as twice the amount of wild type LeAMT1;1 or LeAMT1;2 led to a doubling of ammonium currents (Fig. 3.10). Lower amounts of co-injected mutant cRNA led to lower inhibition of wild type dependent ammonium transport (Data not shown).

To exclude the possibility that the inhibition was due to indirect effects, the LeAMT1;1 mutant G458D was co-expressed with the structurally unrelated amino acid transporter AtAAP6 (Fischer *et al.* 2002). Co-expression of equal amounts of mutant LeAMT1;1 cRNA with AtAAP6 did not influence amino acid currents (Data not shown).

Thus, both non-functional LeAMT1;1-G458D and LeAMT1;2G465D mutant protein specifically inhibited the ammonium conductance of their respective wild-type protein.

3.3.3. Cross-inhibition of LeAMT1;1 and LeAMT1;2 by their point mutants

Furthermore, cross-inhibitory effects on the activities of the wild-type protein by the mutants LeAMT1;1G458D or LeAMT1;2G465D were observed. The LeAMT1;1 mutant G458D, when co-expressed with LeAMT1;2 wild type, reduced LeAMT1;2 currents by $\sim 85\%$ (Fig. 3.10). Conversely, the corresponding mutant in LeAMT1;2 (G465D) reduced LeAMT1;1 wild type activity by $\sim 90\%$. The magnitude of dominant negative inhibition was similar for both pairs of constructs, indicating that mutant polypeptides indiscriminately interacted with either LeAMT1;1 or LeAMT1;2 in oocytes (Fig. 3.10).

3.4. Characterizations of invertase-fusions with membrane protein expressed in yeast

The hetero-oligomeric interaction between LeAMT1;1 and LeAMT1;2 was firmly demonstrated in the oocyte expression system but could not be detected in the split ubiquitin system due to the low signal-to-noise ratio associated with the assay. This prompted us to develop a new yeast-based system, which would be robust enough for monitoring membrane protein interactions. The yeast invertase, encoded by the ScSUC2 gene, was chosen because it was experimentally shown that co-expression of its two split fragments, each containing a secretory signal peptide, led to the reconstitution of enzymatically functional proteins in yeast periplasm (Schonberger, 1996). Similar to the split ubiquitin system, split protein fragments reconstitution had also been found experimentally in many other proteins, e.g. dihydrofolate reductase (DHFR) (Remy *et al.* 1999), beta-galactosidase (Rossi *et al.* 2000), luciferase (Ozawa *et al.* 2001) and beta-lactamase (Wehrman *et al.* 2002), but invertase is unique since it is targeted by a signal peptide to the secretory pathway and functions only in the periplasm where it cleaves sucrose into glucose and fructose, thus enabling yeast to grow on medium containing sucrose as the sole carbon source. This unique feature was utilized in a genetic screen system for cloning secretory or membrane proteins, where a cDNA library was cloned in fusions with the yeast invertase without its indigenous signal peptide and expressed in a yeast *suc2* deletion mutant (Klein *et al.* 1996; Jacobs *et al.* 1997). Therefore, invertase is a favorite tag of membrane or secretory proteins, especially suitable for membrane proteins with extracellular terminus such as LeAMT proteins that possess a predicted extracellular N-terminus (Fig. 3.6).

Yeast invertase exists in two forms: secreted invertase and non-secreted invertase. The secreted form contains at its N-terminal a sequence of 20 amino acids, functioning as a cleavable secretory signal sequence, while the non-secreted form lacks this signal sequence, thus remains intracellular. Both forms are transcribed from the genomic ScSUC2 gene. The ScSUC2 gene could be truncated to discard the signal sequence for artificial expression of the non-secreted invertase, designated as full-length invertase. The N-terminal sequence for the first 377 amino acids of the full-length invertase is designated as a N377 fragment, while the last 211 or 135 amino acid sequence from the C-terminus of the full-length invertase are designated as C211 or C135 fragment, respectively.

To test whether a non-secreted form of full length invertase can be targeted to the periplasm and can function in yeast as a fusion with a membrane protein like LeAMT1;1, several invertase fusion constructs were made for expression under the control of the MET25 promoter in a yeast 2 μ vector, and expressed in the yeast strain YSH2.64 with deleted endogenous *suc2* gene. Since the N-terminus of the LeAMTs was predicted to be extracellular, invertase was fused at the N-terminus of LeAMT1;1 and LeAMT1;2. ScSTE2 encodes the yeast α -factor receptor, a homo-oligomeric seven-transmembrane protein having a pre-

dicted extracellular N-terminus (David, et al. 1997; Yesilaltay et al. 2000). The hTfR gene from human encodes the transferrin receptor, which is a homodimeric type II membrane glycoprotein containing one transmembrane segment with its C-terminus outside the cell (Zerial et al. 1986; Terng et al. 1998). These two proteins of known topology were used as controls and the invertase was fused to the N-terminus of ScSTE2 and the C-terminus of hTfR, respectively, so that the invertase moieties could be extracellular when the fusions were expressed.

As expected, yeast cells expressing the ScSUC2 gene and the invertase-fusion of LeAMT1;1 or ScSTE2 were able to grow on medium with sucrose as the sole carbon source, while those expressing non-secreted form and the N-terminus fragment (N377) of the invertase permitted no growth (Fig. 3.11). The visible sucrose-dependent growth was consistent with the invertase activities detected using whole cell and lysed cell assay. Whole cell assay enables the measurement of invertase activities on the cell surface, while the lysed-cell invertase activities are the total activities contributed by both the cell-surface and the intercellular invertase. Cell-surface invertase activities produced by cells expressing LeAMT1;1- or

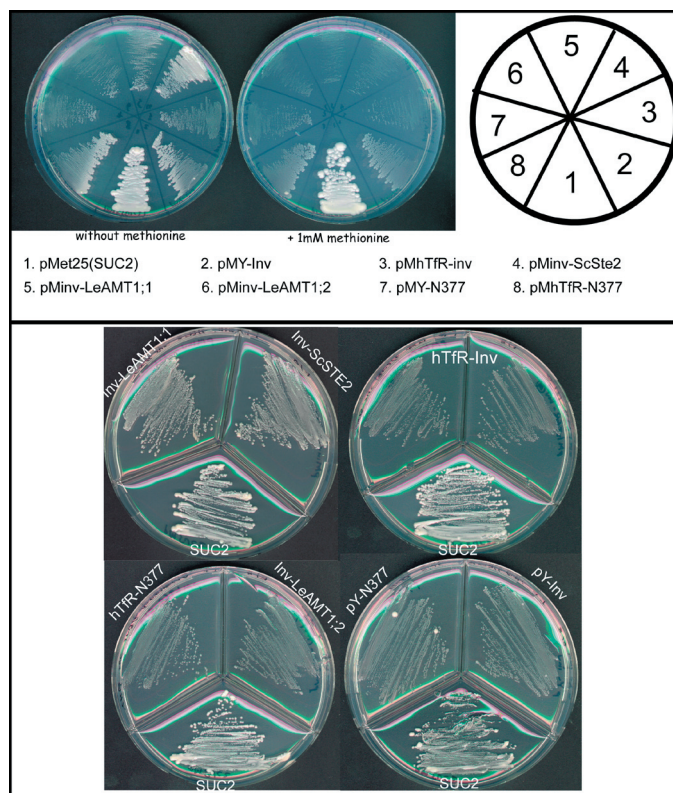


Fig. 3.11 Complementation of sucrose-dependent growth in yeast strain YSH2.64 expressing the secreted invertase (SUC2 gene) and invertase fusion with LeAMT1;1 or ScSTE2 under the control of the repressible Met25 promoter. Single colony was re-streaked on agarose plates containing yeast nitrogen base plus 2% sucrose, 2mg/L Antimycin A and tryptophan and with/without methionine, grown at 28°C for 8 days (**upper panel**) or 16 days (**lower panel**). Longer incubation results in non-specific background growth (lower panel).

ScSTE2-invertase fusion were significantly higher than those produced by the mother strain YSH2.64 or by cells expressing the non-secreted form or fragments (N377) of the invertase, although these activities accounted for only 4% or 20% of their total activities, respectively (Fig. 3.12). Cells expressing wild-type ScSUC2 produced the highest invertase activities, which were found exclusively as a secreted form (Fig. 3.12) and such secreted enzyme cleaved the sucrose in solid medium with very high efficiency, causing a slightly unspecific growth of the neighboring cells on both sides expressing non-secreted invertase and N-fragment invertase (N377)-hTfR fusion (Fig. 3.11, upper panel).

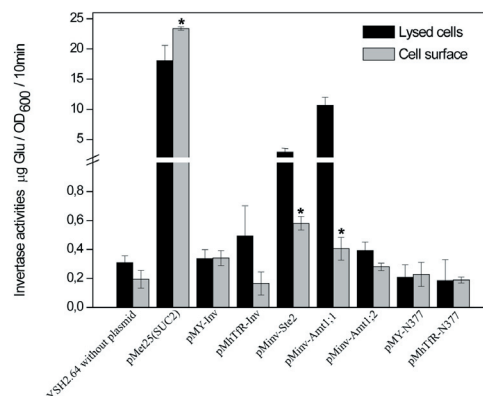
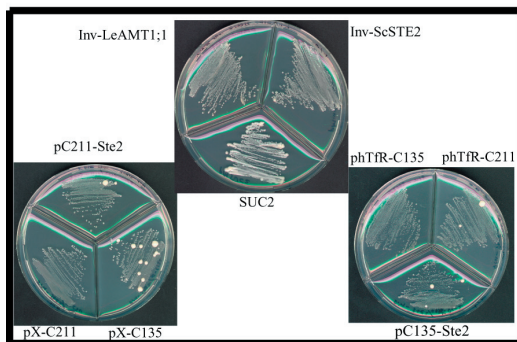


Fig. 3.12 Invertase activities in yeast strain YSH2.64 harboring various plasmids. Enzyme activity was determined as described in Materials and Methods. Data are the average of three independent assays from the same transformants.

A Sucrose-dependent growth assay



B Yeast cell invertase activity assay

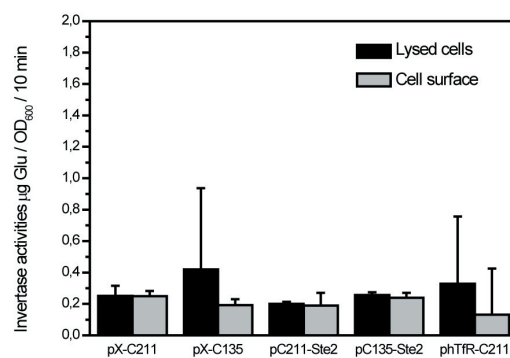


Fig.3.13 Complementation assay of sucrose-dependent growth in yeast strain YSH2.64 co-expressing the invertase fragment fusions, N377-ScSTE2 with either C135-ScSTE2 or C211-ScSTE2, and the corresponding enzymatic activity. **A**, Yeast cells were grown for 16 days on medium containing yeast nitrogen base plus 2% sucrose and 2 mg/L Antimycin A, this prolonged incubation led to non-specific background growth. Cells expressing SUC2 and invertase fusions of LeAMT1;1 and ScSTE2 used as positive control (the upper plate). **B**, Invertase activities were determined on cells coexpressing N377-ScSTE2 fusion and different fusions of C135 or C211 as described in Materials and Methods.

The invertase fusions of LeAMT1;2 and hTfR were unable to complement the yeast growth on sucrose medium (Fig. 3.11), because the detected invertase activities in lysed cells harboring these constructs were not significant as compared to that from the control cells without any plasmids (Fig. 3.12).

To test whether split fragments of the invertase were able to rejoin into its functional complexes *in vivo* when fused with two interacting proteins, the N-terminal fragment of the invertase (N377) was fused to the N-terminus of the ScSTE2 and co-expressed with different ScSTE2 constructs fused to C-terminal fragment C211 or C135, respectively. As shown in Fig. 3.13, neither growth nor invertase activity assays demonstrate functional reconstitution of the split fragments. An additional attempt using the hTfR as a fusion partner was also unsuccessful (data not shown). We do not have explanation for such unexpected results; however, a redesign of the two split fragments based on the protein structural information of the invertase might be helpful for a successful “split invertase system” in the future.

3.5. Transgenic tomato plants overexpressing the mutant LeAMT1;1G458D

The dominant negative effect of the point mutant LeAMT1;1G458D observed in oocytes makes it interesting to analyze *in planta* the effects of this mutation on ammonium uptake, nitrogen metabolism and growth characteristics. Hence, the mutant LeAMT1;1G458D, along with the LeAMT1;1, was subcloned into a binary vector pJH212gck for 35S promoter-driven overexpression in plants and these constructs were used to transform *Lycopersicon esculentum* M. cv. Moneymaker plants by *Agrobacterium*-mediated transformation.



Fig. 3.14 Transgenic tomato plants over-expressing LeAMT1;1 and LeAMT1;1G458D. Picture was taken after three months growth in the greenhouse of the regenerated plantlets. **Left**, wild type plant (*L. esculentum* M. var. moneymaker); **Middle**, transgenic plant of LeAMT1;1G458D; **Right**, transgenic plant of LeAMT1;1.

Using 1000 tomato cotyledons as explants, a calculated transformation efficiency of 10% was achieved based, on a PCR screen of the regenerated kanamycin-resistant shoots using NPTII-specific primers. After three month on MS rooting medium containing claforan, the rooted plantlets were gradually transferred into the greenhouse. The regenerated transgenic plants over-expressing LeAMT1;1 or the mutant LeAMT1;1G458D show no obvious difference under normal growth conditions. Both exhibit a wild type phenotype (Fig. 3.14).

3.6. Yeast two-hybrid screens for putative LeAMT1;1 interaction partners

As described in Section 3.1 (Fig. 3.5) and 3.3, the C-terminal cytoplasmic tail of the LeAMT1;1 was unlikely to be the site for homo-oligomeric interaction of the LeAMT1;1, but rather it might be a regulatory site for the transport activities or a signal transduction site. In fact, the bacterial homolog AmtB was demonstrated to interact with a nitrogen signal transduction protein PII at its cytoplasmic C-terminus, which was 32 amino acids in length. However, no PII homolog is found in the yeast genome and the plant homolog encodes a plastid protein unlikely to be an interacting partner of LeAMT1;1 (Hsieh et al. 1998; Moorhead and Smith 2003). Therefore, we sought to identify putative plant proteins that might interact with the C-terminal cytoplasmic tail of the LeAMT1;1 by using the yeast two-hybrid screens.

Since no self-interaction of the C-terminal cytoplasmic fragment (C45) of LeAMT1;1 was observed in the yeast two-hybrid system (Fig. 3.5), there is a possibility that it interacts with a membrane protein, in which case the interaction would not be detected by the yeast two hybrid system. Therefore, the subcellular localization of this fragment was first examined in *Arabidopsis* protoplasts with a GFP-fusion. GFP fluorescence imaging indicated a typical cytosolic distribution of C45, similar to cells expressing the cytosolic GFP proteins (Fig. 3.15).

The C45 fragment was then subcloned into the vector pGBKT7 fused to the GAL4-DNA binding domain (BD) and the resulting bait plasmid was used for screening of the cDNA library of GAL4-DNA activation domain (AD) fusion from *Arabidopsis* and from tomato, respectively.

The screening of the *Arabidopsis* cDNA library was done by mating of the yeast strain Y187 harboring the C45-BD fusion and the strain AH109 harboring cDNA-AD fusion library (kindly provided by Drs. Ulrike Zentgraf and Ying Miao). Diploid cells able to grow on medium lacking leucine, tryptophan, histidine and adenine were selected. In the first-round screen a total of 31 positive clones were isolated, of which 28 clones were identical, harboring the same cDNA. Another 2 belonged to a single clone. Thus, only 3 different

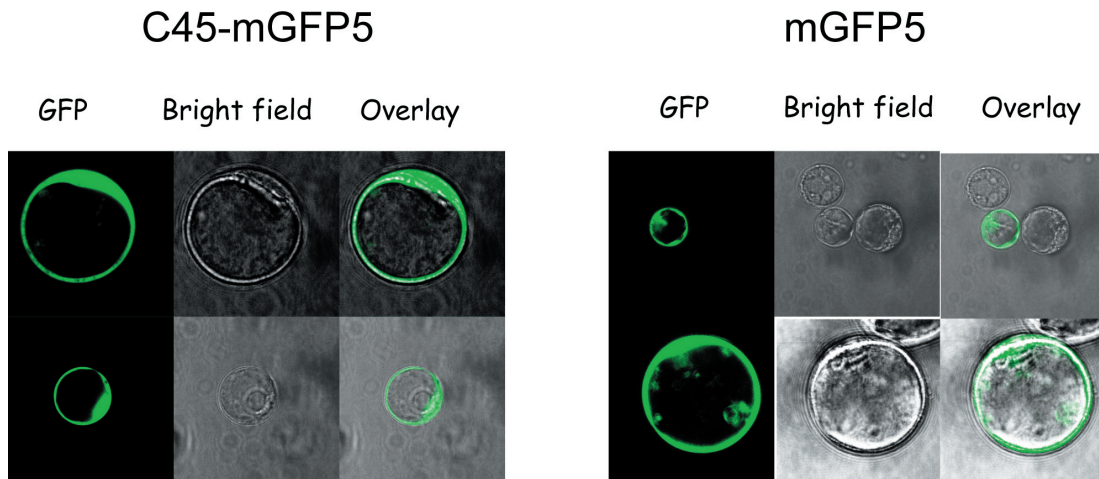


Fig. 3.15 Cytoplasmic localization of the GFP fusion with the C-terminal fragment C45 of LeAMT1;1 in *Arabidopsis* protoplasts. Right panel was the control expressing the GFP alone without any fusion.

clones were actually isolated. Sequencing of these led to the identification of glyceraldehyde-3-phosphate dehydrogenase (At1g42970), gamma-glutamyltransferase (At4g39640) and acetyl-CoA carboxylase (At1g36180). Therefore the first screening was considered unsuccessful, probably due to a low mating efficiency (we did not measure). Further screening with optimized mating efficiency is needed.

Screening of the tomato cDNA library was performed by cotransformation of the yeast strain AH109 with the C45-BD plasmid and the cDNA-AD library plasmids (kindly provided by Rama Panford-Walsh) and isolation of positive colonies on selection medium lacking leucine, tryptophan, histidine and adenine. In the first-round screen, the cotransformation efficiency was approximately 3×10^4 cfu/ μ g DNA, with a total of 40 μ g library cDNA used in the cotransformation. Thus, approximately 1.2×10^6 transformants were screened.

Seven to fourteen days after transformation, approximately 1000 positive colonies of various growing sizes were seen on selection plates and 114 were selected and plasmids isolated. 44 cDNA inserts of the isolated plasmids were sequenced and blast search was done against the GeneBank and the database provide by the Solanaceae Genomics Network. The identified clones included phosphoinositide-specific phospholipase C (3 independent clones), SNF1 (2 independent clones) and pT53 wound-repressed gene product (2 independent clones) (Table 3.1).

Since artifices sometimes arise during a yeast two-hybrid library screen, two isolated clones Le013 and Le018 (Table 3.1) were used in a re-transformation experiment, together with the bait plasmid of BD-C45 fusion or control plasmid of BD alone, to verify the interactions between the C45 and the PI-PLC fragments. In the yeast reporter strain AH109,

co-expression of Le013 or Le018 with the BD-C45 fusion resulted in the activation of the reporter LacZ, giving a positive signal in a beta-galactosidase activity assay, whereas the co-expression of Le013 or Le018 with the BD alone yielded no positive signal in the beta-Gal assay (Fig. 3.16). These results confirm the interactions between the BD-C45 fusion and the AD-fusions of PI-PLC fragments encoded by the isolated cDNAs.

Table 3.1 Identifications of 44 positive clones from a Y2H screen of tomato cDNA-AD library

Clone #	Insert length (c.a. bp by PCR)	BlastN / SGN-EST	ACCESSION	E
Le043	500	S.tuberosum mRNA for phosphoinositide-specific phospholipase C (1464-1679)	X94289.1 STPLC3	2e-97
Le042	650	S.tuberosum mRNA for phosphoinositide-specific phospholipase C(1464-2006)	X94289.1 STPLC3	e-109
Le013	500	S.tuberosum mRNA for phosphoinositide-specific phospholipase C (1464-2006)	X94289.1 STPLC3	e-111
Le018	700	S.tuberosum mRNA for phosphoinositide-specific phospholipase C (1431-1680)	X94289.1 STPLC3	e-112
Le028.1	750	S.tuberosum mRNA for phosphoinositide-specific phospholipase C (1563-1870)	X94289.1 STPLC3	e-124
Le026	850	S.tuberosum mRNA for phosphoinositide-specific phospholipase C (1563-1917)	X94289.1 STPLC3	e-155
Le027.1	600	Lycopersicon esculentum SNF1 (SNF1) mRNA (1030-1776)	AF143743.1	0.0
Le023	350	Lycopersicon esculentum SNF1 (SNF1) mRNA (1030-1278)	AF143743.1	e-109
Le046	700	L.esculentum pT53 mRNA for wound-repressed gene (19-446)	X59883.1 LEPT53	3e-63
Le011	500	L.esculentum pT53 mRNA for wound-repressed gene (25-300)	X59883.1 LEPT53	e-131
Le029.1	300	L.esculentum mRNA for cell wall protein (373-512)	X77373.1 LECEWAPR	1e-69
Le010	150	L.esculentum mRNA for cell wall protein (373-512)	X77373.1 LECEWAPR	1e-69
Le032	700	Tomato chlorophyll a/b-binding protein gene Cab-3C	M14444.1 TOMCBPB	0.0
Le025	600	Lycopersicon esculentum glutamate 1-semialdehyde 2,1-aminomutase mRNA (1331-1620)	L39279.1 TOMGSAAM	e-144

Le017.1	750	<i>Lycopersicon esculentum</i> cystathionine gamma synthase (816-935)	AY508112.1	3e-67
Le021	600	<i>Lycopersicon esculentum</i> plastid mRNA for superoxide dismutase [Fe] (sodb gene) (709-929)	AJ579656.1 LES579656	e-108
Le035	450	<i>Nicotiana tabacum</i> cDNA-AFLP-fragment BT2-M33-00	NTA538899	2e-31
Le012	700	<i>Solanum tuberosum</i> PCS-1 mRNA for cytosolic cysteine synthase	AB029511	4e-30
Le014.2	400	<i>Arabidopsis thaliana</i> expressed protein (At2g29660)	NM_128520.2	4e-14
Le006	600	<i>Oryza sativa</i> (japonica cultivar-group) cDNA clone:001-102-A03 <i>Oryza sativa</i> (japonica cultivar-group) putative cyanase (OSJNBa0079L16.19)		1e-18 2e-17
Le034	750	<i>Arabidopsis thaliana</i> cyanate lyase (CYN) (At3g23490)	NM_113252	5e-18
Le019	700	<i>Vitis vinifera</i> samdc gene for S-adenosylmethionine decarboxylase	AJ567368.1 VVI567368	4e-08
Le033.1	750	<i>Vitis vinifera</i> samdc gene for S-adenosylmethionine decarboxylase (993-1093)	AJ567368.1 VVI567368	6e-08
Le049	650	<i>Arabidopsis thaliana</i> putative cytoplasmic ribosomal protein S15a(At3g46040)	AY091373	5e-12
Le015	300	<i>Arabidopsis thaliana</i> myb family transcription factor (At1g74430)	NM_106103.2	0.12
Le005	700	<i>Arabidopsis thaliana</i> myb family transcription factor (At1g74430)		0.17
Le008	650	<i>Arabidopsis thaliana</i> myb family transcription factor (At1g74430)		0.22
Le036	400	Cloning vector pSG925, HIS4-based plasmid		
Le003	900	no results / ribosomal protein		
Le004	300	no results / unknown protein		
Le007	400	no results / hypothetical protein		
Le009	650	no results / cyanate lyase		
Le020	400	no results / expressed protein		
Le022	750	no results / pathogenesis-related protein Bet VI family		
Le024	400	no results / unknown protein (same as Le004)		

Le030	800	no results / ribosomal protein		
Le031	800	no results / expressed protein		
Le038	600	no results / wound-induced protein Sn-1 vacuolar membrane		
Le054	650	no results / wound-induced protein Sn-1 vacuolar membrane		
Le039	2700	sequencing fail		
Le050	700	sequencing fail		
Le056.1	500	sequencing fail		
Le002.1	100	too short		
Le016	1200	Vector		

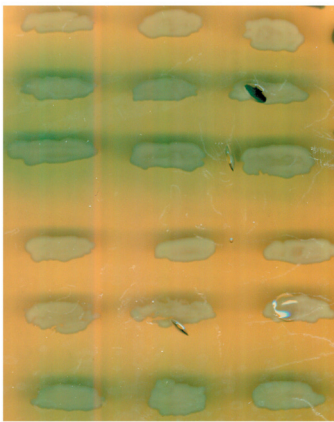
Bait fusion		Prey fusion
DNA BD-C45		DNA AD
DNA BD-C45		DNA AD-Le013
DNA BD-C45		DNA AD-Le018
DNA BD		DNA AD-Le013
DNA BD		DNA AD-Le018
DNA BD-murine p53		DNA AD-T-antigen of SV40

Fig 3.16 The cytoplasmic C-terminal fragment of LeAMT1;1, C45, interacts with cDNAs coding for polypeptides homology to the C2 domain of a plant phosphoinositide-specific phospholipase C (PI-PLC) in a yeast two hybrid assay. Le013 and Le018 are two independent clones isolated by a yeast two hybrid library screen, corresponding to the last 108 and 119 amino acid residues of the homologous potato StPLC3 (Kopka et al. 1998), respectively. Thees two cDNA containing plasmids were used in combination with various bait plasmids for re-transformation of yeast reporter strain AH109. Transformants harboring various bait/prey combinations were re-straked on fresh plates with SD-trp-leu medium and grown for 4 days at 28C. The reporter alpha-galactosidase activity was tested by using overlay agarose containing 24 µg/ml X-alpha-Gal and incubating for 10 hours at 30°C. Murine p53 and SV40 T antigen are known interacting proteins and served as a positive control here.

4. Discussion

4.1. The dominant negative effect of the G458D mutation of LeAMT1;1

A dominant-negative point mutation at the conserved cytoplasmic C-termini of the LeAMT proteins (Fig. 3.6), which corresponds to the reported inhibitory mep1-1 mutation in the yeast ammonium transporter Mep1p, was generated and characterized. This mutation is caused by an amino acid substitution from the conserved glycine to aspartate, and results in non-functional mep1 which trans-inhibits the activities of both Mep1 and Mep2 proteins at the post-transcriptional level (Marini *et al.* 2000). The corresponding mutants of LeAMT1;1 and LeAMT1;2 were non-functional in *Xenopus* oocytes and specifically reduced the activities of co-expressed wild-type LeAMT proteins in a dominant negative manner (Fig. 3.10). When tested in yeast, the LeAMT1;1G458D was also unable to complement the growth defect of the triple null mutant 31019b (Fig. 3.7). These results indicate the importance of the conserved cytoplasmic C-terminal motif in mediating the transport activities of Amt proteins. Moreover, this mutation defect in transport function was not caused by targeting failure since GFP-fusions expressed in plant protoplasts and in yeast showed that, unlike the C-terminal truncated mutant, this mutation did not impair the subcellular localization of the transporter (Fig 3.8 and 3.9). In addition, the mutant and wild type forms of LeAMT1;1 interacted with each other in a split ubiquitin assay (Fig. 3.4). Taken together, these results may suggest that oligomerization of the transporter proteins is necessary for maintaining an efficient transport of NH_4^+ across the plasma membrane. A dysfunctional subunit in a transporter complex would reduce its total activity (Fig. 3.10). A dominant negative effect resulting from oligomeric interaction between mutant and wild type subunits in a protein complex is also observed in other membrane proteins such as *Helicobacter pylori* vacuolating toxin VacA (Vinion-Dubiel *et al.* 1999), *Bacillus anthracis* protective antigen PA63 (Singh *et al.* 2001), inward rectifying renal K^+ channel Kir 1.1a (Flagg *et al.* 1999) and a yeast glucose transporter (Sherwood *et al.* 2000). In these case, the chimaeric protein complexes are localized at the plasma membrane but their activities are reduced by the presence of the mutant subunits. In order to facilitate further analysis of the physiological effects of the dominant-negative mutant of LeAMT1;1, stable transgenic lines of tomato were generated to over-express the wild type and the mutant genes under the 35S promoter.

4.2. Homo-oligomerization of tomato ammonium transporters

The self-interaction of the ammonium transporter LeAMT1;1 was demonstrated in both an optimized split ubiquitin system (Fig. 3.3 and 3.4) and a *Xenopus* oocyte expression system (Fig. 3.10). These results strongly suggest that plant ammonium transporters form homo-

oligomers at the plasma membrane, which is consistent with a protein gel analysis where a high molecular mass protein complex was detected by LeAMT1;1 antibody using plant membrane protein preparations (Ludewig *et al.* 2003). The apparent molecular mass of the complex is 140kDa, which can be converted to 35kDa, an apparent mass of a monomeric form, by heat denaturation. Self-interaction of the LeAMT1;2 was also demonstrated in the oocyte system, where the dominant-negative point mutant LeAMT1;2G465D inhibited the activities of the its wild-type protein co-expressed in the same oocytes, a similar effect as the LeAMT1;1 mutant (Fig. 3.10). An attempt using the split ubiquitin system to detect the self-interaction of LeAMT1;2 was not yet successful due to technical reasons, however a high molecular form of the LeAMT1;2 was also observed in plant membrane protein preparations (von Wiren, personal communication). These results strongly support the idea that plant ammonium transporters, at least in the case of the LeAMT1;1 and LeAMT1;2, exist in oligomeric forms.

In fact, oligomerization appears to be a general feature of Amt/Mep/Rh proteins. The *E. coli* ammonium transporter AmtB exists as a homo-trimer (Blakey *et al.* 2002), whereas the human Rhesus blood-group antigens form hetero-tetrameric protein complexes at the erythrocyte membrane (Eyers *et al.* 1994).

The subunit composition in LeAMT oligomers cannot be defined by the recent data. The apparent mass of 140kDa of the LeAMT1;1, compared with the apparent monomeric mass of 35kDa, implies two possibilities: a homo-tetramer (4 x 35kDa) or a homo-trimer, associating with additional protein like the bacterial AmtB, which interacts with the soluble protein GlnK (Coutts *et al.* 2002).

The biological significance of a membrane transporter being an oligomeric protein complex is not fully understood, however, some important mechanisms can be proposed. (i) Maintaining the substrate specificity. As in the symmetric tetrameric potassium channels, the 4 subunits interact in such a way that they form a central interior pore, where the atomic interactions between the residues in the amino acid side chains allow specific binding of the K⁺ but not Ca²⁺ or other cations and the physical space of the pore retains an efficient conductance of K⁺ (Doyle *et al.* 1998). (ii) Enhancing a more stable conformation in the lipid environment. Helix-helix and helix-lipid interactions are important determinants for stabilizing a membrane protein in the lipid bilayer. A number of recently solved crystallized structures of membrane transporters have revealed that co-packing of subunits is thermodynamically preferred (Levi *et al.* 2002; Cristian *et al.* 2003). The observation that hybrid protein complexes of wild-type and mutant forms of the LeAMT1;1 or LeAMT1;2 have a reduced activity may be a reflection of this (Fig. 3.10). (iii) Facilitating interactions with other proteins that might possess a similar oligomeric status. An example is the trimeric AmtB, which interacts at its cytoplasmic C-terminus with the trimeric regulatory protein GlnK (Coutts *et al.* 2002).

4.3. LeAMT1;1 forms hetero-oligomers with LeAMT1;2

The cross-inhibition of ammonium transporters by dominant negative AMT mutants suggests that heterooligomeric complexes can exist, at least in oocytes (Fig. 3.10). Co-expressed LeAMT1;1 and LeAMT1;2 ammonium transporters formed functional hetero-oligomers with intermediate properties, which may be explained by simple superposition of individual transport properties (Ludewig *et al.* 2003). Although it is still not shown whether LeAMT1;1 and LeAMT1;2 co-assemble at the plant plasma membrane, the co-expression in the same cell types, such as root hairs, may suggest that they interact to form complexes in plants (von Wiren *et al.* 2000). The interaction between LeAMT1;1 and LeAMT1;2 is not required for their individual activity since each transporter can function independently to complement the yeast triple mutant 31019b (*mep1* Δ *mep2* Δ *mep3* Δ) (von Wiren *et al.* 2000).

The results of this work do not allow determination on what may be the physiological roles of Amt oligomerization. Each subunit may form a pore, thus constituting a functional transport system. In this case, oligomerization may play a role in yet unknown regulatory phenomena. It may also be that individual ammonium transporter subunits form hetero-oligomers, similar to heteromeric sucrose transporters, in which separately expressed halves from different paralogs can reconstitute a functional transporter with intermediate affinity (Reinders *et al.* 2002). It is also possible that LeAMT-oligomers may form one common pore as in the case of potassium channels. In this case also, a transport system with intermediate function is probably generated. Structural analysis, together with dissection of the domains relevant for interaction using the split ubiquitin system, and biochemical analyses will help to determine the structure and function of the complexes. However, irrespective of the structure, electrophysiological analysis of oocytes expressing equal amounts of cRNA of LeAMT1;1 and LeAMT1;2 suggest that hetero-oligomers do not create a novel property but rather behave as a superposition of individual transporters (Ludewig *et al.* 2003).

4.4. LeAMT1;1 interacts at its cytoplasmic C-terminus with a signaling protein PI-PLC

The cytoplasmic C-terminus of the LeAMT1;1 or LeAMT1;2 is the largest region (48 amino acids in LeAMT1;1) of these proteins predicted to face the cytoplasmic side of the plasma membrane (Fig. 3.6). The region contains a highly conserved motif, which is present in most members of the Amt/Mep family. This region does not seem to be a prominent self-interaction domain for LeAMT1;1 because the corresponding C-terminal fragment does not self-interact in a yeast two hybrid assay. Rather, it is more likely to serve as a regulatory site, as implicated by the following observations. (i) A point mutation of the conserved glycine within that region renders the transporter non-functional and exhibits a dominant-

negative effect (Fig. 3.10; Marini *et al.* 2000). (ii) This dominant-negative mutant is still able to interact with its wild-type proteins in the split ubiquitin system (Fig. 3.4). (iii) The corresponding C-terminal region in the bacterial homolog AmtB interacts with a cytoplasmic signal transduction protein GlnK (PII-like protein), which in turn regulates the activity of the AmtB in response to the cellular nitrogen status (Coutts *et al.* 2002).

If this C-terminal region of plant Amts is important for the regulation of ammonium transport, it must contain a binding site for a signal transduction protein or proteins linking to the nitrogen sensory/regulatory network. Since the only known plant GlnK-homolog is a chloroplast protein (Moorhead and Smith 2003), the putative interaction partners of the plant ammonium transporter are likely to be other cytoplasmic proteins rather than the PII-like proteins.

A number of putative interaction partners from a tomato cDNA library were isolated by a yeast two hybrid screen with the C-terminal cytoplasmic fragment of LeAMT1;1 as a bait (Table 3.1). Of particular interest are the three independent prey clones harboring cDNAs that encode products with high homology to StPLC3 (Kopka *et al.* 1998), a potato homolog of phosphoinositide-specific phospholipase C (PI-PLC). The closest homolog in Arabidopsis is AtPLC2, which like StPLC3 is expressed constitutively in vegetative and floral tissue (Hirayama *et al.* 1997).

Phosphoinositide-specific phospholipase C isozymes are soluble multidomain proteins found only in eukaryotes where they hydrolyse the membrane phospholipids phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DG). IP₃ triggers an intracellular Ca²⁺ release, whereas DG activates protein kinase C. PI-PLCs play important roles in various cellular signal transduction pathways upon activation by membrane receptors or sensors. Although PI-PLCs comprise a large family of proteins, including at least β , γ and δ subgroups, only the δ subgroup is found in plants, and in simple organisms such as yeast and cellular slime modes a single δ -related gene is present (review in Rebecchi and Pentylala, 2000). The plant PI-PLCs contain from N-terminus to the C-terminus the EF, X, Y and C2 domains, which are all functionally conserved among the δ -subgroup (Mueller-Roeber and Pical, 2002).

The three tomato PI-PLC fragments interacting with LeAMT1;1 map to the C2 domain of StPLC3. Thus, the region of the C2 domain in the PI-PLC appears to contain binding site for the C-terminus of LeAMT1;1.

The biological mechanism underlying the putative interaction between LeAMT1;1 and the PI-PLC is currently unknown. There exist two possibilities: (i) LeAMT1;1 may function as an ammonium sensor and generate signals to the downstream transduction cascade by interacting with the PI-PLC, thus regulating gene expressions and cellular processes in response to ammonium availabilities (Fig.4.1A). In this case, LeAMT1;1 may resemble

the yeast Mep2p, which is shown to regulate pseudohyphal differentiation in response to ammonium limitation (Lorenz and Heitman, 1998). The putative sensory function of Mep2p requires Gpa2p, an α subunit of the GTP-binding protein, for downstream signaling. Direct interaction between Mep2p and Gpa2p has not been found, however Gpa2p is shown to associate with a complex of the Gpr1p (a GTP binding protein-coupled receptor) and the Plc1p (the yeast ortholog of PI-PLC) (Ansari *et al.* 1999). Thus, it would be interesting to see whether Mep2p also interacts with the yeast Plc1p. (ii) PI-PLC acts as a regulator of LeAMT1;1 by binding to its C-terminus (Fig.4.1B). In this case, PI-PLC is somehow similar in function to the GlnK in *E. coli* where AmtB activities are regulated by the interacting GlnK (Coutts *et al.* 2002). PI-PLC may be activated by a yet unknown membrane sensor of external ammonium or by a cellular component sensing internal nitrogen metabolites such as glutamine and glutamate, thus regulating the LeAMT1;1 activities accordingly. While in yeast the Plc1p is found to physically interact with the Gpr1p that is required for the pseudohyphal differentiation under nitrogen starvation independent of Mep2p (Ansari *et al.* 1999), it would be interesting to test if plant nitrogen response also involves the G protein signaling components. Further experiments would provide detailed information in this respect.

Crosstalk between sugar- and nitrogen-response signaling pathways has been well documented in plants (Coruzzi and Zhou, 2001). This is obvious since nitrogen assimilation needs carbon skeletons and energy produced by the carbon metabolism. The putative interaction between LeAMT1;1 and a plant SNF1-related protein kinase (Table 3.1), which plays a central role in sugar-response pathways (Halford *et al.* 2003), may suggest a direct link between ammonium transport activity and the cellular carbon availability.

Therefore, the putative interactions between LeAMT1;1 and the tomato PI-PLC and the SNF1-related protein kinase, as revealed by the yeast two hybrid assay, provide a helpful starting point to further dissect the ammonium sensory and signaling network.

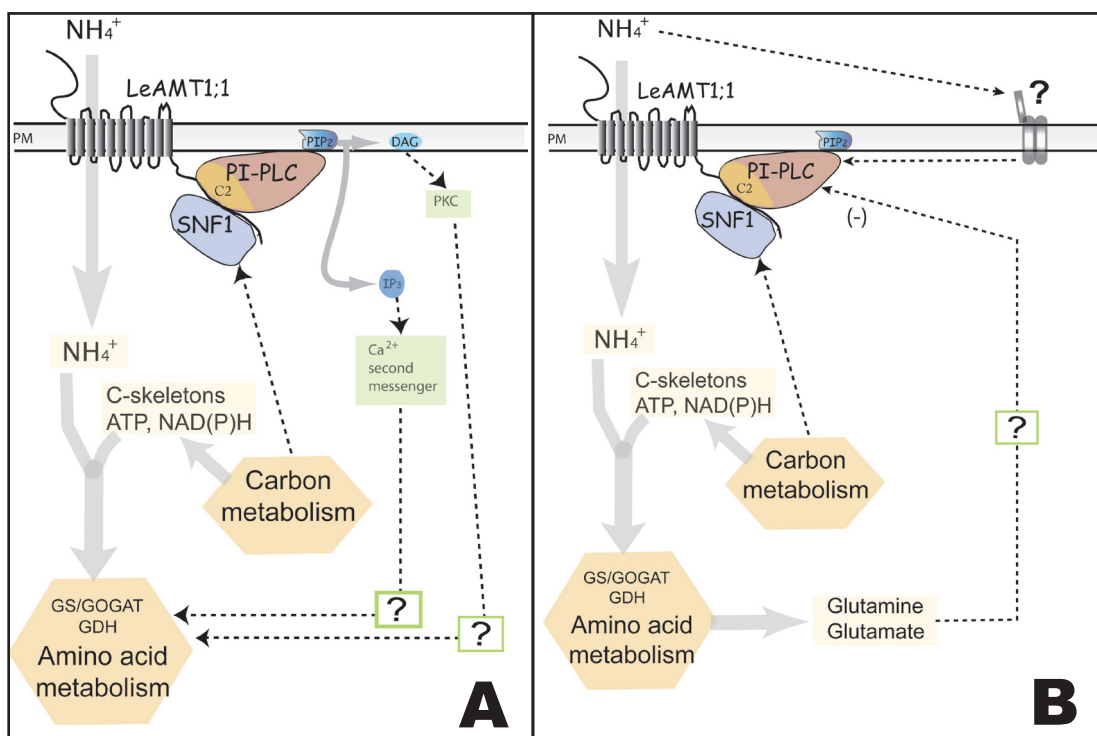


Fig 4.1 Hypothetical models of ammonium sensory/regulatory pathways involving the interactions between LeAMT1;1 and the PI-PLC and SNF1-related protein in higher plants. **A.** LeAMT1;1 functions as an ammonium sensor by generating signal in response to external ammonium and transducing the signal to the PI-PLC, which in turn hydrolyzes the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) thus activates the downstream signal cascade, leading to the regulation of ammonium transport and assimilation activities. **B.** An alternative model where the PI-PLC is a regulator of LeAMT1;1. It modulates the transport activity of LeAMT1;1 by binding to its C-terminus, in response to signals generated by a yet unknown ammonium sensor in the plasma membrane or by an intracellular glutamine/glutamate receptor. In both model, the plant SNF1-related protein kinase may act as a mediator of ammonium transport in connection to the cellular carbohydrate metabolism, which provides C-skeletons and energy used in the assimilation of ammonium into amino acids. Dash lines indicate the putative signaling pathways.

5. References

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