Ammonium and urea transporter inventory of the Selaginella and Physcomitrella genomes

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INTRODUCTION

Of all mineral elements required by plants, nitrogen is quantitatively the most important and thus often growth-limiting for many plants. Nitrogen is found in many organic compounds such as amino acids, nucleic acid, and consequently in proteins, as well as in nucleic acids. In most soils, nitrogen is heterogeneously distributed and found in various forms such as ammonium, nitrate, urea, amino acids, peptides, and even in water-insoluble forms (Jackson et al., 1990). Among the different nitrogen sources, ammonium is often preferred by plants, since its assimilation requires less energy. Moreover, ammonium is the predominant nitrogen form in anoxic soils, such as paddy rice fields, and it is abundantly used as fertilizer.

Due to the impermeability or poor permeability of the lipid bilayer of the plasma membrane to most nutrients including nitrogen compounds, uni and multicellular organisms developed a suite of nitrogen transporters. In 1979, electrophysiological studies in the unicellular algae Chara australis showed a positive inward current across the plasma membrane induced by ammonium addition (Walker et al., 1979). Fifteen years later, the first ammonium transporter, AtAMT1;1 from Arabidopsis, was cloned by yeast complementation (Ninnemann et al., 1994), in parallel with the yeast MEP orthologs (Marini et al., 1994). Since then, many para and orthologs were isolated either by yeast complementation or via homology cloning (Lauter et al., 1996; Gazzarrini et al., 1999; Simon-Rosin et al., 2003; Sonoda et al., 2003). Homologs have been found in bacteria, fungi, algae, plants, and animals (Marini et al., 1997, 2000; Gonzalez-Ballester et al., 2004) and the transport of the charged form of ammonium (NH₄⁺) was confirmed for the plant ammonium transporter AMT1;1 by electrophysiological characterization in Xenopus oocytes (Ludewig et al., 2002; Wood et al., 2006; Loqué et al., 2009).

Ammonium transporters fall into three subfamilies, Rhesus, Mep/AMTB (also called AMT2), and AMT1. Plant ammonium transporters belong to the two subclasses: AMT1 and AMT2 (Sohlenkamp et al., 2002). The AMT2 members are sequence-wise more closely related to fungal and bacterial ammonium transporters (Mep and AMTB) forming the Mep/AMTB/AMT2 subfamily (Ludewig et al., 2001). Bacterial AMTs, from Escherichia coli and from the archaeabacterium Archaeoglobus fulgidus, have been crystallized, revealing a trimeric structure (Khademi et al., 2004; Andrade et al., 2005; Conroy et al., 2007).

In both Eubacteria and Archaea, AMT activity is regulated by the interaction with GlnK proteins (Coutts et al., 2002). Importantly, at least the bacterial AMTs, similar to the plant nitrate transporter CHL1 and the yeast glucose and amino acid transporters RGT2, SNF1, and GAP1 have a dual function as sensors and transporters, and thus can be defined as transceptors (Hyde et al., 2007; Ho et al., 2009; Thevelein and Voordeckers, 2009). Therefore, at least the bacterial AMTs link energy and nitrogen status of the cell with ammonium uptake to control ammonium-dependent transcription (Tremblay and Hallenbeck, 2009). Plants also contain GlnK homologs, however these are confined to the chloroplast (Hsieh et al., 1998). In plants, plasma membrane AMT activity is regulated by an allosteric auto-inhibition driven by the conserved cytosolic C-terminal tail, which connects the adjacent members in the trimeric complex (Loqué et al., 2007, 2009; Neuhauser et al., 2007; Lanquar et al., 2009; Lanquar and Frommer, 2010). An increase in the concentration of extracellular ammonium leads to the phosphorylation of a specific threonine residue...
in the cytosolic C-terminus of AtAMT1;1, resulting in closure of the pores of the transporter complex (Loqué et al., 2007; Lanquar et al., 2009). Since the C-terminus, and this regulatory threonine in particular, is highly conserved in plant AMT1s, it is conceivable that this feedback mechanism represents a common feature of plant ammonium transporters. We recently demonstrated that the *Archaeoglobus* AMT1 also uses a transport allosteric mechanism involving the conserved C-terminus, indicating that this unique regulatory system was developed early in evolution and has been maintained. This was surprising, because point mutations in the pore region were found that release the transporter from the tight dependence on *trans*-activation (Loqué et al., 2009). Such point mutations must have occurred during evolution, but apparently have not been fixed, suggesting a strong pressure to retain this regulatory mechanism, potentially to protect against accumulation of toxic levels of ammonium.

Urea is another nitrogen source used by plants that is commonly available in nature. Urea is mainly derived from decomposition of nitrogen-containing molecules and from urine secretion by animals. Additionally, urea is widely used in agriculture as a fertilizer due to its stability, low toxicity and cost–effective production.

Urea transporters are present in most organisms and belong to different classes, some functioning as passive channels, others as secondary active transporters. Interestingly, urea transporters commonly found in animals and bacteria (UT, UreI, Yut) are absent in plant and fungal genomes (Wang et al., 2008). Plant and fungal genomes encode a different family of urea transporters, called DUR3. In 1993 the first plant urea transporter was cloned from *Arabidopsis* by homology to the yeast transporter DUR3 (Elberry et al., 1993). DUR3 proteins are secondary active transporters, structurally belonging to the sodium solute symporter (SSS) superfamily (Wang et al., 2008). Electrophysiological studies showed that a proton, rather than sodium, is co-transported with urea by AtDUR3 (Liu et al., 2003). In planta characterization showed that DUR3 genes from *Arabidopsis* and rice are induced by nitrogen deficiency (Liu et al., 2003; Wang et al., 2011). DUR3s have an important role in high affinity urea transport under nitrogen limiting growth conditions (Kojima et al., 2007; Wang et al., 2011). DUR3 is not the only mechanism for urea uptake, since several aquaporins in the TIP and NOD26 families have been shown to facilitate urea import through the plasma membrane (for review, see Kojima et al., 2006). Additionally, ABC transporters are known to transport urea in cyanobacteria (Valladares et al., 2002). Typically, plant genomes contain > 100 ABC transporter homologs; whether any of these transport urea remains an open question.

The genome sequence of the lycophyte *Selaginella moellendorffii* (Banks et al., 2011) provides a valuable tool for comparative genomic studies in plants. The lineage of this species arose ~400 million years ago early after vascular plants had evolved, and shares many primitive traits with bryophytes. This species adds to the increasing list of plants for which genome sequences have become available, including the moss *Physcomitrella patens* (Rensing et al., 2008).

Here, we compared the ammonium and urea transporter homologs of two dicots (*Arabidopsis* and poplar), one monocot (rice) a lycophyte (*Selaginella*), a bryophyte (*Physcomitrella*), and a unicellular alga (*Chlamydomonas reinhardtii*) with the aim of gaining more insight into the evolution of these important transporters. For *Arabidopsis*, poplar, rice and *Chlamydomonas, AMT* and *DUR3* members have already been annotated and characterized elsewhere, although in the case of poplar annotation is still not complete (Table 1; Loqué and von Wirén, 2004; Cuturier et al., 2006; Fernandez and Galvan, 2007; Wang et al., 2008; Li et al., 2009). Our results indicate a progressive reduction of the number of genes encoding urea transporters during higher plant evolution, possibly suggesting a shift in nutrient form preference of angiosperms for other nitrogen sources. Analysis of number and distribution of ammonium transporters between the AMT1 and AMT2 families revealed that most species, with the exception of *Physcomitrella*, present a bias for one or the other subfamily (*Arabidopsis*: AMT1, *Selaginella* and rice: AMT2).

**Materials and Methods**

Protein sequences of the six members of *Arabidopsis* AMTs and AtDUR3 were used to retrieve homologs of the ammonium and

| Gene function | Gene family | Protein used as query | Arabidopsis | Poplar* | Rice | Selaginella | Physcomitrella | Chlamydomonas |
|---------------|-------------|-----------------------|-------------|---------|------|-------------|----------------|---------------|
| Ammonium transport | AMT1 | AtAMT1;1 | 5 | 7 + 1 pseudo | 3 | 1 | 6 | 8 |
| Ammonium transport | AMT2 | AtAMT2 | 1 | 8 + 3 putative + 11 pseudo | 9 + 1 pseudo | 3 | 7 | 0 |
| Ammonium transport | Rh | HsRhAG | 0 | 0 | 0 | 0 | 0 | 2 |
| Urea transport | Dur3 | AtDUR3 | 1 | 1 | 1 | 1 | 2 | 3 |

*The genome of poplar contains several short pseudogenes for AMTs and three full length putative AMT2 genes which have not been annotated yet.*
Ammonium urea transporters in *Selaginella* and *Physcomitrella*

The genome of the moss *P. patens* revealed a higher and more diversified number of ammonium transporters, with six members of the AMT1 subfamily and seven members of the AMT2 subfamily (*Table 1*). AMT paralogs often differ with respect to affinities for ammonium and regulation. The existence of multiple paralogs may either help to increase transport capacity, ensure flexibility for regulation, and help cover a wider range of ammonium levels. In both AMT1 and AMT2 clades, the members of the *Physcomitrella* AMTs group closely together, suggesting a recent origin of the paralogs within the group (*Figure 1; Figure A3 in Appendix*). Three of the AMT1 members (named *PpAMT1;1, PpAMT1;2,* and *PpAMT1;3,* highly similar, correspond to recent gene duplications as they occur in tandem at the same genetic locus. The persistence of several paralogs in *Physcomitrella* is not limited to the AMT family, and in fact it seems to be a common feature derived from genome duplication events (Lang et al., 2005; Rensing et al., 2007).

The unicellular alga *C. reinhardtii* has eight AMTs, all belonging to the AMT1 family, and sharing only 31–46% identity with AMTs of *Arabidopsis* or *Selaginella*. Two of them (*CrAMT-E* and *-G*) also show alternative splicing (Fernandez and Galvan, 2007). Regarding phylogeny, they fall into three subclades, separated from other plant genes. This divergence is emphasized also by the lack of the conserved regulatory threonine in the C-terminus (Loqué et al., 2007). Other residues, usually conserved in AMT1s but missing or misplaced in some *Chlamydomonas* AMTs are the N-terminal cysteines, which have been shown to be important to promote interactions among monomers in the trimeric complex

### Table 2 | List of predicted ammonium and urea transporters in *Selaginella* and *Physcomitrella*.

| Species       | Gene and allele name | Locus identifier in Ensembl Genome | Protein (aa) |
|---------------|----------------------|-----------------------------------|--------------|
| *Selaginella* | *SmAMT1*             | SELMODRAFT_163770 SELMODRAFT_169094 | 513          |
|               | *SmAMT2.1*           | SELMODRAFT_93278                  | 443          |
|               | *SmAMT2.2*           | SELMODRAFT_108685                 |              |
|               | *SmAMT2.3*           | SELMODRAFT_120477 SELMODRAFT_84585 | 486          |
| *Physcomitrella* | *PpAMT1.1*     | XM_001758548                       | 504          |
|               | *PpAMT1.2*           | XM_00178547                       | 503          |
|               | *PpAMT1.3*           | XM_001758551                       | 505          |
|               | *PpAMT1.4*           | XM_001785448                       | 505          |
|               | *PpAMT1.5*           | XM_001786003                       | 495          |
|               | *PpAMT1.6*           | XM_001758559                       | 511          |
|               | *PpAMT2.1*           | XM_001762751                       | 475          |
|               | *PpAMT2.2*           | XM_001752462                       | 490          |
|               | *PpAMT2.3*           | XM_001754186                       | 493          |
|               | *PpAMT2.4*           | XM_001770002                       | 498          |
|               | *PpAMT2.5*           | XM_001754764                       | 494          |
|               | *PpAMT2.6*           | XM_001781152                       | 495          |
|               | *PpAMT2.7*           | XM_001778469                       | 461          |
| *Selaginella* | *SmDUR3*             | SELMODRAFT_135570 SELMODRAFT_172808 | 678/679      |
| *Physcomitrella* | *PpDUR3A*   | XM_001779344                       | 713          |
|               | *PpDUR3B*            | XM_001784492                       | 678          |
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FIGURE 1 | Unrooted phylogenetic tree of the ammonium transporters in Arabidopsis thaliana (At), Populus trichocarpa (Pt), Oryza sativa (Os), Selaginella moellendorffii (Sm), Physcomitrella patens (Pp), and Chlamydomonas reinhardtii (Cr). Red indicates Selaginella AMTs, blue Physcomitrella. The tree was constructed by aligning the protein sequences by ClustalW and generated with the Neighborhood-Joining method.

(Graff et al., 2011). It will be interesting to study how AMTs in this organism assemble and are regulated. Apart from the AMT genes, the genome of Chlamydomonas also contains homologs of the animal Rhesus ammonia transporters (Rh), that are distantly related to the AMT family (Soupene et al., 2004). Rh genes are however absent in Selaginella, Physcomitrella, and in any plant species sequenced so far.

An interesting observation is that the proportion of AMT1 and AMT2 genes in the various species is different (Table 1). The Arabidopsis genome possesses five AMT1 members and one AMT2, but in rice the proportion is inverted (three AMT1s and nine AMT2s). Similarly, the Selaginella genome is more enriched in AMT2s (three members) and a single AMT1. Similar to poplar, Physcomitrella is more balanced, with six AMT1 and seven AMT2 types; finally, Chlamydomonas is so far the species containing more AMT1 members (eight) and totally lacking the AMT2 type. It is interesting to observe that the C-terminus of the AMT1 family is highly conserved also in Selaginella and Physcomitrella (but not Chlamydomonas) as is the equivalent of threonine-460 in the C-terminus AtAMT1;1, which is involved in ammonium-dependent allosteric regulation (Loqué et al., 2007; Lanquar et al., 2009), suggesting that the regulatory mechanism via phosphorylation of AMT1 members is maintained in all multicellular plants.

UREA TRANSPORTER GENE FAMILY

Analysis of the Selaginella genome suggests the presence of a unique DUR3 homolog (named SmDUR3), similar to the one found in Arabidopsis, poplar, or rice genomes (Table 1). Whereas vascular plants have a single urea transporter, the moss Physcomitrella possesses two DUR3 homologs (PpDUR3A and B), with 85% identity between each other. Going further back in the evolutionary tree, Chlamydomonas has three different copies, named DUR3A, B and C, more closely related to each other (about 80% identity) than with the DUR3 members of multicellular plants (Figure 2; Figure A4 in Appendix). Other algae, as well as fungi, are known to have additional copies of DUR3 genes (Kakinuma et al., 2008; Morel et al., 2008).

The progressive loss of genes for urea transporters could indicate that the evolution of the vascular system shifted the preference...
for other nitrogen sources, such as ammonium, nitrate, and amino acids, all present in the xylem sap of vascular plants (Smirnoff and Stewart, 1985; Atkins, 2000; Schojerring et al., 2002).

CONCLUSION

The comparative analysis of ammonium transporters revealed that different lineages of plants vary in the number of AMTs belonging to either family, and in many cases they show a bias toward one of the two AMT subfamilies, with some organisms particularly enriched in the plant-specific AMT1 clade (Arabidopsis, Chlamydomonas), while others rely more on the AMT2 family, typical for bacteria and fungi. The physiological and evolutionary relevance of these shifts remains to be determined. The number of urea transporters decreased during evolution, maybe indicating a preference of vascular plants for other nitrogen sources or the evolution of yet uncharacterized urea transporters.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
### APPENDIX

![Figure A1](image-url)

**FIGURE A1** | Continued
De Michele et al. Ammonium urea transporters in Selaginella and Physcomitrella

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FIGURE A1 | Continued
FIGURE A1 | Continued
FIGURE A1 | Alignment of protein sequences of ammonium transporters by ClustalW. In gray squares are the regions excluded by analyses.
FIGURE A2 | Continued
FIGURE A2 | Alignment of protein sequences of urea transporters by ClustalW. In gray squares are the regions excluded by analyses.
FIGURE A3 | Unrooted phylogenetic tree of the ammonium transporters in Arabidopsis thaliana (At), Populus trichocarpa (Pt), Oryza sativa (Os), Selaginella moellendorffii (Sm), Physcomitrella patens (Pp), and Chlamydomonas reinhardtii (Cr). Red indicates Selaginella AMTs, blue Physcomitrella. The tree was constructed by aligning the protein sequences by ClustalW and generated with the Maximum-Likelihood method.
FIGURE A4 | Unrooted phylogenetic tree of the urea transporters in Arabidopsis thaliana (At), Populus trichocarpa (Pt), Oryza sativa (Os), Selaginella moellendorffii (Sm), Physcomitrella patens (Pp), and Chlamydomonas reinhardtii (Cr). Red indicates Selaginella DUR3, blue Physcomitrella. The tree was constructed by aligning the protein sequences by ClustalW and generated with the Maximum-Likelihood method.