Duplication of *NRAMP3* gene in poplars generated two homologous transporters with distinct functions

*Mathieu Pottier^{1,3}, Van Anh Le Thi^{1,4}, Catherine Primard-Brisset¹, Jessica Marion¹, Michele Bianchi¹, Cindy Victor¹, Annabelle Déjardin², Gilles Pilate², Sébastien Thomine¹**

¹Institut de Biologie Intégrative de la Cellule, CNRS, Avenue de la Terrasse, 91198 Gif-sur-Yvette, France

²INRAE, ONF, BioForA, Orléans, France

³Current affiliation: Institute for Molecular Physiology, Heinrich-Heine-University Düsseldorf, Düsseldorf 40225, Germany

⁴Current affiliation: Graduate University of Science and Technology (GUST), Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi 10000, Vietnam

*Author for correspondence

Sébastien Thomine

Sebastien.thomine@i2bc.paris-saclay.fr

ABSTRACT

Transition metals are essential for a wealth of metabolic reactions, but their concentrations need to be tightly controlled across cells and cell compartments, as metal excess or imbalance has deleterious effects. Metal homeostasis is achieved by a combination of metal transport across membranes and metal binding to a variety of molecules. Gene duplication is a key process in evolution, as emergence of advantageous mutations on one of the copies can confer a new function. Here, we report that the poplar genome contains two paralogues encoding NRAMP3 metal transporters localized in tandem. All *Populus* species analyzed had two copies of NRAMP3, whereas only one could be identified in *Salix* species indicating that duplication occurred when the two genera separated. Both copies are under purifying selection and encode functional transporters, as shown by expression in the yeast heterologous expression system. However, genetic complementation revealed that only one of the paralogues has retained the original function in release of metals stored in the vacuole previously characterized in *A. thaliana*. Confocal imaging showed that the other copy has acquired a distinct localization to the Trans Golgi Network (TGN). Expression in poplar indicated that the copy of NRAMP3 localized on the TGN has a novel function in the control of cell-to-cell transport of manganese. This work provides a clear case of neo-functionalization through change in the subcellular localization of a metal transporter as well as evidence for the involvement of the secretory pathway in cell-to-cell transport of manganese.

Keywords: manganese, metal, recycling, Golgi apparatus, vacuole, Arabidopsis, *Salix*, *Populus*, selective pressure, evolution, apoplasmic transport, symplasmic transport, nutrition, micronutrient.

INTRODUCTION

Several transition metals are essential cofactors for a wealth of metabolic reactions in all living organisms. Iron (Fe) and copper (Cu) are for example needed in large amounts for the respiratory electron transfer chains and ATP production in bacteria and mitochondria. Transition metals are also important for DNA synthesis, proteolysis and the control of reactive oxygen species (ROS). Photosynthetic organisms have an additional specific requirement for manganese (Mn) for light energy conversion and water splitting (Shen 2015). Although they

are essential, transition metal concentrations need to be tightly controlled across cells and cell compartments, as excess or imbalance between different metals has deleterious effects. Metal homeostasis is achieved by a combination of metal transport across membranes and metal binding to a variety of molecules, including proteins, small peptides, amino acids, organic acids and more specialized metabolites, such as phytochelatins or nicotianamine in plants (Seregin and Kozhevnikova 2020).

Many families of transporters are implicated in the maintenance of metal homeostasis, either for metal uptake, distribution of metals to organs within organisms and organelles within a cell, or for removal and sequestration of excess metal. For example, in *Arabidopsis thaliana*, Mn is taken up by AtNRAMP1 (Natural Resistance Associated Macrophage Protein 1) in the roots and distributed within cells by AtNRAMP2 (Cailliatte et al. 2010; Alejandro et al. 2017; Gao et al. 2018). AtMTP8 (Metal Tolerance Protein 8) and AtMTP11, which belong to a different transporter family, are responsible for loading Mn from the cytosol into the vacuole or the Trans Golgi Network (TGN), respectively (Delhaize et al. 2007; Peiter et al. 2007; Eroglu et al. 2016). The vacuole is used to store Mn excess and prevent its toxicity. However, when this element becomes scarce, other NRAMP family members, namely AtNRAMP3 and AtNRAMP4, allow the retrieval of Mn from the vacuole (Lanquar et al. 2010). Mn is needed in the secretory system as a cofactor of glycosyl transferases involved in protein glycosylation (Alejandro et al. 2020). It also plays important roles as a cofactor of Super Oxide Dismutase in mitochondria and peroxisomes (Alejandro et al. 2020). Moreover, Mn is essential for oxygenic photosynthesis as a component of the Mn_4CaO_5 cofactor of the water splitting complex, which is bound to photosystem II (PS II) at the inner side of the thylakoid membranes (Shen 2015). CMT1 (Chloroplast Manganese Transporter 1) and PAM71 (Photosynthesis-affected mutant 71), two transporters belonging to the GDT1 family, have been shown to allow the import of Mn across the inner membrane of the chloroplast envelope and the thylakoid membrane, respectively (Schneider et al. 2016; Eisenhut et al. 2018; Zhang et al. 2018). Recently, another member of the GDT1/UPF0016 family was shown to play a crucial role in loading Mn in the Golgi apparatus, where it is needed as a cofactor of glycosyl transferases involved in cell wall formation (Yang et al. 2021). The networks of transporters that mediate uptake, storage and distribution of other essential metals, such as Fe, Zn and Cu, have also been described. Interestingly, these networks are interconnected, as some transporters, as well as ligands, are able to transport a broad range of metal cations (Mathieu Pottier et al. 2015b; Seregin and Kozhevnikova 2020).

This is well illustrated when looking at the functions of transporters of the NRAMP family. This family was first identified in the context of resistance to intracellular pathogens, such as *Mycobacterium tuberculosis*, in mammals. Murine NRAMP1 was shown to limit the growth of intracellular pathogens by depleting essential metals from the phagosomes where they reside (Vidal et al. 1993; Wessling-Resnick 2015). Mammalian NRAMP2 plays a central role in Fe absorption in the intestine and recycling. In yeast, the NRAMP members SMF1 and SMF2 are involved in Mn absorption and distribution, similar to *A. thaliana* NRAMP1 and NRAMP2 (Portnoy et al. 2000; Cailliatte et al. 2010; Alejandro et al. 2017; Gao et al. 2018), while SMF3 allows the release of Fe from the vacuole, similar to *A. thaliana* NRAMP3 and NRAMP4 (Portnoy et al. 2000; Lanquar et al. 2005). In *A. thaliana*, NRAMP1 does not only allow high affinity Mn uptake but also plays a role in low affinity Fe uptake (Castaings et al. 2016). Besides Fe, AtNRAMP3 and AtNRAMP4 also allow the release of Mn from the vacuoles (Lanquar et al. 2005; Lanquar et al. 2010), clearly illustrating that these transporters connect Fe and Mn homeostasis.

While many studies have addressed the molecular mechanisms of metal homeostasis in *A. thaliana* and rice, there are only a limited number of reports on this topic in poplar. Poplars are both a model trees for which the genome of several species has been sequenced (Tuskan et al. 2006; Zhang et al. 2019, Lin et al. 2018), and an industrially important crop for wood production. Poplars display outstanding growth yield among tree species and their wood is mostly used by the peeling industry to produce light packaging and plywood. Moreover, poplars are often used in the rehabilitation of polluted areas because it is highly tolerant to heavy metals and other contaminants (Krämer 2005a; Pottier et al. 2015a). Poplar MTP family members have been functionally investigated. In the first sequenced poplar species *Populus trichocarpa* (Tuskan et al., 2006), it has been shown that PotriMTP1 and PotriMTP11 could be the functional homologues of AtMTP1 and AtMTP11 and are involved in Zn loading into the vacuole and Mn loading into the TGN, respectively (Blaudez et al. 2003; Kramer 2005b; Peiter et al. 2007). Copper homeostasis has also been investigated in the context of photosynthetic efficiency (Ravet et al. 2011). In addition, overexpression of genes involved in Zn and Cd chelation and homeostasis has been undertaken in an attempt to increase tolerance and accumulation of these metals (Adams et al. 2011; He et al. 2015; Wang et al. 2019). Several studies have mined poplar genomic data and established lists of metal transport proteins in this species, analyzed the expression pattern of the corresponding genes and sometimes demonstrated the transport function using yeast complementation (Migeon et al. 2010; Li et al. 2015; Gao et al. 2020).

These studies have often highlighted the presence of duplication in metal homeostasis genes, which is a prevalent feature in poplar genome (Tuskan and et al 2006). However, they have not investigated in detail the function of the duplicated copies.

Gene duplication is a key process in evolution. It occurs through two major processes: either whole genome duplication or local duplication by unequal cross over or transposition (Conant and Wolfe 2008). Gene duplication underlies several key events in evolution such as variation in gene copy number, the generation of new regulatory networks and the appearance of novel functions. After gene duplication occurs, relaxation of selective pressure opens the door to several scenarios. Most of the time, one of the copies undergoes non-functionalization through accumulation of deleterious mutations due to the lack of selective pressure on this copy. In other cases, having multiple copies of the same gene provides advantages and several functional identical genes are therefore actively maintained (Hanikenne et al. 2013). Often the copies can also undergo sub-functionalization: the preduplication function is maintained but partitioned among the two copies. Typically, the expression pattern of the ancestral gene is covered by the two copies which are expressed in different organs and involved in distinct regulatory networks (Tuskan and et al 2006). Finally, in rare cases, emergence of advantageous mutations on one of the copies can also confer a new function, which is commonly known as neo-functionalization (Moriyama et al. 2016).

In this study, we have investigated the function of poplar *NRAMP3*. We found that the poplar genome contains two paralogues of *NRAMP3* in tandem. One of the paralogues has conserved the original function in release of metals stored in the vacuole characterized in *A. thaliana*, whereas the other paralogue has acquired a distinct localization to the TGN. Analysis of the function of this gene in transgenic poplars indicates that it has a novel function in the control of cell-to-cell transport of Mn in this species. Therefore, the functional analysis of the two paralogues of *PotriNRAMP3* provides a clear case of neo-functionalization through change in the subcellular localization of a transporter and evidence for the involvement of the secretory pathway in cell-to-cell transport of Mn.

RESULTS

***PotriNRAMP3.1* and *PotriNRAMP3.2* are a tandem gene pair encoding homologous proteins**

Plant NRAMPs are distributed into three different phylogenetic groups according to their protein sequence identities and exon-intron structures (Migeon et al. 2010). The eleven NRAMPs retrieved from *P. trichocarpa* genome V4.1 follow the same distribution (supplementary tables S1, S2 and fig. S1A). PotriNRAMP1, PotriNRAMP6.1 and PotriNRAMP6.2 as well as PotriNRAMP7.1, PotriNRAMP7.2 and PotriNRAMP7.3 belong to group I, which also includes AtNRAMP1 and AtNRAMP6. PotriNRAMP2, PotriNRAMP3.1 and PotriNRAMP3.2 are in group II, which includes AtNRAMP2, AtNRAMP5, AtNRAMP3 and AtNRAMP4. PotriEIN2.1 and PotriEIN2.2 are located in the group III as AtEIN2 (supplementary fig. S1A).

Interestingly, *PotriNRAMP3.1* and *PotriNRAMP3.2* genes are localized in close vicinity in *P. trichocarpa* genome. They are positioned in a 32 kb area of chromosome 7 and encode 88.2% identical proteins (supplementary fig. S1B-C). Dot plot analyses performed on genomic DNA including *PotriNRAMP3.1* and *PotriNRAMP3.2* genes show similarity specifically between their coding sequences and their 3'UTR but not between their introns and the surrounding genomic sequences (supplementary fig. S1C-D).

***NRAMP3.1* and *NRAMP3.2* are present in all sequenced poplar species but not in closely related species**

To determine how widespread the duplication of *NRAMP3* observed in *P. trichocarpa* genome is, sequences similar to *PotriNRAMP3.1* and *PotriNRAMP3.2* were retrieved from nine genomes and four transcriptomes and *Populus* species, covering the five main *Populus* sections, i.e., Tacahamaca, *Populus*, *Leucoïdes*, *Turanga*, *Aigeiros* and *Abaso* (Zhang et al. 2019; Wang et al. 2020). We could unequivocally identify two distinct sequences similar to *PotriNRAMP3.1* or *PotriNRAMP3.2*, in all investigated *Populus* genomes, i.e., *P. alba*, *P. cathayana*, *P. simonii*, *P. lasiocarpa*, *P. maximowiczii*, *P. euphratica*, *P. ussuriensis*, *P. nigra*, *P. deltoides*, *P. tremula*, *P. tremuloides* and *P. grandidentata* (supplementary table S1 and supplementary data S1) (Zhang et al. 2019). Evidence for two distinct sequences similar to *PotriNRAMP3.1* or *PotriNRAMP3.2* was also observed in *P. mexicana*, the single living species of the ancestral poplar section *Abaso*, even though its genome is not fully sequenced yet (supplementary table S1 and data S2) (Wang et al. 2020). This result suggests that distinct *PotriNRAMP3.1* and *PotriNRAMP3.2* homologues are present in all *Populus* species. In contrast, reciprocal blasting of *PotriNRAMP3.1* and *PotriNRAMP3.2* on the genome sequences of three *Salix* species (*S.*

purpurea, *S. suchowensis* and *S. brachista*), and on transcript sequences of five other *Salix* species (*S. viminalis*, *S. sachalinensis*, *S. eriocephala*, *S. fargesii* and *S. dasyclados*) that belong to the closest phylogenetic group to *Populus* genus, identified a single sequence similar to *NRAMP3* in each species (supplementary table S1 and data S1) (Chen et al. 2019). These results suggest that the duplication that gave rise to *NRAMP3.1* and *NRAMP3.2* genes coincided with the divergence between *Populus* and *Salix* about 52 million years ago (Ma) (Hou et al. 2016). However, the chromosomal rearrangements that distinguish *Populus* and *Salix* phylum did not affect chromosome 7, which carries the *NRAMP3* loci (Hou et al. 2016). Moreover, gene collinearity is maintained around *NRAMP3* loci in *P. trichocarpa* and *S. purpurea* (fig. 1). Using the corresponding protein sequences of all the *Populus* and *Salix* *NRAMP3* homologues identified during this analysis (supplementary data S3), we constructed a detailed phylogenetic tree. The *A. thaliana* *NRAMP2*, *NRAMP3* and *NRAMP4*, and *P. trichocarpa* and *S. purpurea* homologues of *AtNRAMP2* were used as outgroup (supplementary table S3). This analysis showed that *Populus* *NRAMP3.1* and *NRAMP3.2* protein sequences form distinct phylogenetic groups (fig. 2). The *Salix* homologues clearly cluster together with *Populus* *NRAMP3.2* suggesting that it corresponds to the ancestral copy, whereas *Populus* *NRAMP3.1* sequence diverged.

To further analyze the evolutionary history of *Populus* *NRAMP3.1* and *NRAMP3.2* sequences, we calculated the ratio of non-synonymous (dN) vs synonymous codons (dS), between all *NRAMP3.1*s, between all *NRAMP3.2*s, and between all *NRAMP3.1*s and *NRAMP3.2*s together. Low global dN/dS around 0.2 were obtained for *NRAMP3.1* and *NRAMP3.2* indicating that all genes are under purifying selection. A sliding window analysis revealed that the low global dN/dS values obtained among *NRAMP3.1* or among *NRAMP3.2* sequences result from homogeneously low ratios values along their open reading frames (supplementary fig. S2). In contrast, comparing *NRAMP3.1*s with *NRAMP3.2*s revealed heterogeneous values along the open reading frame, with ratios above or close to 1 in the N and C termini, as expected from divergent sequences. These results indicate that relaxation of selective pressure led to sequence diversity after the duplication event, but that both genes are now under purifying selection. Fixed Effects Likelihood (FEL) method was then employed to investigate site specific selective pressures specifically applied to either *NRAMP3.1*s or *NRAMP3.2*s. In this way, 21 and 4 residues under purifying selection ($p < 0.05$) were identified in *NRAMP3.1*s and *NRAMP3.2*s, respectively (supplementary tables S4 and S5). These residues are highlighted on an alignment between the consensus sequence of *Populus* *NRAMP3.1* and that of *Populus*

NRAMP3.2 generated from all the *Populus* NRAMP3.1 and NRAMP3.2 sequences retrieved in this study (fig. 3; supplementary data S1 and fig. S3). Note that the four residues under purifying selection in NRAMP3.2s are also under purifying selection in NRAMP3.1s. Moreover, with the exception of V491, amino acids under purifying selection in NRAMP3.1s are conserved in NRAMP3.2s. These results suggest essential roles of these residues in the basal NRAMP3 function, common to both NRAMP3.1 and NRAMP3.2. In contrast, distinct residues were found to be under positive selection in both NRAMP3.1 (positions 3 and 437, $p < 0.05$) and NRAMP3.2 (positions 161 and 265, $p < 0.05$) (fig. 3; supplementary tables S4 and S5). Thus, NRAMP3.1 and NRAMP3.2 sequences both continue to diversify, even though they are globally under strong purifying selection. Moreover, these analyses indicate that NRAMP3.1 and NRAMP3.2 are not under non-functionalization.

Both *PotriNRAMP3.1* and *PotriNRAMP3.2* encode functional metal transporters

We then went on to examine the functions of the two paralogues. We cloned the corresponding cDNAs from *P. trichocarpa* and expressed them in yeast. Since their *A. thaliana* closest sequence homologues AtNRAMP3 and AtNRAMP4 are involved in Mn transport, we investigated PotriNRAMP3s ability to complement the *smf1* strain, which is deficient in Mn uptake (Supek et al. 1996). The *smf1* yeast strain fails to grow on synthetic medium containing high concentrations of the divalent cation chelator EGTA unless it is supplemented with Mn (Supek et al. 1996; Cohen et al. 2000). Expression of *PotriNRAMP3.2* restored the ability of *smf1* to grow on EGTA to the same extent as the positive control *AtNRAMP1* (Thomine et al. 2000). In contrast, PotriNRAMP3.1 complemented *smf1* phenotype with lower efficiency (fig. 4A; supplementary fig. S4). This result may be due to lower expression of *PotriNRAMP3.1* compared to *PotriNRAMP3.2* in yeast, a different subcellular localization or a lower efficiency of Mn transport. To test the hypothesis that a different localization accounts for the poor complementation of *smf1* by PotriNRAMP3.1, we complemented *smf2*, a yeast strain lacking an intracellular NRAMP involved in Mn supply to mitochondria and Golgi (Cohen et al. 2000; Luk and Culotta 2001). As *smf1*, this strain is unable to grow on high EGTA unless Mn is supplemented. We found that both PotriNRAMP3.1 and PotriNRAMP3.2 fully complement the *smf2* phenotype as efficiently as the positive control AtNRAMP2 (fig. 4B; supplementary fig. S5) (Alejandro et al. 2017). Moreover, the exposure to toxic Mn concentration in the absence of EGTA decreased the growth of the *smf2* strains transformed by *AtNRAMP2*, *PotriNRAMP3.1*

and *PotriNRAMP3.2* whereas it did not strongly affect the growth of *smf2* transformed by *GUS* (fig. 4B; supplementary fig S5). This increase of sensitivity to Mn was more pronounced for *PotriNRAMP3.1*. To further analyze their ability to transport Mn, cation accumulation was measured in *smf2* yeast expressing *AtNRAMP4*, *PotriNRAMP3.1* or *PotriNRAMP3.2*. Consistent with their ability to complement yeast Mn transport mutants (Thomine et al. 2000), Mn concentration was higher in yeast expressing *AtNRAMP4* as a positive control, or *PotriNRAMP3.1* and *PotriNRAMP3.2* compared to yeast expressing *GUS* as a negative control. This result confirms that both *PotriNRAMP3.1* and *PotriNRAMP3.2* proteins are able to transport Mn (fig. 4C).

As their *A. thaliana* closest sequence homologues *AtNRAMP3* and *AtNRAMP4* are also able to transport Fe, we tested the ability of *PotriNRAMP3.1* and *PotriNRAMP3.2* to complement the *fet3fet4* strain, which is deficient for both low- and high-affinity Fe uptake systems and requires a high Fe concentration for growth (Eide et al. 1996). Expression of *AtNRAMP4* and to a lesser extent that of *AtNRAMP1* were previously shown to restore *fet3fet4* growth on a low Fe medium (Thomine et al. 2000). We found that expression of *PotriNRAMP3.1* and *PotriNRAMP3.2* rescues *fet3fet4* growth under Fe limitation at a level intermediate between *AtNRAMP4* and *AtNRAMP1*, indicating that they are both able to transport Fe (fig. 5; supplementary fig. S6).

Taken together, this work shows that both copies of *PotriNRAMP3* have retained Mn and Fe transport ability, definitively excluding non-functionalization. Besides, these results also suggest that these two proteins have distinct subcellular localizations.

***NRAMP3.1* and *NRAMP3.2* are both expressed in poplar roots, stems, leaves and buds**

To test whether *Populus NRAMP3.1* and *NRAMP3.2* had undergone sub-functionalization by expression in distinct organs, their transcript levels were determined using RT-qPCR, in roots, stems, buds and leaves of the poplar hybrid INRA 717-1-B4 (*P. tremula* x *P. alba*) (Leplé et al. 1992). We designed primers that allow specific amplification of *NRAMP3.1* or *NRAMP3.2*, (supplementary table S6). *Populus NRAMP3.1* and *NRAMP3.2* exhibited expression in all studied organs (fig. 6). *NRAMP3.2* mRNA level was overall higher than that of *NRAMP3.1*. *NRAMP3.1* mRNA level was similar in all organs, whereas that of *NRAMP3.2* was higher in stems and leaves compared to roots and buds. These results show that, in the conditions tested,

NRAMP3.1 and *NRAMP3.2* are both expressed in all tested organs, arguing against their subfunctionalization by expression in distinct organs of the plants.

PotriNRAMP3.2*, but not *PotriNRAMP3.1*, complements the *nramp3nramp4* double mutant of *A. thaliana

Because both *PotriNRAMP3*s share high protein sequence identity with *AtNRAMP3* and *AtNRAMP4*, we tested whether they could perform the same function *in planta*. *AtNRAMP3* and *AtNRAMP4* have redundant function in iron remobilization from vacuoles during seed germination. As a consequence, *A. thaliana nramp3nramp4* double mutants are sensitive to iron starvation during their early development (Lanquar et al. 2005). We expressed *PotriNRAMP3.1* and *PotriNRAMP3.2* under the *Ubiquitin 10* (*pUb10*) promoter in the *A. thaliana* Columbia 0 (Col-0) *nramp3nramp4* mutant background (Lanquar et al. 2005; Oomen et al. 2009; Grefen et al. 2010), and used 3 independent homozygous T3 lines expressing transgenes at various levels for further experiments (supplementary fig. S7). After eight days of growth under Fe starvation, lines transformed with *pUb10:PotriNRAMP3.2* exhibited full complementation of the double mutant phenotype: root length and cotyledon greening were indistinguishable from wild-type (fig. 7). Complementation was observed irrespective of the expression level of the transgene, indicating that even low levels are sufficient to restore the wild-type phenotype. In contrast, the expression of *PotriNRAMP3.1* did not improve the growth of the *A. thaliana nramp3nramp4* double mutant under Fe deficient conditions, even in lines showing high expression of the transgene (fig. 7, supplementary fig. S7). Similar results were obtained with the *p35S:PotriNRAMP3.1-GFP* and *p35:PotriNRAMP3.2-GFP* constructs used to study subcellular localization in both *A. thaliana* and poplar (see below), except that *p35S:PotriNRAMP3.1-GFP* partially improved *nramp3nramp4* growth under Fe deficiency (supplementary fig. S8). Even though *PotriNRAMP3.1* is able to transport Fe (fig. 5), it is thus not able to restore Fe remobilization during seed germination.

***PotriNRAMP3.1* and *PotriNRAMP3.2* have distinct subcellular localizations in plant cells**

Change in intracellular localization is one of the mechanisms leading to neofunctionalization (Ren et al. 2014). Because *smf1* complementation results suggest that *PotriNRAMP3.1* and

PotriNRAMP3.2 may have distinct subcellular localizations (fig. 4), *A. thaliana* and poplar transgenic lines expressing C-terminal GFP fusion proteins of these two transporters were generated. Previous studies showed that tagging with GFP at the C-terminal end does not affect NRAMP targeting and function in plants (Lanquar et al. 2005; Cailliatte et al. 2010; Alejandro et al. 2017). The *A. thaliana nramp3nramp4* double mutant (Col-0) was stably transformed with *p35S:PotriNRAMP3.1-GFP* and *p35S:PotriNRAMP3.2-GFP*. Roots of these plants were then observed by confocal microscopy (fig. 8). Interestingly, distinct subcellular localizations were observed for PotriNRAMP3.1-GFP and PotriNRAMP3.2-GFP. While PotriNRAMP3.2-GFP was targeted to the vacuolar membrane, as its homologues in *A. thaliana* and *Noccaea caerulea* (Thomine et al. 2003; Lanquar et al. 2005; Oomen et al. 2009), PotriNRAMP3.1 was localized in intracellular punctuate structures (fig. 8). The localization of PotriNRAMP3.2 on the vacuolar membrane is consistent with its ability to complement the *A. thaliana nramp3nramp4* double mutant. Similar PotriNRAMP3.1 and PotriNRAMP3.2 localizations were observed in poplar root cells (fig. 8) as well as in mesophyll protoplasts and leaf epidermal cells (supplementary fig. S9).

To determine more precisely the subcellular localization of PotriNRAMP3.1, we tested the colocalization of PotriNRAMP3.1-GFP with RFP markers for different cell compartments (Ebine et al. 2011; Uemura et al. 2012; Inada et al. 2016). To this aim, *A. thaliana* lines expressing PotriNRAMP3.1-GFP were crossed with stable lines expressing markers for the trans Golgi apparatus *i.e.*, mRFP-ST (Sialyl Transferase), the TGN *i.e.*, mRFP-Syp43 and two endosomal markers *i.e.*, ARA6-mRFP and ARA7-mRFP. Spinning disk confocal microscopy performed on the F1 seedlings showed an extensive overlap between PotriNRAMP3.1-GFP and mRFP-SYP43 fluorescence (fig. 9A). Interestingly, although PotriNRAMP3.1-GFP fluorescence did not overlap with that of mRFP-ST, it was most often in close vicinity (fig. 9B). In contrast, little or no colocalization was observed with endosomal markers (supplementary fig. S10). These colocalization experiments indicate that PotriNRAMP3.1 resides on the TGN, and that it is present in both Golgi-associated and Golgi-independent TGN compartments (Viotti et al. 2010; Uemura et al. 2019). Together, these results show that the *PotriNRAMP3.2* copy has retained the subcellular localization and function of the *NRAMP3* genes characterized in other species, whereas *PotriNRAMP3.1* has likely acquired a novel function due to mutations that modified its subcellular localization to the TGN. However, *PotriNRAMP3.1* expression in *A. thaliana* did not lead to any phenotypic alteration that could provide hints at this novel function.

PotriNRAMP3.1, but not PotriNRAMP3.2, affects manganese homeostasis in poplar

To investigate the functions of PotriNRAMP3.1 and PotriNRAMP3.2 in poplar, the genes coding these transporters were over-expressed as GFP fusions under the control of the CaMV 35S promoter. For each construct, four independent transgenic lines overexpressing (OE) the *PotriNRAMP3s* at levels 10 to 40 times higher than non-transgenic (NT) control trees were analyzed (supplementary fig. S11). Their physiological characterization highlighted that poplar lines with the highest levels of *PotriNRAMP3.1* expression displayed reduced height as well as interveinal chlorosis on mature leaves compared to NT control trees (fig. 10A, C, F; supplementary fig. S11). In contrast, *PotriNRAMP3.2* OE trees were indistinguishable from NT poplars (fig. 10B, C, H). To better understand the origin of the chlorosis, the maximum quantum yield of PS II was imaged using an Imaging PAM (Walz, Germany) in leaves of control poplars as well as *PotriNRAMP3.1* and *PotriNRAMP3.2* OE lines (fig. 10E, G, I). The chlorotic areas in *PotriNRAMP3.1* OE lines coincided with strongly decreased PS II maximum quantum yield (0.472 ± 0.035). In contrast, PS II efficiency was close to the optimal value of 0.82 in leaves from control (0.756 ± 0.002) and *PotriNRAMP3.2* OE trees (0.751 ± 0.008). As interveinal chlorosis is a symptom of Fe deficiency and decrease in PS II efficiency may be a symptom of Mn deficiency (Connorton et al. 2017; Alejandro et al. 2020), we quantified metals in young, mature and senescent leaves from the different poplar genotypes. These analyses revealed that Mn concentrations in young and mature leaves were significantly lower in *PotriNRAMP3.1* OE lines compared to NT control or *PotriNRAMP3.2* OE lines (fig. 11A, B, C). In contrast, no significant difference in Fe or Zn concentrations was detected among the different genotypes (supplementary fig. S12). Interestingly, opposite to what was observed in leaves, Mn concentrations in stems of *PotriNRAMP3.1* OE lines were higher than in NT control or *PotriNRAMP3.2* OE lines (fig. 11D). Note that Mn concentrations were also significantly higher in stems of *PotriNRAMP3.2* OE lines compared to NT control. The defect in Mn distribution observed in *PotriNRAMP3.1* OE lines suggests that the phenotypes observed in these lines are due to a defect in Mn transfer from stems to leaves leading to limited Mn supply to leaves.

To further test this hypothesis, we analyzed Mn distribution in mature leaves from *PotriNRAMP3.1* OE lines compared to NT control. We dissected leaves into vein and lamina, and measured Mn separately (fig. 11E). In *PotriNRAMP3.1* OE lines, the Mn concentration

was lower in lamina compared with veins, whereas the concentrations in these two parts of the leaf were similar in NT control. Moreover, the Mn concentration in the lamina was lower in PotriNRAMP3.1 OE lines than in the NT control line, while the opposite was observed in veins. These results confirm that overexpression of *PotriNRAMP3.1* in poplar perturbs Mn distribution between and within organs. This suggests that PotriNRAMP3.1 is involved in Mn transport between organs and tissues.

To confirm that the leaf chlorosis symptoms observed in PotriNRAMP3.1 OE lines were due to Mn depletion in the lamina, we supplemented the trees with Mn. We grew tree cuttings for 4 weeks and then started watering half of them with 0.5 mM of MnSO₄ for an additional 5 weeks. We observed that chlorosis did not appear in newly formed leaves of Mn treated trees (supplementary fig. S13A). In these leaves, Mn concentrations were higher in lamina and veins, but the treatment did not restore the defect in Mn distribution between these tissues (supplementary fig. S13B). Chlorosis was not reverted in leaves formed prior to the treatment indicating that the treatment prevented chlorosis in newly formed leaves rather than corrected it in older leaves.

Discussion

In this study, we have characterized two poplar metal transporter NRAMP3 using a combination of phylogenetic, cell biological and molecular genetic approaches. We found that poplar genomes harbor two tandem copies of NRAMP3 gene under selection, whereas only one copy is present in *Salix* genus, the closest phylum. Moreover, we demonstrated that the two paralogues encode functional metal transporters. However, they have acquired different functions *in planta*. Whereas PotriNRAMP3.2 has the same function in metal retrieval from the vacuole as AtNRAMP3 and AtNRAMP4, PotriNRAMP3.1 displays a distinct subcellular localization to the TGN as well as a distinct function. Our results show that PotriNRAMP3.1 is involved in Mn distribution in poplar aerial organs. Elemental analyses indicate that poplar lines ectopically expressing *PotriNRAMP3.1* are impaired in Mn transfer from the stem to the leaves and, within the leaves, from the vein to the lamina, resulting in chlorosis and a decreased PS II efficiency. Together our results show that a gene duplication of NRAMP3 specific to the poplar genus gave rise to the neofunctionalization of one of the copies, while the other retained the conserved function described in other species and highlight an unsuspected role of the secretory pathway in cell-to-cell transport of Mn.

Distinct mechanisms for the formation of *NRAMP* gene pairs in *A. thaliana* and poplar

Duplication events are a driving force in evolution, facilitating adaptation to changing environments. Although gene duplication may be followed by accumulation of deleterious mutations and gene elimination, it may also lead to diversification of gene function and sub- or neo-functionalization (Yang et al. 2006). The ancestral angiosperm genome contained only 14000 genes or less (Proost et al. 2011). However, whole genome triplications occurring about 120 Ma, significantly increased the size of the Eudicotyledon genomes.

In *A. thaliana* *AtNRAMP3* and *AtNRAMP4* encode functionally redundant metal transporters (Lanquar et al. 2005). This pair of genes located on two different chromosomes are present in *A. thaliana*, *Arabidopsis lyrata* and *Noccea caerulescens* (Oomen et al. 2009), but not in *Carica papaya* and *Ricinus communis* genomes. Thus, they probably originate from a duplication that took place after the *Carica papaya* divergence that happened 72 Ma. The analysis of the duplicated regions of the *A. thaliana* genome has showed that *AtNRAMP3* and *AtNRAMP4* loci are located on the duplicated block 0204146800380, suggesting that the pair originates from one of these two whole genome duplications that occurred between 70 and 23 Ma in the *A. thaliana* lineage (Lanquar et al. 2005; Ming et al. 2008; Proost et al. 2011).

The poplar lineage has also undergone one whole genome duplication 60-65 Ma *i.e.*, before the *Populus* and *Salix* divergence that took place 52 Ma (Tuskan and et al 2006; Hou et al. 2016). Comparing gene order in *S. purpurea* and in *P. trichocarpa* showed genomic collinearity upstream and downstream *NRAMP3* loci, except that only one copy of *NRAMP3* is found in *S. purpurea* (fig. 1). In contrast, two copies of *NRAMP3* were found in all sequenced poplar genotypes (fig. 2). Thus, it is unlikely that this whole genome duplication account for the emergence of *NRAMP3.1* and *NRAMP3.2* genes specific to *Populus* species since this event happened before the *Populus* and *Salix* divergence. Moreover, gene tandem arrangements generally imply local duplication processes rather than whole genome duplications. However, the genomic sequence surrounding *Populus NRAMP3.1* and *NRAMP3.2* shows many remains of Class I long terminal repeats (LTR) retrotransposon elements (Gypsy) mainly located between the two genes. Retrotransposons can mediate gene duplications. However, such duplications usually create a typical intron-free copy which can be integrated throughout the genome and not specifically close to the initial copy (Freeling 2009). The conservation of intron/exon structure and the tandem arrangement of *Populus NRAMP3.1* and *NRAMP3.2*

suggest another mechanism. Repeated sequences of retrotransposons are known to stimulate intrachromosomal recombination events or unequal crossing over, leading to gene duplication (White et al. 1994; Flagel and Wendel 2009). The genome of poplar which contains significantly more gene tandems than that of *A. thaliana* (Proost et al. 2011), contains also three time more transposons (Ming et al. 2008). Thus, it is most likely that this mechanism accounts for the tandem duplication of *Populus NRAMP3.1* and *NRAMP3.2*. Thus, distinct mechanisms of gene duplication led to *NRAMP* gene pair formation in poplar and *A. thaliana*.

***Populus NRAMP3* copies are subjected to both positive and purifying selections**

Most gene duplications are thought to be rapidly followed by the loss of function of one of the copies. This fate is referred as non-functionalization (Freeling 2009). While one paralogue retains the preduplication function, selective constraints generally relax on the second paralogue which, therefore, accumulates deleterious mutations leading to loss of function (Ohno 1970). However, pseudogene formation is not the only fate of duplicated genes. In few cases, both copies can retain function after a duplication event for different reasons. First, environmental conditions may maintain selective pressure on both genes to retain preduplication function, increasing fitness through dose effect. For example, multiple tandem copies of *HMA4* are maintained in the metal hyperaccumulator species *Arabidopsis halleri* and *N. caerulea* leading to a high expression level essential for Zn and Cd translocation and metal tolerance (Ó Lochlainn et al. 2011; Craciun et al. 2012; Hanikenne et al. 2013).

Alternatively, relaxation of purifying selection allowed by gene duplication can also allow non-synonymous mutations while maintaining function in the two daughter genes. This can occur through two scenarios. One is the subfunctionalization, which is the partitioning of the preduplication function among daughter genes. The other one is the neofunctionalization which leads to the emergence of a new function in one of the gene copies, while preserving the sequence and the function of the ancestral gene in the other copy (Zhang 2003).

In the present study, dN/dS analysis highlights that *Populus NRAMP3.1* and *NRAMP3.2* sequences are mostly under purifying selection (fig. 3; supplementary tables S4 and S5) acting on many residues in the core conserved transmembrane domains of the protein. This is in agreement with the finding that both PotriNRAMP3.1 and PotriNRAMP3.2 have retained metal transport ability. However, they also exhibit a clear relaxation of purifying selection especially on residues localized at the N and C terminal ends of the protein (fig. 3; supplementary fig. S2,

tables S4 and S5). Mutations in the N terminal region, where motives involved in the targeting of AtNRAMP3 and AtNRAMP4 have previously been identified, likely enabled the protein to acquire distinct subcellular localizations (Müdsam et al. 2018). The vacuolar membrane localization of PotriNRAMP3.2 (fig. 8), its transport capacities (fig. 4, 5) and its ability to complement *A. thaliana nramp3nramp4* double mutant phenotypes (fig. 7) indicate that PotriNRAMP3.2 is the functional homologue of AtNRAMP3 and AtNRAMP4. The ability of PotriNRAMP3.1 to transport Fe and Mn in yeast (fig. 4, 5), its distinct intracellular localization (fig. 8, 9), its inability to complement the *nramp3nramp4* double mutant phenotypes (fig. 7) and the signature of purifying selection (fig. 3, supplementary fig. S2, tables S4 and S5) argue in favor of neo-functionalization rather than non-functionalization. The finding that *Populus NRAMP3.1* and *Populus NRAMP3.2* promoter sequences lack significant sequence identities further indicates that the regulation of the two copies has diverged (supplementary fig. S1D). This is in agreement with our previous report showing that *Populus NRAMP3.1* and *NRAMP3.2* belong to different networks of co-expressed genes (Pottier et al. 2015a). We propose a scenario in which after *Populus NRAMP3* gene duplication, relaxation of purifying selection allowed mutations altering PotriNRAMP3.1 subcellular localization. This mutated version of *Populus NRAMP3* was subsequently maintained by purifying selection, probably because it conferred improved fitness. Specific inactivation of *Populus NRAMP3.1* would allow to further test this scenario.

PotriNRAMP3.1 modulates tissue distribution of Mn

To investigate the function of these transporters *in planta*, we generated transgenic poplars over-expressing *PotriNRAMP3.1-GFP* and *PotriNRAMP3.2-GFP*. PotriNRAMP3.1 and PotriNRAMP3.2 subcellular localizations in poplar roots or leaves were similar to those observed in *A. thaliana*. (fig. 8, 9; supplementary fig. S9). Analysis of metal concentrations revealed a specific decrease in Mn concentrations in leaves from lines over-expressing *PotriNRAMP3.1-GFP*. Moreover, lines with the lowest Mn leaf concentrations displayed internervous chlorosis (fig. 10). In agreement with the role of Mn in photosynthesis, PS II efficiency was decreased in the chlorotic parts of *PotriNRAMP3.1* OE leaves (fig. 10). Further analysis showed that the decrease in Mn leaf concentration was associated to an increase in Mn concentration in stems (fig. 11). Furthermore, Mn distribution within leaves is also affected in *PotriNRAMP3.1* OE lines: Mn accumulates at higher levels in the veins while it is depleted in the lamina. Together, these results indicate that PotriNRAMP3.1 is involved in transcellular transport of Mn. This phenotype is unexpected as PotriNRAMP3.1-GFP exhibits a clear

intracellular localization to the TGN. To account with this phenotype, we propose a working model based on two hypotheses (1) Mn cell-to-cell transport requires Mn secretion (2) expression of *PotriNRAMP3.1* limits Mn secretion by allowing the retrieval of Mn from the TGN, which would otherwise be secreted. This model is illustrated in figure 12. The function of PotriNRAMP3.1 in retrieval of Mn from the secretory system is equivalent to the function of AtNRAMP2 (Alejandro et al. 2017; Gao et al. 2018). AtNRAMP2 localizes to the TGN and was proposed to retrieve Mn from this compartment to make it available for uptake into the chloroplasts. Previous work also showed that loss of AtMTP11, which also localizes in the secretory system, leads to an increase in plant Mn concentration (Peiter et al. 2007). As AtMTP11 is involved in Mn loading into the secretory system, the observed phenotype also agrees with the hypothesis that Mn concentrations in plant tissues are, at least in part, controlled by Mn secretion. As SMF2 has also been proposed to retrieve Mn from the secretory system in yeast, the proposed model would also account for the strong decrease in Mn content in the *smf2* mutant (Luk and Culotta 2001). Homologues of AtMTP11 and AtNRAMP2 are also present in poplar and could act in concert with PotriNRAMP3.1 to control the rate of Mn secretion vs intracellular distribution in poplar cells. Interestingly, mRNA levels of *PotriNRAMP2* and *PotriNRAMP3.1* are correlated in leaves (M. Pottier et al. 2015a).

Taken together, our results provide a clear case for neofunctionalization in a tandem of NRAMP genes specific to poplar. It also provides new insights into the cell-to-cell transport of divalent cations by showing the significant contribution of the secretory pathway in the cellular export of Mn. Besides, these results also provide new evidence of the essential role of transporters located at the secretory pathway in the regulation of the cellular storage of Mn. In the future, it would be interesting to find out what is the advantage conferred by the newly functionalized PotriNRAMP3.1 which led to the conservation of this gene in all examined poplar species. It will also be interesting to understand the interplay between PotriNRAMP3.1, PotriNRAMP2 and PotriMTP11 in the control of Mn concentration in the secretory pathway.

Materials and methods

Sequences analysis

Sequences were retrieved from Phytozome V13, PopGenIE V3, NCBI, TAIR and 1KP Project databases as indicated in the supplementary table S1 (Zhang 2003; Tuskan et al. 2006; Sjödin et al. 2009; Goodstein et al. 2012; Lamesch et al. 2012; Dai et al. 2014; Lin et al. 2018; Zhou

et al. 2018; Chen et al. 2019; One Thousand Plant Transcriptomes Initiative 2019; Sayers et al. 2021). To obtain the genomic sequence of *NRAMP3.1* (Potri007G050600) and *NRAMP3.2* (Potri007G050600) in *P. alba*, *P. cathayana*, *P. simonii*, *P. lasiocarpa*, *P. maximowiczii*, *P. euphratica*, *P. ussuriensis*, *P. nigra*, *P. deltoides*, and *P. mexicana*, raw reads were aligned to *P. trichocarpa* reference sequence V4.1 and the obtained genome consensus was blasted with *PotriNRAMP3.1* and *PotriNRAMP3.2* using QIAGEN CLC Genomics Workbench 12.0. *P. trichocarpa* and *S. purpurea* genomic DNA homologies were investigated by Dot-plot analyses performed on Gepard softwares V1.30 and V1.40 (Krumsiek et al. 2007).

Phylogenetic tree construction

Full-length amino acid sequences were aligned by CLUSTALW and imported into the Molecular Evolutionary Genetics Analysis (MEGA) package version 7 (Kumar et al. 2016). Phylogenetic analyses were conducted using the Maximum Likelihood method, based on the JTT matrix-based model (fig. 2) or Le_Gascuel (LG) model (supplementary fig. S1), implemented in MEGA7 (Jones et al. 1992; Le and Gascuel 2008). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the analyzed genes. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, +G, parameter = 0.4725 and parameter = 1.2613 for figure 2 and supplementary figure S1, respectively). Trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% and 90% site coverage were eliminated for figure 2 and supplementary figure S1, respectively.

Selective pressure analysis

To determine the nonsynonymous (dN) and synonymous (dS) substitution rates, and the dN/dS ratios along *NRAMP3* coding sequence, pairwise comparisons were performed among the 13 *NRAMP3.1* sequences, among the 13 *NRAMP3.2* sequences, as well as between the 13 *NRAMP3.1* and the 13 *NRAMP3.2* sequences following codon based alignment using the

JCoDA v1.4 package with the Nei-Gojobori substitution model, with a 20 residue window and a shift of 10 residues (Nei, M and Gojobori, T 1986; Steinway et al. 2010). Positive and purifying selections at individual sites were subsequently inferred using the Fixed Effects Likelihood (FEL) method (Nei, M and Gojobori, T 1986; Steinway et al. 2010). The 26 NRAMP coding sequences were used to generate a phylogenetic tree. A subset of branches encompassing either the *NRAMP3.1s* or the *NRAMP3.2s* were analyzed separately to estimate dS and dN at a site (α and β , respectively). A maximum-likelihood approach was then undertaken to calculate the dN/dS for each codon site (ω ; $p < 0.05$).

Gene expression

Total RNA from 1-month-old poplars or 1 week-old *A. thaliana* grown *in vitro* on half strength Murashige and Skoog (MS) or ABIS medium, respectively, was extracted using RNeasy Plant Mini Kit (Qiagen) as previously described (Pottier et al., 2015a). Five micrograms of DNA-free RNA were used for reverse transcription by the SuperScript III First-Strand kit (Invitrogen). Reverse transcriptions were performed using random hexamers. Gene-specific PCR primers were designed using OligoPerfect™ Designer (<http://tools.lifetechnologies.com>). All primers used for RT-qPCR are listed in supplementary table S6. One-sixth of the cDNAs was used as a template in 10- μ l qPCR reactions performed on a Roche LightCycler 96 using Roche reagents, according to the manufacturer's instructions (<http://www.roche.com>). Primer specificity was confirmed by analysis of the melting curves and sequencing of the PCR products. Relative transcript levels were calculated by normalization to the transcript amount to constitutively expressed genes.

Construction of expression vectors.

PotriNRAMP3.1 and *PotriNRAMP3.2* CDSs were amplified by PCR from cDNA synthesized from leaf RNA of *P. trichocarpa* cv Nisqually-1. Primer PtNramp3aLgtw encompassing the ATG initiation codon was used with PtNramp3aRgtwstp encompassing the TAA stop codon or with PtNramp3aRgtwnostp designed before the TAA stop codon. Primer PtNramp3bLlonggtw encompassing the ATG initiation codon was used with PtNramp3bRlonggtwstp encompassing the TAA stop codon or with PtNramp3bRlonggtwnostp designed before the TAA stop codon. For *PotriNRAMP3.1*, amplification was performed in 2 steps, first with specific primers and

second using the generic U5 and U3 primers. Forward and reverse primers used for *NRAMP* cloning are shown in supplementary table S7. PCRs were performed with the Phusion high-fidelity DNA polymerase (Thermo-Scientific). The gel-purified PCR products were recombined into pDONR207 for *PotriNRAMP3.1* and into pDONR201 for *PotriNRAMP3.2* following the BP Clonase (Invitrogen) manufacturer's instruction.

For the generation of yeast expression vectors, LR reactions were performed using the pDR195gtw vector adapted to Gateway cloning (Rentsch et al. 1995; Oomen et al. 2009) and the pDONR (Invitrogen) containing *PotriNRAMP3.1* and *PotriNRAMP3.2* with stop codon.

For the generation of plant expression vectors, LR reactions, were performed to transfer *PotriNRAMP3s* fragments with or without stop codon from pDONR vectors to the Gateway binary over-expression vectors pB7WGF2, pB7FWG2 (Karimi et al. 2002) and pMDC83 (Curtis and Grossniklaus 2003) that contain the 35S CaMV promoter (p35S) and *GFP* sequence allowing to generate *PotriNRAMP3-GFP* fusion proteins. *PotriNRAMP3s* fragments with stop codon were also transferred in the pUB-DEST vector to drive their expression under control of Ubiquitin 10 promoter (Grefen et al. 2010).

Yeast strains, transformations and media

DEY1453 (*fet3fet4*, Eide et al. 1996), *smf1* (Supek, et al. 1996; Thomine, et al. 2000) and *smf2* (Cohen, et al. 2000) yeast strains were grown at 30°C on Yeast extract/Peptone/Dextrose (before transformation) or synthetic dextrose -ura (after transformation). For the DEY1453 strain, media were supplemented with 0.2 mM FeCl₃. Yeast cells were transformed according to standard procedures (Invitrogen).

Yeast growth assays

Transformed *fet3fet4*, *smf1*, *smf2* yeast mutants were grown overnight in liquid synthetic dextrose -ura (pH 6) supplemented with 0.2 mM FeCl₃ for *fet3fet4* mutants. The cultures were diluted to ODs of 1 to 10⁻³ and spotted on synthetic dextrose-ura plates (pH 6). Transformed *fet3fet4* strains were spotted on media supplemented with 100 µM of the Fe chelator BathoPhenanthroline-Di-Sulfonic acid (BPDS) and 0.2 mM FeCl₃ (+ Fe) or with 100 µM BPDS and 40 µM FeCl₃ (-Fe). Transformed *smf1* strains were spotted on media supplemented with 5 mM ethylene glycolbis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) and 100 µM

MnSO₄ (+ Mn) or with 5 mM EGTA without MnSO₄ (- Mn). Transformed *smf2* strains were spotted on media supplemented with 5 mM EGTA and 100 μM MnSO₄ (+ Mn), with 5 mM EGTA without MnSO₄ (- Mn) or with 10 mM MnSO₄ (+++ Mn).

Confocal imaging

Roots of *in vitro* grown 6-day-old *A. thaliana* seedlings or 2-3 week-old poplar explants were mounted in liquid culture medium, and confocal images of epidermal cells in the elongation zone were obtained by high-speed (100 ms) sequential acquisition of GFP ($\lambda_{\text{ex}} = 490$ nm, $\lambda_{\text{em}} = 500$ –550 nm) and mRFP ($\lambda_{\text{ex}} = 590$ nm, $\lambda_{\text{em}} = 600$ –650 nm, employing a Nipkow spinning disk confocal system equipped with a Prime 95Bcamera (Photometrics) and a Nikon 100X 1.4 aperture oil immersion objective. Super resolution images were generated with a Live-SR module for optically demodulated structured illumination (GATACA Systems). Image processing (cropping, contrast adjustment and background subtraction) was performed with ImageJ 1.45s program (Schneider et al. 2012).

Plant material and plant transformation

The generation of the *nramp3nramp4* double mutants of *A. thaliana* Col-0 was described previously (Molins *et al.* 2012). *PotriNRAMP3.1* and *PotriNRAMP3.2* constructs were introduced in Col-0 *nramp3nramp4* mutants through *A. tumefaciens* (strain AGL0) mediated transformation using the flower dip method (Clough 2005). Independent homozygous *A. thaliana* Col-0 *nramp3nramp4* transformants with a single insertion locus were obtained by plant selection based on Basta resistance and complementation of the *nramp3nramp4* phenotype on low Fe medium.

The poplar INRA 717-1-B4 clone (*P. tremula* x *P. alba*) was transformed as described (Leple et al. 1992). Briefly, stem explants were excised from *in vitro* grown poplar and incubated for 2 days in the dark on M1 medium (Supplementary table S8). The explants were then co-cultivated for 16 h at 24°C on an orbital shaker (180 rpm) with 150 ml of a suspension (OD 0.3) of *Agrobacterium tumefaciens* C58/pMP90 transformed with *p35S:PotriNRAMP3.1-GFP* or *p35S:PotriNRAMP3.2-GFP* constructs in pMDC83. The explants were washed 5 times in sterile water under orbital shaking (140 rpm). After washing, they were transferred on M2 medium containing Ticarpen, cefotaxime and hygromycin B, and incubated at 24°C in darkness

for 21 days (Supplementary table S8). The plates were subsequently transferred to light (16 h 130 $\mu\text{E}\cdot\text{m}^{-2}/8\text{h}$ dark) to recover green calli. The green calli were transferred to M3 agar medium with hygromycin B (Supplementary table S8). The regenerated transformed shoots were then transferred on half strength MS agar medium with hygromycin to allow root regeneration (Supplementary table S8). The transgenic lines were propagated on the same medium until transfer to pots in the greenhouse.

A. thaliana growth conditions

A. thaliana seedlings were grown on ABIS medium plates containing 2.5 mM H_3PO_4 , 5 mM KNO_3 , 2 mM MgSO_4 , 1 mM $\text{Ca}(\text{NO}_3)_2$, MS microelements, 1% sucrose, 1% Phytigel, 1 mM MES adjusted with KOH to pH 6.1 and FeHBED (N,N'-di(2-hydroxybenzyl) ethylenediamine-N,N'-diacetic acid monochloride hydrate; Strem Chemicals, Newburyport, MA, USA) as indicated in figure legends. FeHBED was prepared as described by Lanquar *et al.* (Lanquar, et al. 2005). For low iron sensitivity growth assays, plants were grown for 8 days on plates where Fe was omitted. Plates were placed vertically in environmental growth chambers (Sanyo MLR-350, Morigushi, Japan) at 21°C with a 16 h photoperiod under 120 $\mu\text{mol photon}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Photosystem II maximum quantum yield

Photosystem II maximum quantum yield was determined using Imaging PAM (Walz, Germany). Efficiency of the photosynthetic electron transport (F_v/F_m) was assayed by calculating the ratio of variable fluorescence (F_v) to maximal fluorescence (F_m) after a saturating light pulse (Maxwell & Johnson 2000). Plants were dark adapted for 15 min prior to measurements. Five areas of interest (AOI) in the green part and 5 AOI in the chlorotic parts of each leaf were selected for quantification in three independent leaves of each genotype.

Root length measurements

Plants grown vertically on plates were photographed at the indicated times and root length was determined using the ImageJ 1.45s program and a digitizer tablet (WACOM Europe GMBH, Krefeld, Germany, Intuos 4M). Values are the average of measurements on 10–12 roots.

Metal concentration analysis

For metal analyses in yeast, liquid SD -ura medium containing transformed *smf2* strain growing overnight were diluted to OD 0.3 in liquid SD -ura supplemented with 30 μ M FeCl₃ and 10 μ M MnSO₄. After 30 h of incubation at 30°C under agitation, yeast cells were recovered by centrifugation (3340g, 5 min, 4°C) and washed twice in 50 ml ice cold YNB supplemented with EDTA 20 mM and MES 50 mM pH6, pelleted and then washed in ice cold ultrapure water.

For metal analyses in plants, shoots and roots were harvested separately and washed. Roots were desorbed by incubation in ice-cold buffer containing 5 mM CaCl₂ and 1 mM MES-KOH, pH 5.7, for 10 min.

The dry weight (DW) of the samples (yeasts or plants) was measured after drying at 60°C for 3 days. The dried samples were digested in 2 ml of 70 % nitric acid in a DigiBlock ED36 (LabTech, Italy) at 80°C for 1 h, 100°C for 1 h and 120°C for 2 h. After dilution in trace-metal-free water, the metal content of the samples was determined by atomic absorption spectrometry using an AA240FS flame spectrometer (Agilent, USA) or by atomic emission spectroscopy using a MP AES 1200 spectrometer (Agilent, USA).

Statistical analysis

Data were analyzed with Kruskal-Wallis and Mann-Whitney non-parametric tests for multiple comparisons and pair comparisons, respectively. For multiple comparisons, a Dunn's post hoc test was performed when significant differences were detected. Both tests were performed using GraphPad Prism 7.

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References

- Adams JP, Adeli A, Hsu C-Y, Harkess RL, Page GP, dePamphilis CW, Schultz EB, Yuceer C. 2011. Poplar maintains zinc homeostasis with heavy metal genes HMA4 and PCS1. *J. Exp. Bot.* 62:3737–3752.
- Alejandro S, Cailliatte R, Alcon C, Dirick L, Domergue F, Correia D, Castaings L, Briat JF, Mari S, Curie C. 2017. Intracellular Distribution of Manganese by the Trans-Golgi Network Transporter NRAMP2 Is Critical for Photosynthesis and Cellular Redox Homeostasis. *Plant Cell* 29:3068–3084.
- Alejandro S, Höller S, Meier B, Peiter E. 2020. Manganese in Plants: From Acquisition to Subcellular Allocation. *Front. Plant Sci.* 11:300.
- Blaudez D, Kohler A, Martin F, Sanders D, Chalot M. 2003. Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. *Plant Cell* 15:2911–2928.
- Cailliatte R, Schikora A, Briat JF, Mari S, Curie C. 2010. High-affinity manganese uptake by the metal transporter NRAMP1 is essential for Arabidopsis growth in low manganese conditions. *Plant Cell* 22:904–917.
- Castaings L, Caquot A, Loubet S, Curie C. 2016. The high-affinity metal Transporters NRAMP1 and IRT1 Team up to Take up Iron under Sufficient Metal Provision. *Sci. Rep.* 6:37222.
- Chen J, Huang Y, Brachi B, Yun Q, Zhang W, Lu W, Li H, Li W, Sun X, Wang G, et al. 2019. Genome-wide analysis of Cushion willow provides insights into alpine plant divergence in a biodiversity hotspot. *Nat. Commun.* 10:5230.
- Cohen A, Nelson H, Nelson N. 2000. The family of SMF metal ion transporters in yeast cells. *J. Biol. Chem.* 275:33388–33394.
- Conant GC, Wolfe KH. 2008. Turning a hobby into a job: how duplicated genes find new functions. *Nat. Rev. Genet.* 9:938–950.
- Connorton JM, Balk J, Rodríguez-Celma J. 2017. Iron homeostasis in plants – a brief overview. *Metallomics* 9:813–823.
- Craciun AR, Meyer C-L, Chen J, Roosens N, De Groodt R, Hilson P, Verbruggen N. 2012. Variation in HMA4 gene copy number and expression among *Noccaea caerulescens* populations presenting different levels of Cd tolerance and accumulation. *J. Exp. Bot.* 63:4179–4189.
- Curtis MD, Grossniklaus U. 2003. A Gateway Cloning Vector Set for High-Throughput Functional Analysis of Genes in Planta. *Plant Physiol.* 133:462–469.
- Dai X, Hu Q, Cai Q, Feng K, Ye N, Tuskan GA, Milne R, Chen Y, Wan Z, Wang Z, et al. 2014. The willow genome and divergent evolution from poplar after the common genome duplication. *Cell Res.* 24:1274–1277.
- Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardson AE. 2007. A role for the AtMTP11 gene of Arabidopsis in manganese transport

754 and tolerance. *Plant J* 51:198–210.

755 Ebine K, Fujimoto M, Okatani Y, Nishiyama T, Goh T, Ito E, Dainobu T, Nishitani A,
756 Uemura T, Sato MH, et al. 2011. A membrane trafficking pathway regulated by the plant-
757 specific RAB GTPase ARA6. *Nat. Cell Biol.* 13:853–859.

758 Eide D, Broderius M, Fett J, Guerinot ML. 1996. A novel iron-regulated metal transporter
759 from plants identified by functional expression in yeast. *Proc. Natl. Acad. Sci.* 93:5624–5628.

760 Eisenhut M, Hoecker N, Schmidt SB, Basgaran RM, Flachbart S, Jahns P, Eser T, Geimer S,
761 Husted S, Weber APM, et al. 2018. The Plastid Envelope CHLOROPLAST MANGANESE
762 TRANSPORTER1 Is Essential for Manganese Homeostasis in Arabidopsis. *Mol Plant*
763 11:955–969.

764 Eroglu S, Meier B, von Wiren N, Peiter E. 2016. The Vacuolar Manganese Transporter MTP8
765 Determines Tolerance to Iron Deficiency-Induced Chlorosis in Arabidopsis. *Plant Physiol*
766 170:1030–1045.

767 Flagel LE, Wendel JF. 2009. Gene duplication and evolutionary novelty in plants. *New*
768 *Phytol.* 183:557–564.

769 Freeling M. 2009. Bias in Plant Gene Content Following Different Sorts of Duplication:
770 Tandem, Whole-Genome, Segmental, or by Transposition. *Annu. Rev. Plant Biol.* 60:433–
771 453.

772 Gao H, Xie W, Yang C, Xu J, Li J, Wang H, Chen X, Huang CF. 2018. NRAMP2, a trans-
773 Golgi network-localized manganese transporter, is required for Arabidopsis root growth under
774 manganese deficiency. *New Phytol* 217:179–193.

775 Gao Y, Yang F, Liu J, Xie W, Zhang L, Chen Z, Peng Z, Ou Y, Yao Y. 2020. Genome-Wide
776 Identification of Metal Tolerance Protein Genes in *Populus trichocarpa* and Their Roles in
777 Response to Various Heavy Metal Stresses. *Int. J. Mol. Sci.* 21:1680.

778 Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W,
779 Hellsten U, Putnam N, et al. 2012. Phytozome: a comparative platform for green plant
780 genomics. *Nucleic Acids Res.* 40:D1178–D1186.

781 Grefen C, Donald N, Hashimoto K, Kudla J, Schumacher K, Blatt MR. 2010. A ubiquitin-10
782 promoter-based vector set for fluorescent protein tagging facilitates temporal stability and
783 native protein distribution in transient and stable expression studies: Fluorescence tagging and
784 expression in Arabidopsis. *Plant J.* 64:355–365.

785 Hanikenne M, Kroymann J, Trampczynska A, Bernal M, Motte P, Clemens S, Krämer U.
786 2013. Hard selective sweep and ectopic gene conversion in a gene cluster affording
787 environmental adaptation. *PLoS Genet.* 9:e1003707.

788 He J, Li H, Ma C, Zhang Y, Polle A, Rennenberg H, Cheng X, Luo Z-B. 2015.
789 Overexpression of bacterial γ -glutamylcysteine synthetase mediates changes in cadmium
790 influx, allocation and detoxification in poplar. *New Phytol.* 205:240–254.

791 Hou J, Ye N, Dong Z, Lu M, Li L, Yin T. 2016. Major Chromosomal Rearrangements
792 Distinguish Willow and Poplar After the Ancestral “Salicoid” Genome Duplication. *Genome*
793 *Biol. Evol.* 8:1868–1875.

794 Inada N, Betsuyaku S, Shimada TL, Ebine K, Ito E, Kutsuna N, Hasezawa S, Takano Y,
795 Fukuda H, Nakano A, et al. 2016. Modulation of Plant RAB GTPase-Mediated Membrane
796 Trafficking Pathway at the Interface Between Plants and Obligate Biotrophic Pathogens.
797 *Plant Cell Physiol.* 57:1854–1864.

798 Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices
799 from protein sequences. *Bioinformatics* 8:275–282.

800 Karimi M, Inzé D, Depicker A. 2002. GATEWAYTM vectors for Agrobacterium-mediated
801 plant transformation. *Trends Plant Sci.* 7:193–195.

802 Kramer U. 2005a. Phytoremediation: novel approaches to cleaning up polluted soils. *Curr*
803 *Opin Biotechnol* 16:133–141.

804 Kramer U. 2005b. MTP1 mops up excess zinc in Arabidopsis cells. *Trends Plant Sci.* 10:313–
805 315.

806 Krumsiek J, Arnold R, Rattei T. 2007. Gepard: a rapid and sensitive tool for creating dotplots
807 on genome scale. *Bioinformatics* 23:1026–1028.

808 Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis
809 Version 7.0 for Bigger Datasets. *Mol. Biol. Evol.* 33:1870–1874.

810 Lamesch P, Berardini TZ, Li D, Swarbreck D, Wilks C, Sasidharan R, Muller R, Dreher K,
811 Alexander DL, Garcia-Hernandez M, et al. 2012. The Arabidopsis Information Resource
812 (TAIR): improved gene annotation and new tools. *Nucleic Acids Res.* 40:D1202–D1210.

813 Lanquar V, Lelièvre F, Bolte S, Hames C, Alcon C, Neumann D, Vansuyt G, Curie C,
814 Schroder A, Kramer U, et al. 2005. Mobilization of vacuolar iron by AtNRAMP3 and
815 AtNRAMP4 is essential for seed germination on low iron. *EMBO J* 24:4041–4051.

816 Lanquar V, Ramos MS, Lelievre F, Barbier-Brygoo H, Krieger-Liszkay A, Kramer U,
817 Thomine S. 2010. Export of vacuolar manganese by AtNRAMP3 and AtNRAMP4 is required
818 for optimal photosynthesis and growth under manganese deficiency. *Plant Physiol* 152:1986–
819 1999.

820 Le SQ, Gascuel O. 2008. An Improved General Amino Acid Replacement Matrix. *Mol. Biol.*
821 *Evol.* 25:1307–1320.

822 Leplé J-C, Brasileiro A-C, Michel M-F, Delmotte F., Jouanin L. 1992. Transgenic poplars:
823 expression of chimeric genes using four different constructs. *Plant Cell Rep.* [Internet] 11.
824 Available from: <http://link.springer.com/10.1007/BF00232166>

825 Li D, Xu X, Hu X, Liu Q, Wang Z, Zhang H, Wang Han, Wei M, Wang Hanzeng, Liu H, et
826 al. 2015. Genome-Wide Analysis and Heavy Metal-Induced Expression Profiling of the HMA
827 Gene Family in Populus trichocarpa. *Front. Plant Sci.* 6:1149.

828 Lin Y-C, Wang J, Delhomme N, Schiffthaler B, Sundström G, Zuccolo A, Nystedt B,
829 Hvidsten TR, de la Torre A, Cossu RM, et al. 2018. Functional and evolutionary genomic
830 inferences in *Populus* through genome and population sequencing of American and European
831 aspen. *Proc. Natl. Acad. Sci.* 115:E10970–E10978.

832 Luk EE, Culotta VC. 2001. Manganese superoxide dismutase in *Saccharomyces cerevisiae*

833 acquires its metal co-factor through a pathway involving the Nramp metal transporter, Smf2p.
834 *J. Biol. Chem.* 276:47556–47562.

835 Migeon A, Blaudez D, Wilkins O, Montanini B, Campbell MM, Richaud P, Thomine S,
836 Chalot M. 2010. Genome-wide analysis of plant metal transporters, with an emphasis on
837 poplar. *Cell. Mol. Life Sci. CMLS* 67:3763–3784.

838 Ming R, Hou S, Feng Y, Yu Q, Dionne-Laporte A, Saw JH, Senin P, Wang W, Ly BV, Lewis
839 KLT, et al. 2008. The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya*
840 Linnaeus). *Nature* 452:991–996.

841 Moriyama Y, Ito F, Takeda H, Yano T, Okabe M, Kuraku S, Keeley FW, Koshiba-Takeuchi
842 K. 2016. Evolution of the fish heart by sub/neofunctionalization of an elastin gene. *Nat.*
843 *Commun.* 7:10397.

844 Müdsam C, Wollschläger P, Sauer N, Schneider S. 2018. Sorting of *Arabidopsis* NRAMP3
845 and NRAMP4 depends on adaptor protein complex AP4 and a dileucine-based motif. *Traffic*
846 19:503–521.

847 Nei, M, Gojobori, T. 1986. Simple methods for estimating the numbers of synonymous and
848 nonsynonymous nucleotide substitutions. *Mol. Biol. Evol.* [Internet]. Available from:
849 [https://academic.oup.com/mbe/article/3/5/418/988012/Simple-methods-for-estimating-the-](https://academic.oup.com/mbe/article/3/5/418/988012/Simple-methods-for-estimating-the-numbers-of)
850 [numbers-of](https://academic.oup.com/mbe/article/3/5/418/988012/Simple-methods-for-estimating-the-numbers-of)

851 Ó Lochlainn S, Bowen HC, Fray RG, Hammond JP, King GJ, White PJ, Graham NS,
852 Broadley MR. 2011. Tandem Quadruplication of HMA4 in the Zinc (Zn) and Cadmium (Cd)
853 Hyperaccumulator *Nocca caerulea*. Baxter I, editor. *PLoS ONE* 6:e17814.

854 One Thousand Plant Transcriptomes Initiative. 2019. One thousand plant transcriptomes and
855 the phylogenomics of green plants. *Nature* 574:679–685.

856 Oomen RJFJ, Wu J, Lelièvre F, Blanchet S, Richaud P, Barbier-Brygoo H, Aarts MGM,
857 Thomine S. 2009. Functional characterization of NRAMP3 and NRAMP4 from the metal
858 hyperaccumulator *Thlaspi caerulescens*. *New Phytol.* 181:637–650.

859 Peiter E, Montanini B, Gobert A, Pendas P, Husted S, Maathuis FJ, Blaudez D, Chalot M,
860 Sanders D. 2007. A secretory pathway-localized cation diffusion facilitator confers plant
861 manganese tolerance. *Proc Natl Acad Sci U S A* 104:8532–8537.

862 Portnoy ME, Liu XF, Culotta VC. 2000. *Saccharomyces cerevisiae* expresses three
863 functionally distinct homologues of the Nramp family of metal transporters. *Mol. Cell. Biol.*
864 20:7893–7902.

865 Pottier M., Garcia de la Torre VS, Victor C, David LC, Chalot M, Thomine S. 2015a.
866 Genotypic variations in the dynamics of metal concentrations in poplar leaves: a field study
867 with a perspective on phytoremediation. *Env. Pollut* 199:73–82.

868 Pottier M., Oomen R., Picco C., Giraudat J., Scholz-Starke J., Richaud P., Carpaneto A.,
869 Thomine S. 2015b. Identification of mutations allowing Natural Resistance Associated
870 Macrophage Proteins (NRAMP) to discriminate against cadmium. *Plant J.* 83:625–637.

871 Proost S, Pattyn P, Gerats T, Van de Peer Y. 2011. Journey through the past: 150 million
872 years of plant genome evolution: One hundred and fifty million years of plant genome

873 evolution. *Plant J.* 66:58–65.

874 Ravet K, Danford FL, Dihle A, Pittarello M, Pilon M. 2011. Spatiotemporal analysis of
875 copper homeostasis in *Populus trichocarpa* reveals an integrated molecular remodeling for a
876 preferential allocation of copper to plastocyanin in the chloroplasts of developing leaves.
877 *Plant Physiol.* 157:1300–1312.

878 Ren L-L, Liu Y-J, Liu H-J, Qian T-T, Qi L-W, Wang X-R, Zeng Q-Y. 2014. Subcellular
879 Relocalization and Positive Selection Play Key Roles in the Retention of Duplicate Genes of
880 *Populus* Class III Peroxidase Family. *Plant Cell* 26:2404–2419.

881 Rentsch D, Laloi M, Rouhara I, Schmelzer E, Delrot S, Frommer WB. 1995. *NTR1* encodes a
882 high affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett.* 370:264–268.

883 Sayers EW, Beck J, Bolton EE, Bourex D, Brister JR, Canese K, Comeau DC, Funk K, Kim
884 S, Klimke W, et al. 2021. Database resources of the National Center for Biotechnology
885 Information. *Nucleic Acids Res.* 49:D10–D17.

886 Schneider A, Steinberger I, Herdean A, Gandini C, Eisenhut M, Kurz S, Morper A, Hoecker
887 N, Ruhle T, Labs M, et al. 2016. The Evolutionarily Conserved Protein PHOTOSYNTHESIS
888 AFFECTED MUTANT71 Is Required for Efficient Manganese Uptake at the Thylakoid
889 Membrane in *Arabidopsis*. *Plant Cell* 28:892–910.

890 Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image
891 analysis. *Nat. Methods* 9:671–675.

892 Seregin IV, Kozhevnikova AD. 2020. Low-molecular-weight ligands in plants: role in metal
893 homeostasis and hyperaccumulation. *Photosynth. Res.*

894 Shen JR. 2015. The Structure of Photosystem II and the Mechanism of Water Oxidation in
895 Photosynthesis. *Annu Rev Plant Biol* 66:23–48.

896 Sjödin A, Street NR, Sandberg G, Gustafsson P, Jansson S. 2009. The *Populus* Genome
897 Integrative Explorer (PopGenIE): a new resource for exploring the *Populus* genome. *New*
898 *Phytol.* 182:1013–1025.

899 Steinway SN, Dannenfelser R, Laucius CD, Hayes JE, Nayak S. 2010. JCoDA: a tool for
900 detecting evolutionary selection. *BMC Bioinformatics* 11:284.

901 Supek F, Supekova L, Nelson H, Nelson N. 1996. A yeast manganese transporter related to
902 the macrophage protein involved in conferring resistance to mycobacteria. *Proc. Natl. Acad.*
903 *Sci.* 93:5105–5110.

904 Thomine S, Lelièvre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H. 2003. AtNRAMP3, a
905 multispecific vacuolar metal transporter involved in plant responses to iron deficiency:
906 *NRAMP vacuolar metal transporter*. *Plant J.* 34:685–695.

907 Thomine S, Wang R, Ward JM, Crawford NM, Schroeder JI. 2000. Cadmium and iron
908 transport by members of a plant transporter gene family in *Arabidopsis* with homology to
909 *NRAMP* genes. *Proc Nat Acad Sci USA* 97:4991–4996.

910 Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S,
911 Rombauts S, Salamov A, et al. 2006. The Genome of Black Cottonwood, *Populus trichocarpa*

912 (Torr. & Gray). *Science* 313:1596–1604.

913 Tuskan GA, et al. 2006. The genome of black cottonwood, *Populus trichocarpa* (Torr. &
914 Gray). *Science* 313:1596–1604.

915 Uemura T, Kim H, Saito C, Ebine K, Ueda T, Schulze-Lefert P, Nakano A. 2012. Qa-
916 SNAREs localized to the trans-Golgi network regulate multiple transport pathways and
917 extracellular disease resistance in plants. *Proc. Natl. Acad. Sci.* 109:1784–1789.

918 Uemura T, Nakano RT, Takagi J, Wang Y, Kramer K, Finkemeier I, Nakagami H, Tsuda K,
919 Ueda T, Schulze-Lefert P, et al. 2019. A Golgi-Released Subpopulation of the Trans-Golgi
920 Network Mediates Protein Secretion in Arabidopsis. *Plant Physiol.* 179:519–532.

921 Vidal SM, Malo D, Vogan K, Skamene E, Gros P. 1993. Natural Resistance to Infection With
922 Intracellular Parasites - Isolation of a Candidate For Bcg. *Cell* 73:469–485.

923 Viotti C, Bubeck J, Stierhof Y-D, Krebs M, Langhans M, van den Berg W, van Dongen W,
924 Richter S, Geldner N, Takano J, et al. 2010. Endocytic and Secretory Traffic in *Arabidopsis*
925 Merge in the Trans-Golgi Network/Early Endosome, an Independent and Highly Dynamic
926 Organelle. *Plant Cell* 22:1344–1357.

927 Wang H, Liu Yuanyuan, Peng Z, Li J, Huang W, Liu Yan, Wang X, Xie S, Sun L, Han E, et
928 al. 2019. Ectopic Expression of Poplar ABC Transporter PtoABCG36 Confers Cd Tolerance
929 in Arabidopsis thaliana. *Int. J. Mol. Sci.* 20.

930 Wang M, Zhang L, Zhang Z, Li M, Wang D, Zhang X, Xi Z, Keefover - Ring K, Smart LB,
931 DiFazio SP, et al. 2020. Phylogenomics of the genus *Populus* reveals extensive interspecific
932 gene flow and balancing selection. *New Phytol.* 225:1370–1382.

933 Wessling-Resnick M. 2015. Nramp1 and Other Transporters Involved in Metal Withholding
934 during Infection. *J. Biol. Chem.* 290:18984–18990.

935 Whelan S, Goldman N. 2001. A General Empirical Model of Protein Evolution Derived from
936 Multiple Protein Families Using a Maximum-Likelihood Approach. *Mol. Biol. Evol.* 18:691–
937 699.

938 White SE, Habera LF, Wessler SR. 1994. Retrotransposons in the flanking regions of normal
939 plant genes: a role for copia-like elements in the evolution of gene structure and expression.
940 *Proc. Natl. Acad. Sci.* 91:11792–11796.

941 Yang Chang-Hong, Wang C, Singh S, Fan N, Liu S, Zhao L, Cao H, Xie W, Yang Chengwei,
942 Huang C. 2021. Golgi-localised manganese transporter PML3 regulates *Arabidopsis* growth
943 through modulating Golgi glycosylation and cell wall biosynthesis. *New Phytol.*:nph.17209.

944 Yang X, Tuskan GA, Cheng (Max) Zong-Ming. 2006. Divergence of the Dof Gene Families
945 in Poplar, Arabidopsis, and Rice Suggests Multiple Modes of Gene Evolution after
946 Duplication. *Plant Physiol.* 142:820–830.

947 Zhang B, Zhang C, Liu C, Jing Y, Wang Y, Jin L, Yang L, Fu A, Shi J, Zhao F, et al. 2018.
948 Inner Envelope CHLOROPLAST MANGANESE TRANSPORTER 1 Supports Manganese
949 Homeostasis and Phototrophic Growth in Arabidopsis. *Mol Plant* 11:943–954.

950 Zhang B, Zhu W, Diao S, Wu X, Lu J, Ding C, Su X. 2019. The poplar pangenome provides

951 insights into the evolutionary history of the genus. *Commun. Biol.* 2:215.

952 Zhang J. 2003. Evolution by gene duplication: an update. *Trends Ecol. Evol.* 18:292–298.

953 Zhou R, Macaya-Sanz D, Rodgers-Melnick E, Carlson CH, Gouker FE, Evans LM, Schmutz
954 J, Jenkins JW, Yan J, Tuskan GA, et al. 2018. Characterization of a large sex determination
955 region in *Salix purpurea* L. (Salicaceae). *Mol. Genet. Genomics* 293:1437–1452.

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Figure legends

Fig. 1. Collinearity between *Salix purpurea* and *Populus trichocarpa* genomes in the area surrounding *NRAMP3* loci. Gene collinearity between *P. trichocarpa* and *S. purpurea* is maintained around *NRAMP3* loci. (A) Dot-plots performed on Gepard software V1.40 (Krumsiek *et al.*, 2007), using 107 kb and 140 kb sequences of *S. purpurea* and *P. trichocarpa* genomes centered on *NRAMP3* loci, respectively. Sequence homology between *S. purpurea* and *P. trichocarpa* are indicated by black dots. Red arrows indicate homology between *SapurNRAMP3* and *PotriNRAMP3.2* as well as between *SapurNRAMP3* and *PotriNRAMP3.1*. (B) Schematic representation of the genomic sequence around *NRAMP3* locus in *P. trichocarpa* and *S. purpurea*.

Fig. 2. Phylogenetic tree of *NRAMP3* homologues in *Populus* and *Salix* species. *Arabidopsis thaliana* *NRAMP2*, *NRAMP3* and *NRAMP4* as well as *NRAMP2* genes from *Populus trichocarpa* and *Salix purpurea* are shown as an outgroup. \$ indicates incomplete protein sequences. Phylogenetic analyses were conducted as described in Materials and Methods. Protein sequences used for this tree are listed in supplementary data S3.

Fig. 3. Alignment of the consensus sequences of *Populus* *NRAMP3.1* and *NRAMP3.2*. The consensus sequences were determined from the *NRAMP* alignments shown in supplementary figure S3. Asterisk indicates less than 90% of conservation within *Populus* *NRAMP3.1* or *NRAMP3.2* cluster. Identical, similar and different residues between the two consensus sequences are indicated in black, blue, and red, respectively. Frames indicate residues under purifying selection and green arrows indicate positive selection in either *Populus* *NRAMP3.1* or *NRAMP3.2* cluster according to the FEL method ($p < 0.05$). Note that *NRAMP3.1* sequences contain an insertion of one residue at position 4 leading to a gap (-) in *NRAMP3.2* sequences.

Fig. 4. *PotriNRAMP3.1* and *PotriNRAMP3.2* encode functional Mn transporters. Functional complementation of *smf1* (A) and *smf2* (B) yeast mutants. Yeast cells were transformed with pDR195gtw vector containing the cDNA of *GUS*, *AtNRAMP1*, *AtNRAMP2*, *PotriNRAMP3.1* or *PotriNRAMP3.2*. Transformed yeasts were grown overnight in liquid synthetic dextrose -ura.

The cultures were diluted to ODs of 1 to 10^{-3} and spotted on synthetic dextrose -ura plates. Transformed *smf1* (A) strains were grown on medium supplemented with 5 mM EGTA and 100 μ M MnSO₄ (+ Mn) or with 5 mM EGTA without MnSO₄ (- Mn). Transformed *smf2* (B) strains were grown in medium supplemented with 10 mM EGTA and 100 μ M MnSO₄ (+ Mn), with 5 mM EGTA without MnSO₄ (- Mn) or with 10 mM MnSO₄ (+++ Mn). The plates were incubated at 30°C for 5 days (*smf1*) or 2 days (*smf2*) before photography. White lines indicate cropping, unmodified pictures are shown as supplementary figures S4 and S5. (C) Expression of *PotriNRAMP3.1* or *PotriNRAMP3.2* increases Mn accumulation in yeast cells. Liquid SD -ura medium containing *smf2* cells expressing *GUS*, *AtNRAMP4*, *PotriNRAMP3.1* or *PotriNRAMP3.2* were grown overnight and diluted to OD 0.3 in liquid SD -ura supplemented with 30 μ M FeCl₃ and 10 μ M MnSO₄. After 30 hrs of incubation at 30°C, yeast cells were washed twice, and metal concentrations were determined by atomic absorption spectrometry. Error bars represent the SD. Asterisks indicate significant differences with control cells expressing *GUS* according to a Mann–Whitney test ($p < 0.05$).

Fig. 5. *PotriNRAMP3.1* and *PotriNRAMP3.2* transport Fe. Functional complementation of the *fet3fet4* yeast iron uptake mutant by *PotriNRAMP3.1* and *PotriNRAMP3.2*. *fet3fet4* yeast cells were transformed with pDR195gtw vector containing the cDNA of *GUS*, *AtNRAMP1*, *AtNRAMP4*, *PotriNRAMP3.1* and *PotriNRAMP3.2*. Transformed *fet3fet4* yeasts were grown overnight in liquid synthetic dextrose -ura supplemented with 0.2 mM FeCl₃. Cultures were diluted to ODs of 1 to 10^{-3} and spotted on synthetic dextrose -ura plates supplemented with 100 μ M of the Fe chelator BathoPhenanthroline-Di-Sulfonic acid (BPDS) and 0.2 mM FeCl₃ (+ Fe) or with 100 μ M BPDS and 40 μ M FeCl₃ (- Fe). The plates were incubated at 30°C for 4 days before photography.

Fig. 6. *PotriNRAMP3.1* and *PotriNRAMP3.2* are both expressed in all organs. *PotriNRAMP3.1* and *PotriNRAMP3.2* transcript levels were determined by RT-qPCR using specific primers for each isoform and normalization by three reference genes, *i.e.*, *UBIQ*, *EF1* and *PP2A*. The graph shows means $\pm$ SD of three replicates on different individuals.

Fig. 7. *PotriNRAMP3.2* but not *PotriNRAMP3.1* expression complements the Arabidopsis *nramp3nramp4* double mutant growth defects under iron starvation. (A) Representative pictures of *nramp3nramp4 pUb10:PotriNRAMP3.1* and *pUb10:PotriNRAMP3.2 T3* Arabidopsis lines together with wild-type (Col-0) as positive control and *nramp3nramp4* as negative control grown vertically in ABIS supplemented with 50 μ M FeHBED for 8 days (control, bottom panel) or without iron for 8 days (- Fe, top panel). (B) Quantification of main root lengths of wild-type (Col-0), *nramp3nramp4* and 3 independent *nramp3nramp4 pUb10:PotriNRAMP3.1* and *pUb10:PotriNRAMP3.2 T3* lines. Values represent mean of 10–12 roots and bars represent SD. Different letters reflect significant differences according to a Kruskal-Wallis test followed by Dunn’s test for multiple comparison ($p < 0.01$). No significant differences among genotypes were detected in the presence of Fe (B).

Fig. 8. *PotriNRAMP3.1* (A) and *PotriNRAMP3.2* (B) localize to different cellular compartments in both Arabidopsis and Poplar. GFP translational fusions of *PotriNRAMP3.1* (A) and *PotriNRAMP3.2* (B) were imaged by spinning disk confocal microscopy in transgenic Arabidopsis (Left) and Poplar (Right). *NRAMP3.1*-GFP labels granular cytoplasmic structures at the cell periphery, in both Arabidopsis and Poplar. In contrast, *NRAMP3.2*-GFP is present on the vacuolar membrane. Images represent vacuolar planes of root epidermal cells (early elongation zone). Transmitted-light (differential interference contrast, DIC) and fluorescence (GFP) acquisitions are shown. Scale bar : 10 μ m.

Fig. 9. *PotriNRAMP3.1*-GFP localizes to the TGN compartment. (A) Colocalisation of *PotriNRAMP3.1*-GFP with the Trans-Golgi Network (TGN) marker mRFP-SYP43. (B) Juxtaposition of *PotriNRAMP3.1*-GFP with the trans-Golgi apparatus marker mRFP-ST. Note how *PotriNRAMP3.1*-GFP signal either faces the center of the toroidal structure of the trans-Golgi or is present as Golgi-independent structures (white asterisks). On the merged images the overlap of GFP (green) and mRFP (magenta) channels appears white. Spinning disk confocal images were acquired in the cortical planes of root epidermal cells in the early elongation zone of Arabidopsis F1 seedlings. Scale bar : 10 μ m. Super resolution acquisitions (*Super Res.*) are 10 μ m wide.

Fig. 10. The ectopic over expression of *PotriNRAMP3.1* but not that of *PotriNRAMP3.2* leads to phenotypic alterations in poplar. Overview of transgenic poplar expressing *PotriNRAMP3.1-GFP* (A, purple tags) or *PotriNRAMP3.2-GFP* (B, orange tags) along with NT control (A and B, white tag), 2 months after transfer from *in vitro* to soil. (C) Mean heights of poplar from the different genotypes. Error bars represent SE (n = 4-7). Asterisks denote significant difference with respect to NT control according to a Mann-Whitney test (*: $p < 0.05$, **: $p < 0.01$). (D-I) Leaf phenotypes of representative trees. (D,E) NT control, (F,G) *PotriNRAMP3.1-GFP* line 9 (H,I) *PotriNRAMP3.2-GFP*, line 12. (D, F, H) pictures; (E, G, I) PS II maximum quantum yield measured with imaging Pulse-Amplitude-Modulation. Relative *PotriNRAMP3.1* and *PotriNRAMP3.2* mRNA levels of OE lines are shown in supplementary figure S11.

Fig. 11. Ectopic over expression of *PotriNRAMP3.1-GFP* but not that of *PotriNRAMP3.2-GFP* perturbs Mn distribution in poplar leaves. Mn concentrations in senescent (A), mature (B), young leaves (C) and young stems (D) of poplars 2 months after transfer from *in vitro* to soil was determined using Atomic Emission Spectroscopy. Mean Mn concentrations of *PotriNRAMP3.1-GFP* or *PotriNRAMP3.2-GFP* OE lines were compared with NT control. Error bars represent SD (n = 4-7). Asterisks denote significant differences with the NT control according to a Mann-Whitney test (*: $p < 0.05$, **: $p < 0.01$). (E) Mean Mn concentrations in veins and lamina of leaves from NT control and 4 *PotriNRAMP3.1-GFP* OE lines. Error bars represent SD (n = 3-6). Asterisks denote significant differences between veins and lamina according to a Mann-Whitney test (*: $p < 0.05$, **: $p < 0.01$). Different letters denote significant differences among genotypes in the lamina (lowercase) or in the veins (capitals) according to a Kruskal-Wallis test followed by Dunn's test for multiple comparison ($p < 0.01$).

Fig. 12. Working model to account of *PotriNRAMP3.1* role in Mn transport from cell to cell. The model is based on the hypothesis that Mn moves through the transcellular pathway, being secreted in the apoplast via exocytosis by the cells proximal to the veins and taken up by the cells that are distal to the veins. According to this hypothesis, the transporters loading (*PotriMTP11*) or unloading (*PotriNRAMP3.1* as well as *PotriNRAMP2* assuming function conservation with Arabidopsis *AtNRAMP2*) Mn from the secretory pathway would determine the amount of Mn made available by proximal cells for uptake by distal cells. In this context,

- 1083 efficient removal of Mn from the secretory pathway in the proximal cells by PotriNRAMP3.1
1084 overexpression would limit Mn availability for the distal cell.

Fig. 1. Collinearity between *Salix purpurea* and *Populus trichocarpa* genomes in the area surrounding *NRAMP3* loci. Gene collinearity between *P. trichocarpa* and *S. purpurea* is maintained around *NRAMP3* loci. (A) Dot-plots centered on *NRAMP3* loci, performed on Gepard software V1.40 (Krumstiek *et al.*, 2007), using 107 kb and 140 kb sequences of *S. purpurea* and *P. trichocarpa* genomes, respectively. Sequence homology between *S. purpurea* and *P. trichocarpa* are indicated by black dots. Red arrows indicate homology between *SapurNRAMP3* and *PotriNRAMP3.2* as well as between *SapurNRAMP3* and *PotriNRAMP3.1*. (B) Schematic representation of the genomic sequence around *NRAMP3* loci in *P. trichocarpa* and *S. purpurea*.

Fig. 2. Phylogenetic tree of NRAMP3 homologues in *Populus* and *Salix* species. *A. thaliana* NRAMP2, NRAMP3 and NRAMP4 as well as *P. trichocarpa* and *S. purpurea* NRAMP2 genes are shown as an outgroup. \$ indicates incomplete protein sequences. Phylogenetic analyses were conducted as described in Materials and Methods. Protein sequences used for this tree are listed in supplementary data S3.

Fig. 3. Alignment of the consensus sequences of *Populus* NRAMP3.1 and NRAMP3.2. The consensus sequences were determined from the NRAMP alignments shown in supplementary figure S3. Asterisk indicates less than 90% of conservation within *Populus* NRAMP3.1 or NRAMP3.2 cluster. Identical, similar and different residues between the two consensus sequences are indicated in black, blue, and red, respectively. Frames indicate residues under purifying selection and green arrows indicate positive selections in either *Populus* NRAMP3.1 or NRAMP3.2 cluster according to the FEL method ($p < 0.05$). Note that NRAMP3.1 sequences contain an insertion of residue at position 4, leading to a gap (-) in NRAMP3.2 sequences.

Fig. 4. *PotriNRAMP3.1* and *PotriNRAMP3.2* encode functional Mn transporters. Functional complementation of *smf1* (A) and *smf2* (B) yeast mutants. Yeast cells were transformed with pDR195gtw vector containing the cDNA of *GUS*, *AtNRAMP1*, *AtNRAMP2*, *PotriNRAMP3.1* or *PotriNRAMP3.2*. Transformed yeasts were grown overnight in liquid synthetic dextrose -ura. The cultures were diluted to ODs of 1 to 10⁻³ and spotted on synthetic dextrose -ura plates. Transformed *smf1* (A) strains were grown on medium supplemented with 5 mM EGTA and 100 µM MnSO₄ (+ Mn) or with 5 mM EGTA without MnSO₄ (- Mn). Transformed *smf2* (B) strains were grown in medium supplemented with 10 mM EGTA and 100 µM MnSO₄ (+ Mn), with 5 mM EGTA without MnSO₄ (- Mn) or with 10 mM MnSO₄ (+++ Mn). The plates were incubated at 30°C for 5 days (*smf1*) or 2 days (*smf2*) before photography. White lines indicate cropping, unmodified pictures are shown as supplementary figures S4 and S5. (C) Expression of *PotriNRAMP3.1* or *PotriNRAMP3.2* increase Mn accumulation in yeast cells. Liquid SD -ura medium containing *smf2* cells expressing *GUS*, *AtNRAMP4*, *PotriNRAMP3.1* or *PotriNRAMP3.2* were grown overnight and diluted to OD 0.3 in liquid SD -ura supplemented with 30 µM FeCl₃ and 10 µM MnSO₄. After 30 hrs of incubation at 30°C, yeast cells were washed twice, and metal concentrations were determined by atomic absorption spectrometry. Error bars represent the SD. Asterisk indicate significant differences with control cells expressing *GUS* according to a Mann-Whitney test ($p < 0.05$).

Fig. 5. PotriNRAMP3.1 and PotriNRAMP3.2 transport Fe. Functional complementation of the *fet3fet4* yeast iron uptake mutant by *PotriNRAMP3.1* and *PotriNRAMP3.2*. *fet3fet4* yeast cells were transformed with pDR195gtw vector containing the cDNA of *GUS*, *AtNRAMP1*, *AtNRAMP4*, *PotriNRAMP3.1* and *PotriNRAMP3.2*. Transformed *fet3fet4* yeasts were grown overnight in liquid synthetic dextrose -ura supplemented with 0.2 mM FeCl₃. Cultures were diluted to ODs of 1 to 10⁻³ and spotted on synthetic dextrose -ura plates supplemented with 100 μM of the Fe chelator BathoPhenanthroline-Di-Sulfonic acid (BPDS) and 0.2 mM FeCl₃ (+ Fe) or with 100 μM BPDS and 40 μM FeCl₃ (- Fe). The plates were incubated at 30°C for 4 days before photography.

Fig. 6. *PotriNRAMP3.1* and *PotriNRAMP3.2* are both expressed in all organs. *PotriNRAMP3.1* and *PotriNRAMP3.2* transcript levels were determined by RT-qPCR using specific primers for each isoform and normalization by three reference genes, i.e., *UBQ*, *EF1*, and *PP2A*. The graph shows means $\pm$ SD of 4 replicates on different individuals. Different letters denote significant differences among organs for *PotriNRAMP3.1* (lowercase) or *PotriNRAMP3.2* (capitals) according to a Kruskal-Wallis test followed by Dunn's test for multiple comparison ($p < 0.01$).

Fig. 7. *PotriNRAMP3.2* but not *PotriNRAMP3.1* expression complements the Arabidopsis *nramp3nramp4* double mutant growth defects under iron starvation. (A) Representative pictures of *nramp3nramp4 pUb10:PotriNRAMP3.1* and *pUb10:PotriNRAMP3.2* T3 Arabidopsis lines together with wild-type (Col-0) as positive control and *nramp3nramp4* as negative control grown vertically for 8 days in ABIS supplemented with 50 μ M FeHED (control, bottom panel) or without iron (- Fe, top panel). (B) Quantification of main root lengths of wild-type (Col-0), *nramp3nramp4* and 3 independent *nramp3nramp4 pUb10:PotriNRAMP3.1* and *pUb10:PotriNRAMP3.2* T3 lines. Values represent mean of 10–12 roots and bars represent SD. Different letters reflect significant differences according to a Kruskal-Wallis test followed by Dunn's test for multiple comparison ($p < 0.01$). No significant differences among genotypes were detected in the presence of Fe, left panel in (B).

Fig. 8. PotriNRAMP3.1 (A) and PotriNRAMP3.2 (B) localize to different cellular compartments in both Arabidopsis and Poplar. GFP translational fusions of PotriNRAMP3.1 (A) and PotriNRAMP3.2 (B) were imaged by spinning disk confocal microscopy in transgenic Arabidopsis (*Left*) and Poplar (*Right*). NRAMP3.1-GFP labels granular cytoplasmic structures at the cell periphery, in both Arabidopsis and Poplar. In contrast, NRAMP3.2-GFP is present on the vacuolar membrane. Images represent vacuolar planes of root epidermal cells (early elongation zone). Transmitted-light (differential interference contrast, *DIC*) and fluorescence (*GFP*) acquisitions are shown. Scale bar : 10 μ m.

Fig. 9. PotriNRAMP3.1-GFP localizes to the Trans-Golgi Network (TGN) compartment. (A) Colocalisation of PotriNRAMP3.1-GFP with the TGN marker mRFP-SYP43. (B) Juxtaposition of PotriNRAMP3.1-GFP with the trans-Golgi apparatus marker mRFP-ST. Note how PotriNRAMP3.1-GFP signal either faces the center of the toroidal structure of the trans-Golgi or is present as Golgi-independent structures (white asterisks). On the merged images the overlap of GFP (green) and mRFP (magenta) channels appears white. Spinning disk confocal images were acquired in the cortical planes of root epidermal cells in the early elongation zone of Arabidopsis F1 seedlings. Scale bar : 10 μ m. Super resolution acquisitions (*Super Res.*) are 10 μ m wide.

Fig. 10. The ectopic over expression of *PotriNRAMP3.1* but not that of *PotriNRAMP3.2* leads to phenotypic alterations in poplar. Overview of transgenic poplar expressing *PotriNRAMP3.1-GFP* (A, purple tags) or *PotriNRAMP3.2-GFP* (B, orange tags) along with NT control (A and B, white tag), 2 months after transfer from *in vitro* to soil. (C) Mean heights of poplar from the different genotypes. Error bars represent SE (n = 4-7). Asterisks denote significant difference with respect to NT control according to a Mann-Whitney test (*: $p < 0.05$, **: $p < 0.01$). (D-I) Leaf phenotypes of representative trees. (D,E) NT control, (F,G) *PotriNRAMP3.1-GFP* line 9 (H,I) *PotriNRAMP3.2-GFP*, line 12. (D, F, H) pictures; (E, G, I) PS II maximum quantum yield measured with imaging Pulse-Amplitude-Modulation. Relative *PotriNRAMP3.1* and *PotriNRAMP3.2* mRNA levels of over-expressing lines are shown in supplementary figure S11.

Fig. 11. Ectopic over expression of *PotriNRAMP3.1-GFP* but not that of *PotriNRAMP3.2-GFP* perturbs Mn distribution in poplar leaves. Mn concentrations in senescent (A), mature (B), young leaves (C) and young stems (D) of poplars 2 months after transfer from *in vitro* to soil were determined using Atomic Emission Spectroscopy. Mean Mn concentrations of *PotriNRAMP3.1-GFP* or *PotriNRAMP3.2-GFP* OE lines were compared with NT control. Error bars represent SD ($n = 4-7$). Asterisks denote significant differences with the NT control according to a Mann-Whitney test (*: $p < 0.05$, **: $p < 0.01$). (E) Mean Mn concentrations in veins and lamina of leaves from NT control and 4 *PotriNRAMP3.1-GFP* OE lines. Error bars represent SD ($n = 3-6$). Asterisks denote significant differences between veins and lamina according to a Mann-Whitney test (*: $p < 0.05$, **: $p < 0.01$). Different letters denote significant differences among genotypes in the lamina (lowercase) or in the veins (capital) according to a Kruskal-Wallis test followed by Dunn's test for multiple comparison ($p < 0.01$).

Fig. 12. Working model to account of PotriNRAMP3.1 role in Mn transport from cell to cell. The model is based on the hypothesis that Mn moves through the transcellular pathway, being secreted in the apoplast via exocytosis by the cells proximal to the veins and taken up by the cells that are distal to the veins. According to this hypothesis, the transporters loading Mn (PotriMTP11) or unloading Mn (PotriNRAMP3.1 as well as PotriNRAMP2 assuming function conservation with Arabidopsis AtNRAMP2) from the secretory pathway would determine the amount of Mn made available by proximal cells for uptake by distal cells. In this context, efficient removal of Mn from the secretory pathway in the proximal cells by PotriNRAMP3.1 overexpression would limit Mn availability for the distal cell.