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# Importing Manganese into the Chloroplast: Many Membranes to Cross

Manganese (Mn) plays a wealth of roles in living systems. It is ubiquitously involved in the antioxidant response because of its ability to catalyze the dismutation of superoxide anions. In eukaryotic cells, Mn also plays a role in protein glycosylation in the secretory system. However, arguably, in photosynthetic organisms, the most important role of Mn is in the oxygen evolving complex at photosystem II (PS II). At the global level, atmospheric oxygen is derived from the splitting of water, a reaction that relies strictly on the tetra-manganese cluster ( $Mn_4O_5Ca$ ) bound to photosystem II at the lumen side of the thylakoid membrane. The unique redox properties of this cluster allow the storage of oxidation potential arising from the successive absorption of four photons. This high oxidation potential is required for the energetically highly demanding reaction oxidizing water into molecular oxygen and protons. Achieving this reaction within a plant cell requires efficient manganese import into the chloroplast and further into the thylakoid lumen. Until recently, the molecular mechanisms allowing Mn import into plastids had been completely unknown. Two articles published this year provide the first candidates for the long sought pathway for Mn transport into plastids (Eisenhut et al., 2018; Zhang et al., 2018).

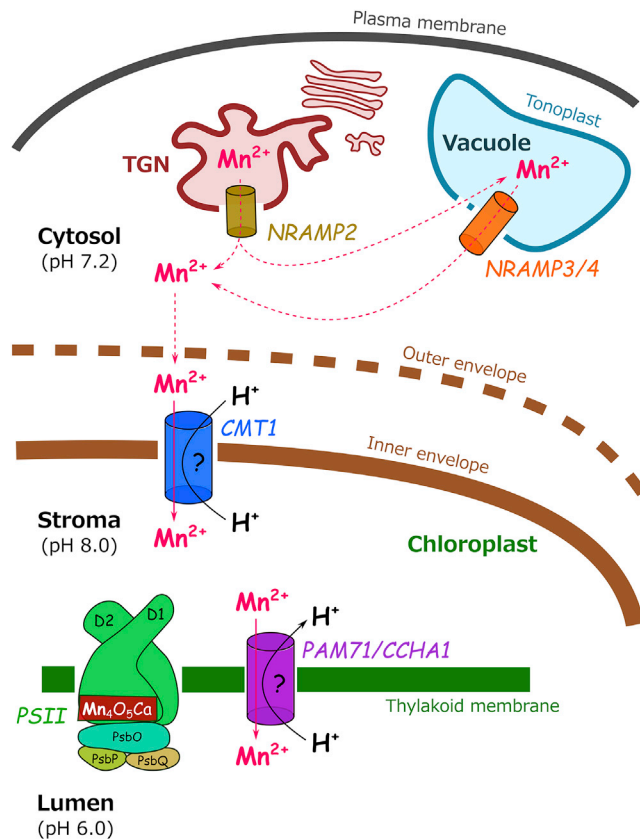
The two studies characterize *CMT1* (*Chloroplast Manganese Transporter 1*), which encodes a protein localized in the inner envelope of chloroplasts. Loss of *CMT1* function leads to consequences expected when Mn is not adequately delivered to PS II: chloroplast Mn concentration, PS II supercomplex formation, photosystem II efficiency, and oxygen evolution are severely decreased. Combined with impaired growth and a pale leaf phenotype, these observations point to an essential role of *CMT1* in photosynthesis. In addition, the lack of *CMT1* leads to distinct phenotypes among individual chloroplasts. Some chloroplasts show normal ultrastructure with well-organized grana stacks while others have no grana and resemble senescent plastids. This heterogeneity may be due to the fact that some chloroplasts retain the ability to acquire Mn above a threshold concentration required for normal chloroplast development. Accordingly, the observation that abundant supply rescues *cmt1* indicates that Mn can enter the chloroplasts through an alternative low affinity Mn import pathway or a poorly selective cation transporter.

To access the thylakoid lumen, Mn needs to cross three membranes: the outer envelope, the inner envelope, and the thylakoid membrane (Figure 1). The outer membrane is non-selectively permeable to most ions, while the other two membranes need specific transport systems. Recently, a genetic approach identified *PAM71*, which encodes a membrane protein homologous to *CMT1* and is responsible for Mn import into the thylakoid lumen (Schneider et al., 2016). In *pam71* mutant, photosystem II function is strongly impaired. In *Arabidopsis* five members of the *PAM71* family (including *PAM71* and *CMT1*) are encoded in the genome, while in cyanobacteria only one member of this

family called *Mnx* or *SynPAM71* is present. *Mnx/SynPAM71* has been shown to be essential for Mn tolerance to excess Mn and to be localized in thylakoids (Brandenburg et al., 2017) and in the plasma membrane (Gandini et al., 2017). In *Arabidopsis*, *PAM71* was also independently identified as *CCHA1*, which has been shown to be involved in calcium transport and pH regulation in thylakoids, raising the question of the selectivity and transport mechanism of this transporter (Wang et al., 2016). *CMT1* and *PAM71/CCHA1* belong to a family of transporters previously implicated in Ca and Mn import into the Golgi apparatus in yeast and mammalian cells (Demaegd et al., 2013; Potelle et al., 2016). Besides *CMT1* and *PAM71/CCHA1*, the other three members present in the *Arabidopsis* genome may be involved in ion transport in the secretory system. These proteins were shown to function as proton-coupled cation transporters. Although such a mechanism would account for the effect on pH of *PAM71/CCHA1* inactivation, both reports suggest that *CMT1* mutation affects chloroplast Mn homeostasis specifically. There is a strong proton gradient across the thylakoid membrane in the light, which may be used to drive the import of Mn into thylakoids through an antiport mechanism. In contrast, Mn import across the envelope would rather require a symport mechanism since the pH gradient across the envelope is supposed to be weaker, with the cytosol (pH 7.2) being slightly more acidic than the stroma (pH 8). Further studies are necessary to clarify the selectivity and transport mechanism of *CMT1* and *PAM71/CCHA1*.

*CMT1* and *PAM71/CCHA1* are expected to function sequentially to bring Mn to the thylakoid lumen, where the tetra-manganese clusters are assembled at PS II in a light-dependent process called photoactivation. The phenotype of *cmt1* mutants is more severe than that of *pam71* mutant, which may indicate that, besides the oxygen evolving complex, other essential Mn-requiring enzymes reside in the stroma. Although enzymes strictly relying on Mn for their activity have not been identified in the stroma, Mn activates Rubisco. Interestingly, however, the double mutant has a slightly less severe phenotype than the single *cmt1* mutant, suggesting that the presence of *PAM71/CCHA1* enhances the dysfunction due to the lack of *CMT1*.

How does Mn reach the chloroplast? Recent reports have characterized several steps in the intracellular transport of Mn to the plastids. In the photosynthetic cell, Mn can be stored in the vacuole, which occupies most of the cell volume. Under Mn deficiency, *NRAMP3* and *NRAMP4* vacuolar metal efflux transporters are required to remobilize Mn from the vacuole so that it can be provided to plastids (Lanquar et al., 2010). More recently, another member of the *NRAMP* family *NRAMP2* was shown to



**Figure 1. The Transport Pathway to Supply Manganese to the Oxygen Evolving Complex of Photosystem II**

Mn can be released in the cytosol from intracellular stores. NRAMP2 allows release from the *trans* Golgi network (TGN), NRAMP3 and NRAMP4 allow release from the vacuole. Cytosolic Mn is thought to permeate non-selectively through the outer envelope of the chloroplast. CMT1 transporter allows Mn to cross the inner membrane of the chloroplast envelope. Stromal Mn is then imported into the thylakoids where the oxygen evolving complex resides by a CMT1 homolog PAM71/CCHA1. Whether transport through CMT1 is coupled to protons needs to be further investigated.

play a critical role in intracellular Mn distribution. NRAMP2 localizes to the Golgi apparatus. In mutants with low NRAMP2 levels, Mn concentration in chloroplasts is lower and PS II function is decreased (Alejandro et al., 2017). Loss of NRAMP2 function leads to a pleiotropic phenotype as this protein is also important to provide Mn to other compartments. Despite the increased knowledge on Mn transporters, many intermediate steps required for the transport of Mn from the root cells to the photosynthetic cells remain to be identified.

The discovery of CMT1 fills an important gap in the knowledge of the molecular mechanism allowing Mn supply for its crucial function in the oxygen evolving complex of PS II. However, many questions remain open concerning Mn homeostasis in chloroplasts. On the one hand, PAM71/CCHA1/CMT1 family members use free Mn cations as substrate, while on the other hand Mn is believed to be quantitatively bound to proteins or phosphate groups inside the cell. This raises the question of the relative concentrations of free Mn versus bound Mn in the different compartments. Mn may also be delivered to transporters or its targets via

chaperone, as shown in the case of copper (Cu). Cu, which is the cofactor of plastocyanin, the electron donor for PS I, also need to sequentially cross the chloroplast envelope and the thylakoid membrane (Abdel-Ghany et al., 2005). Cu is pumped into the stroma by a P-type ATPase of the HMA (heavy metal ATPase) family, PAA1. In the stroma, Cu is bound to a chaperone called CCS (copper chaperone for superoxide dismutase) to prevent the formation of hydroxyl radicals induced by free Cu ions. In turn, CCS delivers Cu either to the CuZnSOD or to a second Cu pumping P-type ATPase localized in the thylakoid membrane PAA2, which passes Cu on to plastocyanin. Manganese, however, is much less toxic than Cu and the concentration of the free metal does not have to be that strictly controlled. It is currently unknown whether Mn is bound to a chaperone or whether it is complexed by metabolites containing bi- or tri-phosphate groups, such as ATP or fructose bisphosphate, which are abundant in the stroma. Likewise, there is no strong evidence for a chaperone capturing Mn when it is released in the thylakoid lumen. However, PsbP, a subunit of the PS II localized at the luminal site, has been shown to bind Mn and to accelerate the assembly of a functional PS II (Bondarava et al., 2005; Cao et al., 2015). Another question concerns the fate of free Mn inside the lumen and Mn bound to PS II, respectively. In the light, PS II is constantly degraded and reassembled through photoinhibition and repair. Mn is released during this process and the oxygen evolving complex is restored in repaired PS II. The question remains if the released Mn is recycled during this process or if “fresh” Mn has to be imported into the lumen for photoactivation. The discovery and characterization of CMT1 will trigger further research addressing the open questions in Mn transport, storage, and speciation.

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