Characterizing the NTMC2T5 proteins at ER-plastid contact sites

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ABSTRACT: Membrane contact sites (MCS) are specialized regions where the membranes of two distinct organelles come into close proximity (10-30 nm) without fusing. These regions facilitate lipid exchange between chloroplasts and the endoplasmic reticulum (ER), playing a key role in plant glycerolipid synthesis.

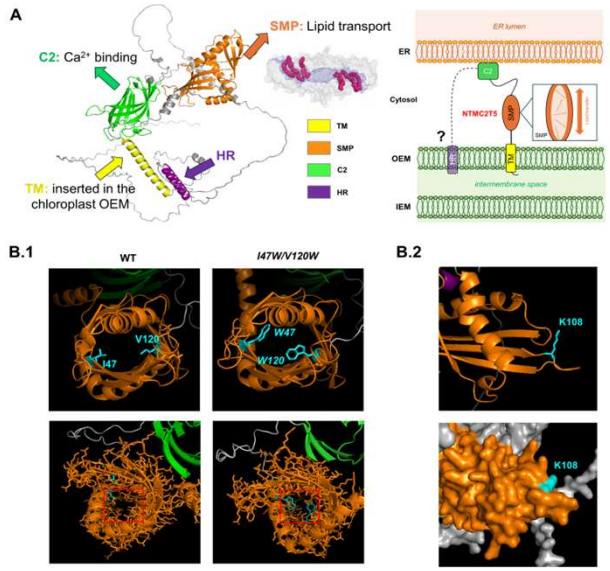
Our research focuses on MCS-associated proteins containing an SMP domain, a conserved lipid-binding module implicated in lipid transfer. We have identified NTMC2T5 proteins, which putatively localize to the outer envelope membrane of chloroplasts and establish contacts with the ER, although their precise molecular function remains largely unknown.

To investigate NTMC2T5 role, we generated *A. thaliana ntmc2t5.1/ntmc2t5.2* double mutants via CRISPR-Cas9. These lines exhibit a distinct post-germination cotyledon yellowing, mirroring the phenotype previously observed in *N. benthamiana ntmc2t5* mutants, and confirming the protein's role in plastid development.

To further characterize NTMC2T5, we expressed truncated versions of the *N. benthamiana* protein NbNTMC2T5 in *E. coli,* comprising its functional domains (SMP and/or C2) and the hydrophobic C-terminal region (HR), which will be used for lipid transfer assays.

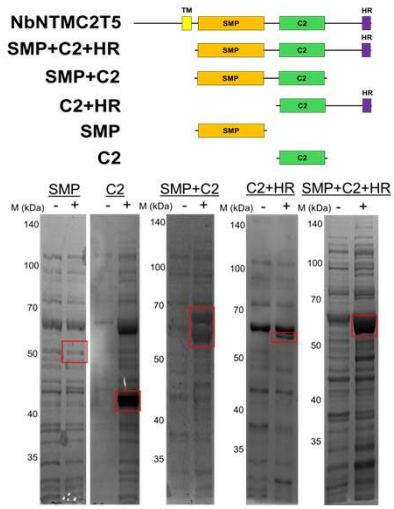
Furthermore, we have conducted BiFC assays, which support that NTMC2T5 proteins localize to the outer chloroplast envelope, although the precise positioning of their HR remains unclear. Together, these approaches provide a foundation for dissecting the molecular function of NTMC2T5 at ER-plastid MCS.

1. Structural modeling reveals SMP and C2 Domains in NbNTMC2T5



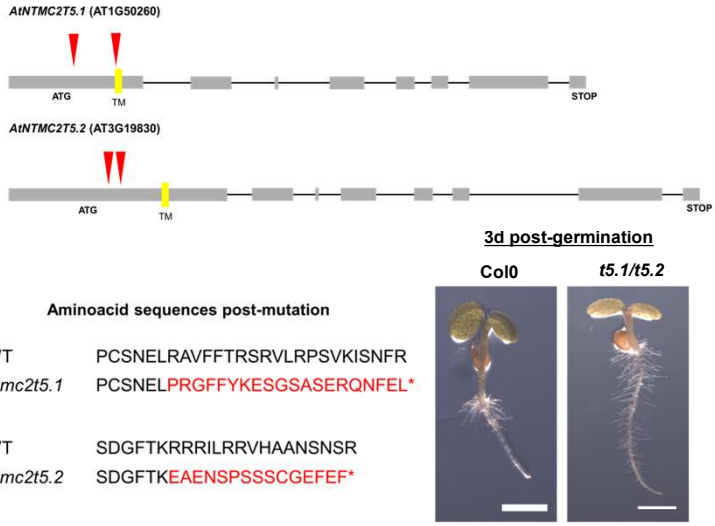
(A) AlphaFold predicts that NbNTMC2T5 contains a transmembrane domain (TM) likely inserted into the chloroplast outer envelope membrane (OEM), together with SMP and C2 domains. A C-terminal hydrophobic region (HR) is also predicted, although its localization remains uncertain. **(B.1)** Modeling of the predicted SMP domain suggests that substitution of I47 and V120 with tryptophan (W) could hinder lipid transport by partially blocking the hydrophobic tunnel. **(B.2)** Alignment of the predicted NbNTMC2T5 SMP domain with other SMP domains suggests that K108 may contribute to lipid binding.

2. NbNTMC2T5 truncated versions were succesfully expressed in the *E. coli* ArcticExpress (DE3) strain



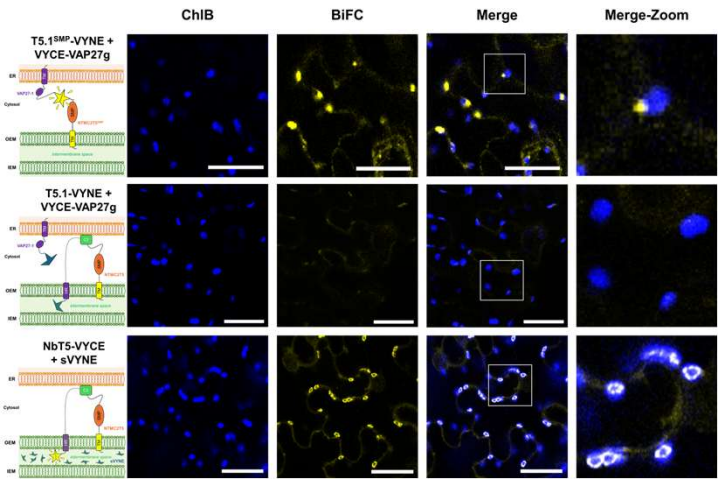
To study SMP-mediated lipid transport and C2-dependent membrane anchoring, truncated NbNTMC2T5 variants are being generated. Except for the isolated SMP domain, constructs were successfully expressed and solubilized in *E. coli* ArcticExpress (DE3) as N-terminal GST-6xHis fusions.

3. *Arabidopsis thaliana ntmc2t5.1/t5.2* double mutant lines have been generated via CRISPR-Cas9



CRISPR-Cas9–generated *A. thaliana* double mutant lines were confirmed by sequencing, revealing frameshift mutations in the *AtNTMC2T5.1* and *AtNTMC2T5.2* coding sequences. These mutants display a yellowing phenotype in cotyledons during the first days after germination. *Scale bar: 1.5 mm.*

4. BiFC Supports Chloroplast OEM Localization of NTMC2T5, but leaves HR localization unresolved



BiFC assays suggest that NTMC2T5 proteins are inserted into the OEM, with the SMP domain facing the cytosol. However, the localization of the HR remains unresolved. Co-expression of NbNTMC2T5-VYCE with the soluble cytosolic N-YFP fragment (sVYNE) produced a strong fluorescence signal within chloroplasts, suggesting that sVYNE may access the chloroplast intermembrane space. *Scale bar: 40 µm.*

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