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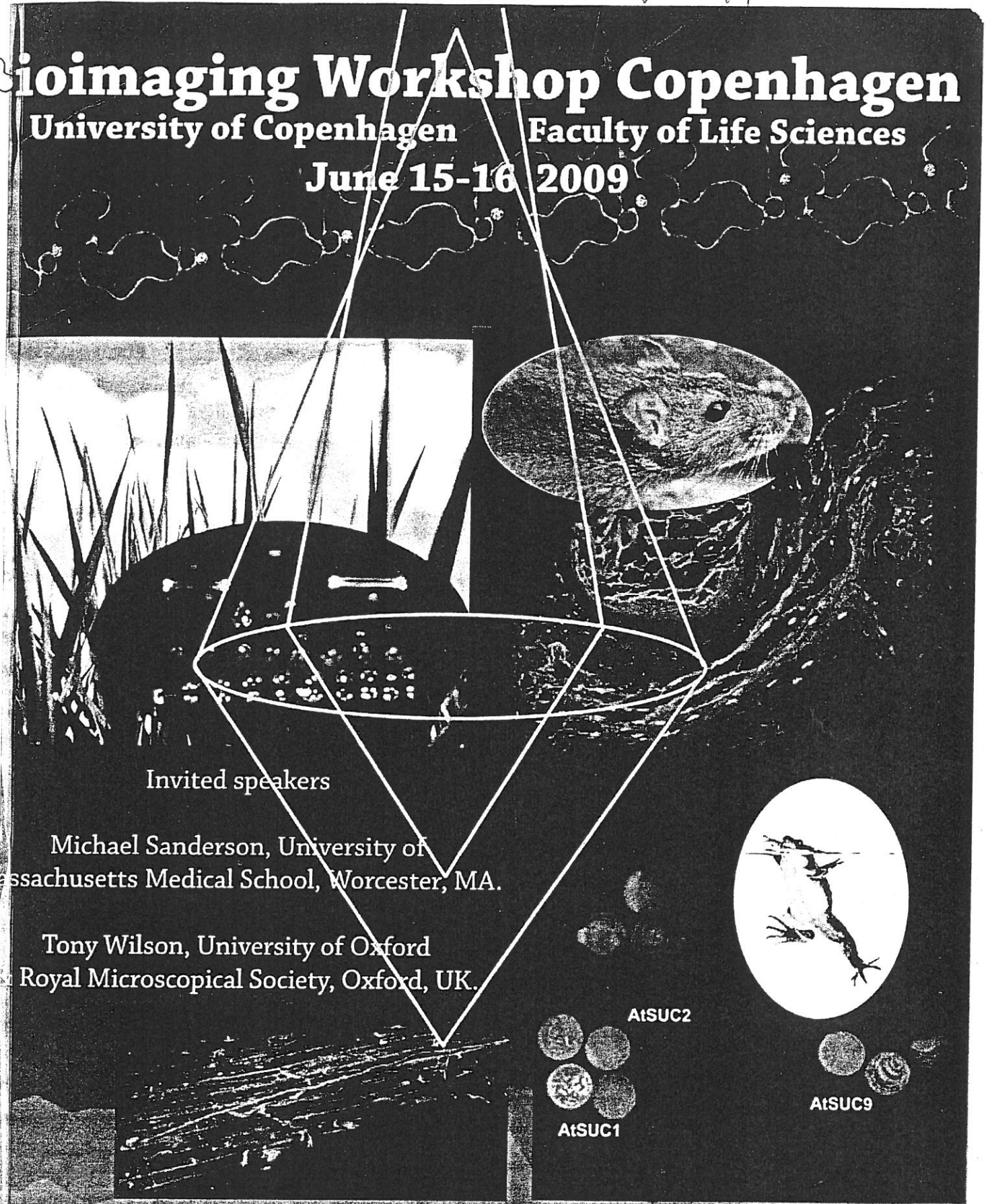

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Identification of Membrane Topology of Golgi-localized Proteins in Plants

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Integral membrane proteins exhibit specific topology in and across the membrane. Membrane topology is of particular importance because it determines the subcellular compartment in which the proteins exert their functions. This is particularly true for enzymes involved in the biosyntheses of *N*-glycan and cell wall polysaccharides (i.e. pectin and hemicelulose), which are known to occur in the lumen of the Golgi apparatus. In general, prediction of membrane topologies of integral membrane proteins is often performed by using bioinformatics-based analysis. This approach, however, often leads to inconsistent results depending on the database used, and experimental validation of the predicted topologies is essential for unequivocal determination of the membrane topologies. In the present work, a novel bioimaging-based method has been developed to test the topologies of integral membrane proteins *in situ* in living cells.

A truncated rat sialyltransferase (ST) that is know to localize in Golgi was recombinantly fused at its N and C-terminus with split domains of a yellow fluorescent protein (YFP) (Yn: 1-155 AA; Yc:156-238AA). These constructs were heterologously expressed in *Nicotiana benthamiana*, and were studied for bimolecular fluorescent complementation (Hu et al, 2002) under a confocal scanning-laser microscopy. Matrix-based interaction analysis showed that Yn/Yc fusions of ST complement fluorescence only when the split YFP domains are exposed to the same side of the membranes. It was also shown that ST-Yn/Yc fusions interact randomly with various Golgi-localized glycosyltransferases tested so far. Therefore it was concluded that Yn/Yc fusion of ST can serve as a marker for membrane topology in the Golgi apparatus. Determination of the membrane topologies of glycosyltransferases (type II membrane proteins, multiple membrane spanning proteins) are currently in progress and will be presented.

Reference:

Hu, Chang-Deng; Chinenov, Yurii and Kerppola, Tom K. Visualization of Interactions among bZIP and Rel Family Proteins in Living Cells Using Bimolecular Fluorescence Complementation. Molecular Cell, Vol 9, 789-798, 2002

DEPARTMENT OF PLANT BIOLOGY AND BIOTECHNOLOGY
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Pro-Active Plants

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Introduction

Integral membrane proteins exhibit specific topology in and across the membrane. Membrane topology is of particular importance because it determines the subcellular compartment in which the proteins exert their functions. This is particularly true for enzymes involved in the biosynthesis of *N*-glycan and cell wall polysaccharides (i.e. pectin and hemicellulose), which are known to occur in the lumen of the Golgi apparatus.
In general, prediction of membrane topologies of integral membrane proteins is performed by using bioinformatics-based analysis. This approach, however, often leads to inconsistent results depending on the database used, and experimental validation of the predicted topologies is essential for unequivocal determination of the membrane topologies. **In the present work, a novel bioimaging-based method has been developed to test the predicted topologies of integral membrane proteins *in situ* in living cells.**

Conclusions

- We have developed a rapid bioimaging-based method, "PROMTO", for examining the membrane topology of Golgi proteins *in vivo*

Perspectives

1. Further validation of the membrane topologies of STtmd-YFP and YFP-STtmd is in progress.
2. Membrane topologies of single- and multiple-membrane-spanning proteins (i.e. cellulose synthase like proteins, nucleotide-sugar conversion enzymes, glycosyltransferases) involved in cell wall biosynthesis will be analyzed using the PROMTO system.
3. PROMTO may be applied for determination of membrane topologies of proteins in other eukaryotic systems including yeast and mammals.

Testing Bioinformatic Prediction Programs

We tested the reliability of publically available databases for prediction of subcellular localization of proteins. Previously a list of putative plant glycosyltransferases (140) have been selected as plant cell wall biosynthetic enzymes localized in the secretory pathway based on the following criteria: i) the presence of glycosyltransferase domains based on Carbohydrate Active enZyme (CAZy) database, and ii) the presence of at least one transmembrane domain based on TmHMM program (WallNet). This dataset was subjected to membrane topology and subcellular localization predictions using the ARAMEMNON plant membrane protein database (Schwacke et al, 2003) that utilizes results from multiple prediction programs to obtain a consensus prediction for each gene.

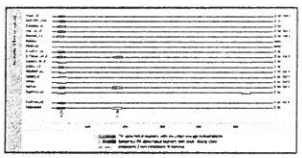

Figure 1: Example of ARAMEMNON Transmembrane Spanning Domain (TMD) Prediction
An ARAMEMNON entry for a putative glycosyltransferase (At5g19670) illustrating that the number of predicted TMDs varies depending on prediction programs.

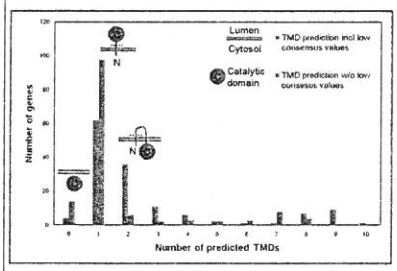

Figure 2: TMD Prediction Affects Membrane Topology of Proteins and Subcellular Localization of Catalytic Domains.
Two sets of results are shown: one set with the full consensus prediction (blue column) and the second set (red column) where the TMDs with low consensus values (0.3) shown in Figure 1 are excluded.

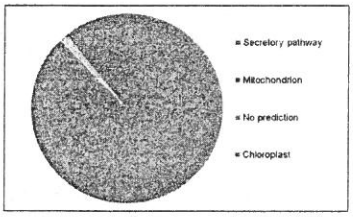

Figure 3: Prediction of Subcellular Localization of the Selected Glycosyltransferases by ARAMEMNON
65 % of the proteins were predicted to be targeted via the secretory pathway as expected from a selection of putative glycosyltransferases, while, 21 % of the proteins were predicted to be targeted to the mitochondrion and 11 % to the chloroplast.

Prediction programs are highly unreliable and an efficient *in vivo* experimental method for identifying protein membrane topology is needed.

Development of a Rapid *in vivo* Experimental Technique "Protein Membrane Topology (PROMTO)" for determination of Membrane Topology of Golgi proteins

We want to developed a tool ("PROMTO") that can be used to test membrane topology of Golgi-localizing proteins by using N-terminal transmembrane domain of rat sialyltransferase ("STtmd" thereafter). STtmd was chosen for this study because it is a well established Golgi marker in yeast, mammals, and plant.

STtmd was recombinantly fused at its N and C-terminus with a full length yellow fluorescent protein (YFP) using the USER (Nour-Eldin et al, 2006) and Gateway (Walhout et al, 2000) cloning systems. These constructs were heterologously expressed in *Nicotiana benthamiana* and their Golgi localization was confirmed by confocal scanning-laser microscopy (Figure 4).

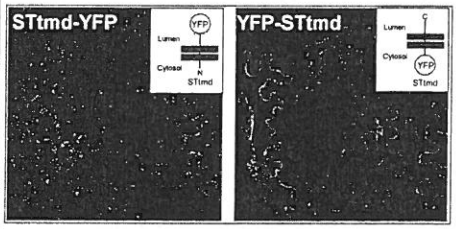

Figure 4: Tagging of Either Terminus Results In Golgi Localization of STtmd
The granular structure of the signal indicates a Golgi localization.

Next, STtmd was recombinantly fused at its N- and C-terminus with split domains YFP (Yn: 1-155 AA; Yc: 156-288 AA) and Bimolecular Fluorescent Complementation (BiFC, Hu et al., 2002) was carried out. We have observed that STtmd-Yn interacted with all of the nine Golgi-localizing proteins tested so far (data not shown). Therefore we concluded that STtmd-BiFC fusion proteins can interact with a wide range of BiFC fusion proteins and can serve as a suitable membrane topology marker as illustrated in Figure 5.

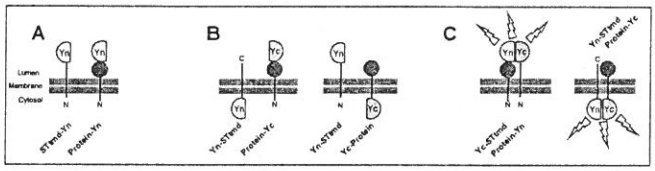

Figure 5: Illustration of "PROMTO" for Membrane Topology Analysis
Fluorescent complementation occurs when the two complementary domains of YFP are present on the same side of the membrane (C).

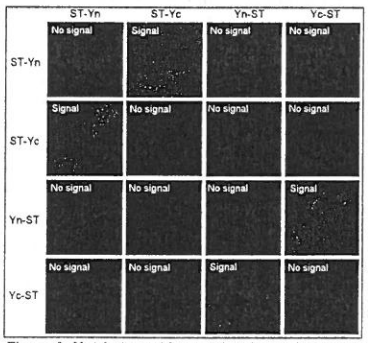

Figure 6: Matrix-based Interaction Analysis Between the STtmd-BiFC Fusion Proteins
The results demonstrate that STtmd-BiFC fusion proteins show specific interactions based on their membrane topologies.

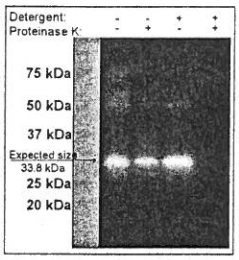

Figure 7: Proteinase K Protection Assay of STtmd-YFP Protein
A Western blot against the YFP epitope using microsomal fractions from *N. benthamiana*. YFP epitope is only degraded in the presence of both Triton X-100 and Proteinase K. This indicates that the YFP is oriented towards the Golgi lumen (preliminary data). YFP-STtmd protection assay is currently being performed

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Acknowledgements

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