Quantitative Analysis of Endomembrane Dynamics in *Tobacco* Pollen

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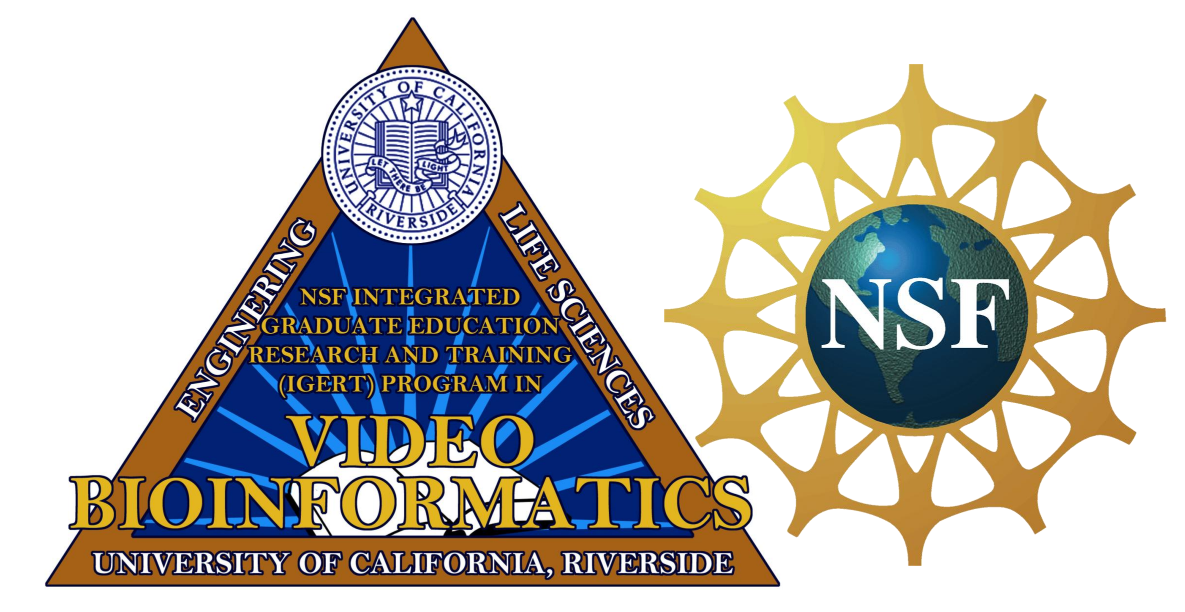

Abstract / Introduction

Endomembrane trafficking is a dynamic process that is required for essential biological processes such as signaling, stress response, defense, development, and polar growth. Recently, the intersection of synthetic chemistry and biology has lead to the use of chemical genomics as a tool to study biological processes. In this study, tobacco pollen was used in a semi-automated high-throughput imaging process to identify novel compounds that disrupt membrane cycling and, thus, pollen germination and morphology. Of more than 46,000 compounds screened, 360 were found to be bioactive in pollen leading to inhibition of germination or growth. In examining the endomembrane trafficking of a specific protein in tobacco pollen, seven compounds were found to cause defects in the localization or movement of RAB2:GFP (Cheung et al, 2002, Plant Cell 14:945), an endomembrane marker for ER to Golgi trafficking, and were termed "RAB2 effectors". The most common RAB2:GFP phenotype observed was the formation of RAB2 agglomerations or "RAB2 bodies". The size and movement of these bodies was quantified using an existing image analysis software package. Taken together, these techniques demonstrate the utility of this high-throughput screen in identifying compounds that alter the movement or localization of endomembrane proteins, the translatability of this method between systems and the advantage of using chemical genomics to dissect dynamic processes.

Chemical Libraries Show a High Rate of Translatability

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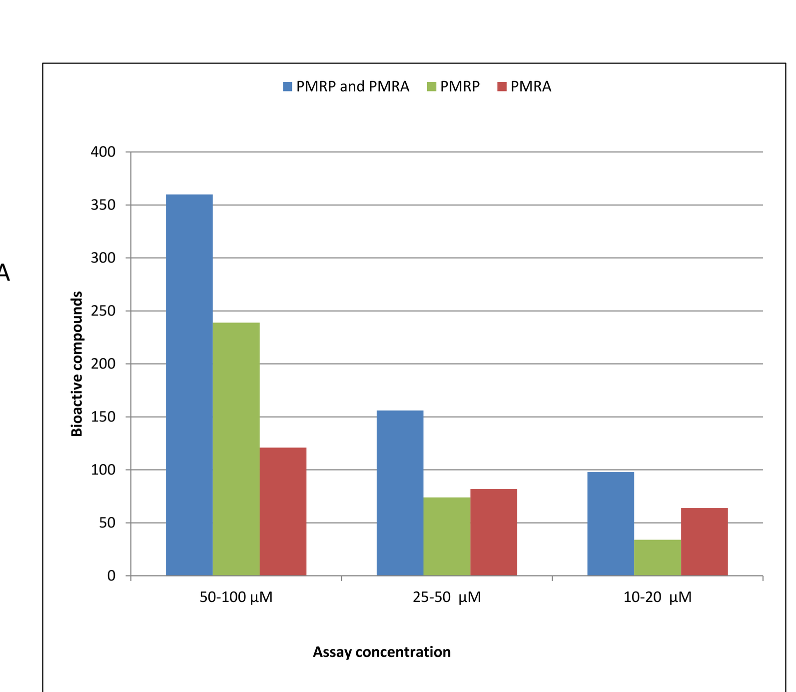
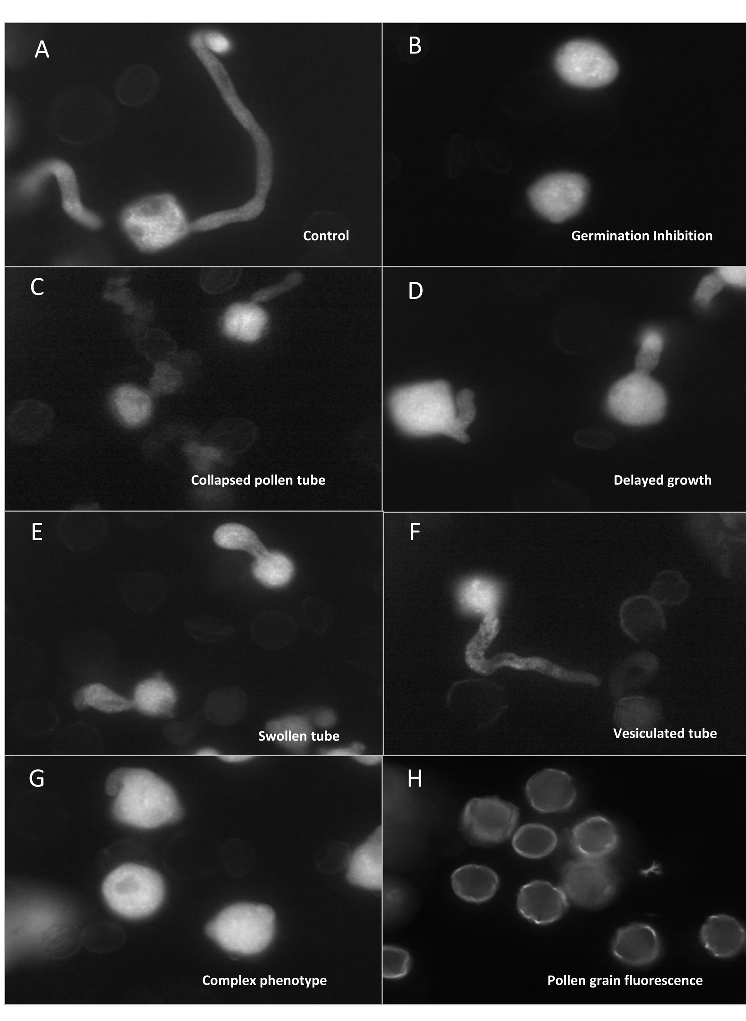


PMRA Bioactivity	25-50 µM	% of total	10-20 µM	% of total
Seedlings	78 compounds	64%	32 compounds	26%
Pollen	82 compounds	68%	64	53%
PMRP Bioactivity	**25-50 µM**	**% of total**	**10-20 µM**	**% of total**
Pollen	74 compounds	31%	34 Compounds	14%

Figure 1. From about 46,000 compounds previously screened on pollen, two sub-libraries were created. One contained compounds that caused defects in endomembrane recycling in seedling roots and was termed the Plasma Membrane Recycling Arabidopsis (PMRA) library. The other altered the morphology only of pollen and was termed the Plasma Membrane Recycling in Pollen (PRMP) library. As expected, the number of bioactive compounds decreased with concentration.

Bioactive Compounds Cause Developmental Defects That Fall Into Eight Classes



Phenotype	50-100 µM	25-50 µM	10-20 µM
Germination Inhibition	112	76	37
Rough grain	48	0	0
Collapsed Tube	51	27	12
Delayed growth	40	30	23
Swollen tube	4	9	5
Vesiculated tube	8	0	8
Complex phenotype	27	14	13
TOTAL	290	156	98

Figure 2. To discover compounds that disrupt endomembrane trafficking RAB2, a small GTPase that regulates the secretory pathway from the endoplasmic reticulum to the Golgi, was fused to green fluorescent protein (GFP). Bioactive compounds caused developmental and morphological phenotypes and were grouped into eight classes based on similar morphological effects. The table shows the number of compounds of each class observed at different concentrations.

Six RAB2 Effectors Cause Changes in Localization and Transport of RAB2:GFP

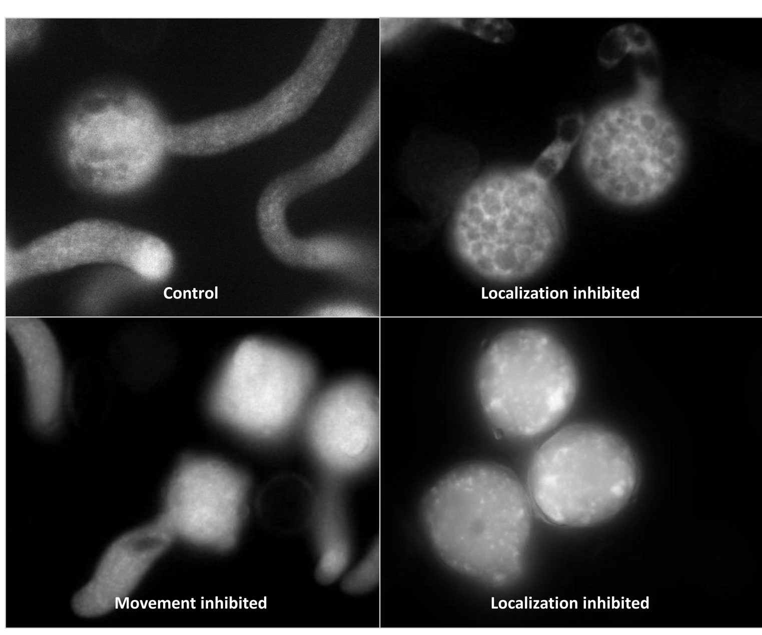


Figure 3. Six compounds caused either a change in localization or movement of RAB2:GFP and were designated "RAB2 effectors." Mislocalization of RAB2:GFP manifested in larger than normal RAB2 vesicles, thought to be agglomerations of multiple vesicles termed "RAB2 bodies." Some RAB2 effectors caused slowed movement of RAB2:GFP while others appeared to halt movement altogether.

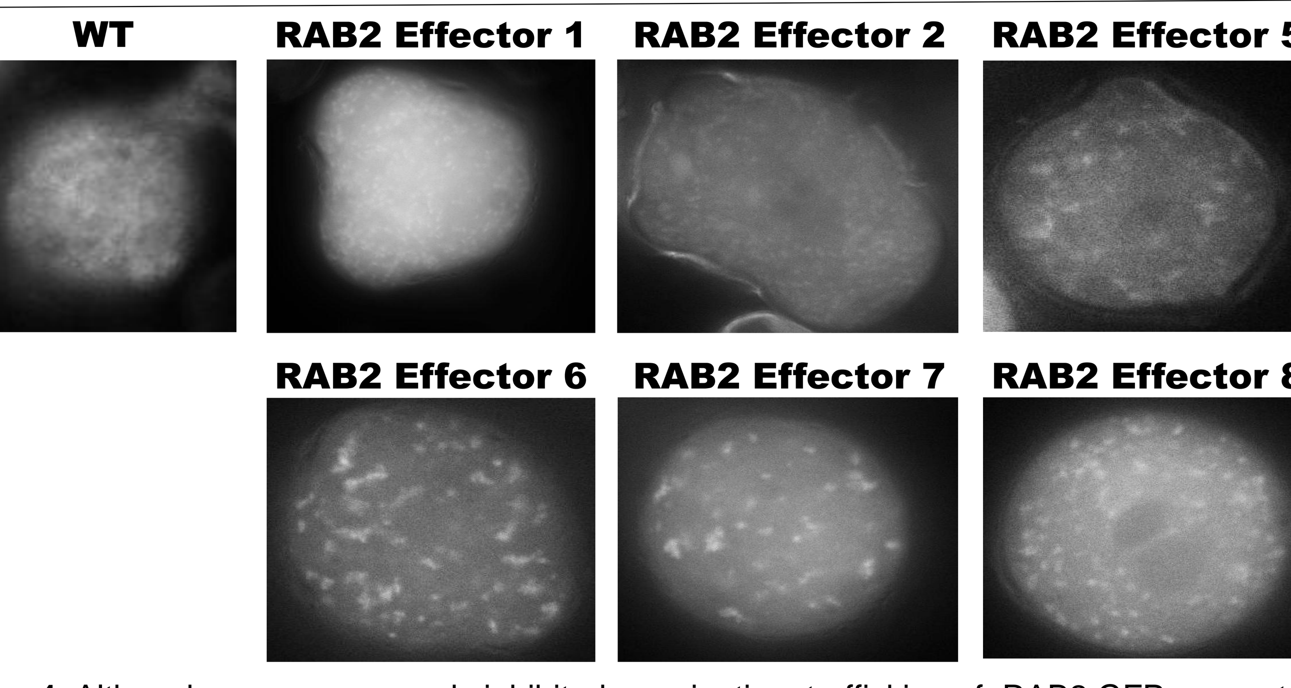


Figure 4. Although many compounds inhibited germination, trafficking of RAB2:GFP was not visibly affected. Initial inspection found that RAB2 effectors 1 and 2 slowed movement of RAB2:GFP and caused small agglomerations. RAB2 effectors 5,6,7 and 8 caused larger bodies while appearing to halt all movement; however video analysis shows a slight movement which is quantified below.

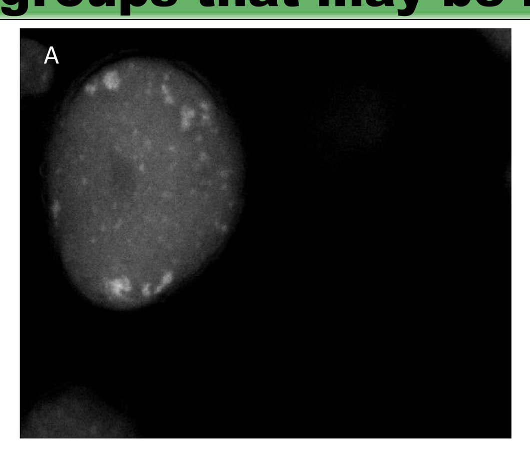
Quantitative Analysis Reveals Discrete Differences in RAB2:GFP Localization and Speed

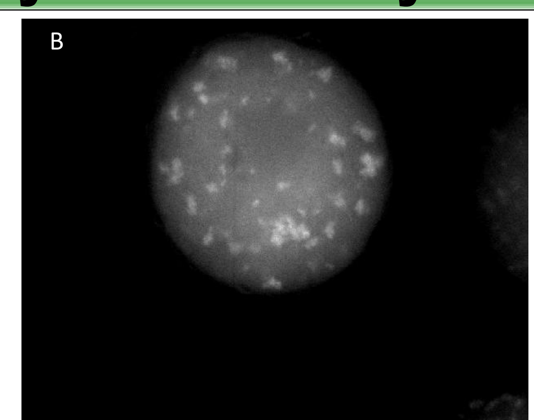
Quantification allows grouping of compounds based on size and speed of Rab2 bodies

Compound	Structure	Average net area of RAB2 Bodies(um^2)	% Area of WT RAB2 Bodies	Average net Speed of RAB2 Bodies (um/s)	% speed of WT of RAB2 bodies (um/s)
RAB2 effector 1		3.075	215.47%	0.288925164	31.7%
RAB2 effector 2		4.312	302.13%	0.307939452	33.8%
RAB2 effector 5		3.800	266.25%	0.108983246	11.9%
RAB2 effector 6		6.75	472.93%	0.045914441	5.0%
RAB2 effector 7		10.053	704.38%	0.047703853	5.2%
RAB2 effector 8		3.575	250.52%	0.067812802	7.4%
WT	No compound	1.427	100%	0.910476627	100%

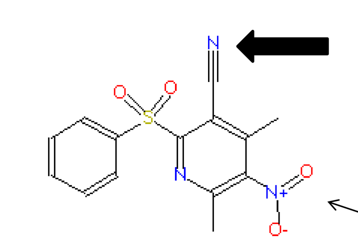
Figure 5. To confirm and quantify the differences in size and movement of the RAB2 bodies, image and video analysis was used on still and time lapse movies. Compound-induced RAB2 bodies were up to seven times the diameter of wild type organelles but moved at 5% of the speed of wild type RAB2 organelles. Quantification shows similar RAB2 body sizes and speeds of RAB2 bodies for effectors 6 and 7. Such variations may indicate distinct targets or modes of action.

RAB2 effectors 6 and 7 share similar functional groups that may be necessary for bioactivity





C **RAB2 effector 6**



D **RAB2 effector 7**

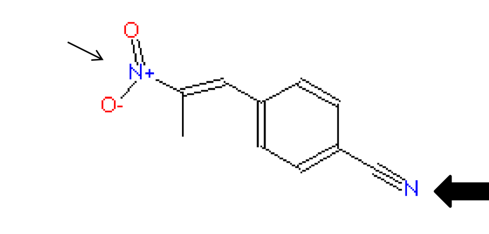
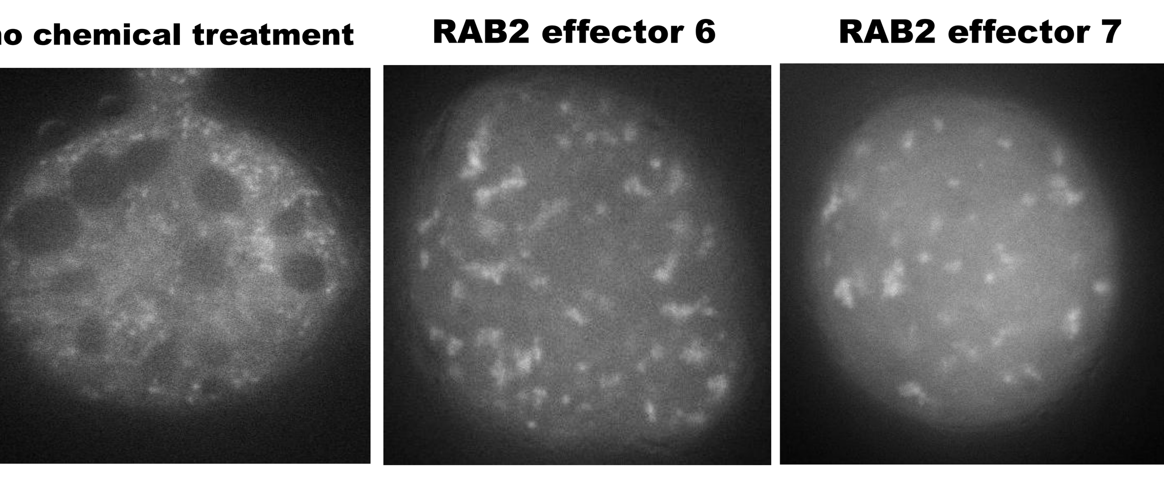


Figure 6. In an effort to determine the functional groups that could be responsible for causing the disruption in movement we analyzed the structure activity relationship of each RAB2 effector. Although there did not appear to be structural similarities among the six compounds, effectors 6 and 7 did have two functional groups in common. Analogs of each compound were tested and although each inhibited germination, none caused bodies or disrupted movement. The analogs were missing either the nitro or nitrile group suggesting they are required for RAB2 bodies and to halt movement.

RAB2 effectors do not effect the cytoskeleton



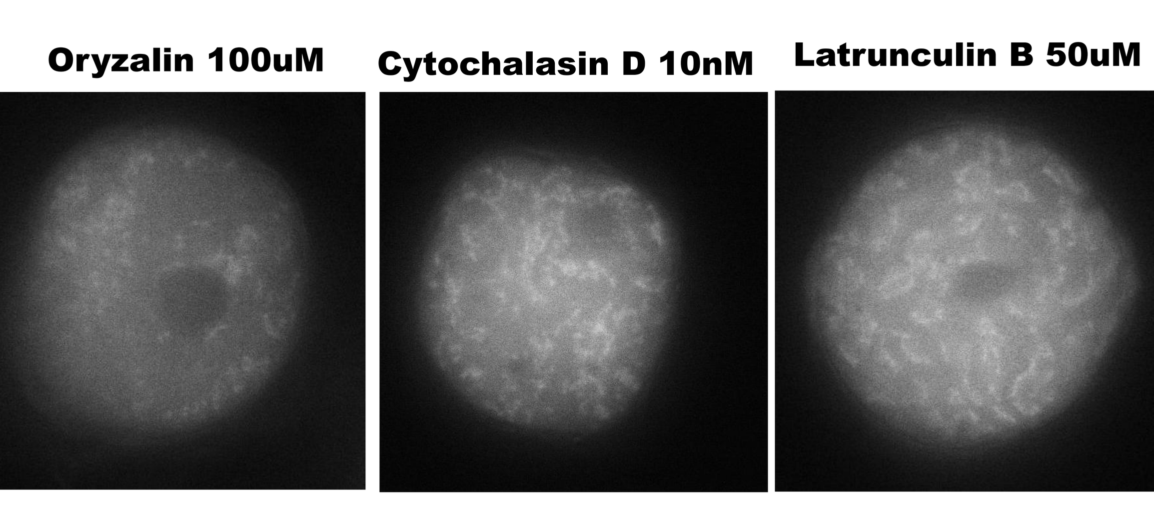


Figure 7. Because movement was halted, it was possible that the RAB2 effectors targeted cytoskeletal elements. To test this hypothesis, RAB2:GFP transformed pollen grains were treated with Oryzalin which depolymerizes microtubules, and Cytochalasin D and Latrunculin B that, by different mechanisms, alter or inhibit actin organization. All drugs halted movement and perturbed the localization of RAB2:GFP but do not cause the formation of RAB2 bodies indicating that RAB2 effectors do not directly disrupt actin or microtuble polymerization.

Conclusions

- **This high-throughput quantitative method is an efficient way of screening chemical libraries for compounds affecting trafficking.**
- **The tested libraries exhibit a high rate of translatability from pollen to seedlings.**
- **Six RAB2 effecters were discovered that perturbed RAB2:GFP localization and movement.**
- **The size and movement of the resulting RAB2 bodies were quantified and indicated that while RAB2 effectors 1,2,5 and 8 induced relatively small RAB2 bodies, Rab2 effectors 6 and 7 induced relatively large RAB2 bodies. Likewise, RAB2 effectors 1 and 2 induced RAB2 bodies that traveled more rapidly than RAB2 effectors 5,6,7 and 8.**
- **Possible bioactive functional groups were identified.**
- **RAB2 effectors are not directly altering microtubules or actin.**