UC Riverside

UC Riverside Electronic Theses and Dissertations

Title

Identification and Characterization of the Endosomal Recycling Inhibitor Endosidin2

Permalink

https://escholarship.org/uc/item/8nc0h2vn

Author

Brown, Michelle

Publication Date

2011

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Identification and Characterization of the Endosomal Recycling Inhibitor Endosidin2

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Genetics, Genomics, and Bioinformatics

by

Michelle Ardella Brown

June 2011

Dissertation Committee:

Dr. Natasha V. Raikhel, Chairperson
Dr. Zhenbiao Yang
Dr. Thomas Girke

Copyright by
Michelle Ardella Brown
June 2011

The Dissertation of Michelle Ardella Brown is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

ACKNOWLEDGEMENTS

I would like to thank my dissertation committee, Natasha Raikhel, Zhenbiao Yang, and Thomas Girke for their helpful discussions, advice, guidance and time. I would especially like to thank my PI and dissertation chair, Natasha Raikhel. I truly appreciate the opportunities she has provided me to grow both personally and scientifically. Encouraging interactive collaborations within the laboratory as well as with members from other institutions, Natasha created a wonderful environment for me to explore my scientific interests and of course, is a wonderful role model for all scientists. I would also like to thank Thomas Girke, my IGERT co-PI, who patiently worked to help me understand R computer programming given my limited computer programming background.

The pollen screen was the brainchild of Academic Coordinator Glenn Hicks and he has been a consistent and invaluable resource for guidance, questions and suggestions. Academic Coordinator David Carter, with his microscopy expertise and willingness to help work out challenges, has helped me to push the imaging boundaries with the current equipment on campus.

I wish to thank all of the Raikhel lab members, past and present, for their guidance, encouragement and support. I would especially like to thank Marci Surpin for always taking the time to provide feedback and constructive criticism, Abel Rosado for keeping me on track and sane in these last few months of finishing, Georgia Drakakaki and Stephanie Robert for encouraging me and challenging me to defend my ideas as we worked with the initial chemical screens, Marcela Rojas-Pierce and Eu-Ju (Julie) Sohn

for always taking the time to explain things and encouraging me to apply to graduate school in the first place, April Agee and Jocelyn Brimo for teaching me that there is tremendous joy to be had, Peng Liu for continuing the ES2 story and all of its challenges, Nolan Ung for being willing to take up the reins of many imaging projects as he begins his journey through graduate school (I know he'll succeed!), and Mien van de Ven for being a constant and patient resource to fix issues, deal with paperwork, and for the amazing baked goods on my birthday.

My collaborators and colleagues have provided great assistance with many aspects of this work. I would like to thank Gloria Muday of Wake Forest University for allowing me to do a brief internship in her lab, providing additional insight into the problems I was working on and her generous hospitality while I was there. I would also like to thank her students Jonathan Isley, who recently finished his Masters degree, and Ryan Hughes, who recently finished his Bachelors degree, for their work on the dynamic gravity assays and auxin transport assays. Thanks also go to Byung-Ho Kang, of the University of Florida, for his efforts to resolve the location of ES2 bodies.

I would like to thank the ChemGen NSF-IGERT and its co-directors Julia Bailey-Serres and Sean Cutler for funding and the opportunity to do some extraordinary things during my graduate career. I would also like to thank the Genetics, Genomics, and Bioinformatics program for funding.

On a personal note, I would like to thank my friends Samer Elkashef, Charles Jang, Matt Collin, Melissa Scranton, and Mindy Rodriguez-Salus for reveling in the joys of geekdom with me. To my sons Arik and Tyler, thank you for understanding when I

had to spend time engaged in research activities and know that I love you both dearly. To my husband Charlie, thank you for your support, understanding and love as I faced the challenges of graduate school. I would like to thank my grandparents, Dorothy and Frank Briggs, for always believing in me. Finally, I would like to thank my parents, Rita and Don Davis, who taught me that I can do anything I put my mind to, to stand up for my beliefs, and that success is inevitable if one is willing to learn from their failures. My love and thanks to all of you for your support and encouragement during the completion of my PhD studies.

ABSTRACT OF THE DISSERTATION

Identification and Characterization of the Endosomal Recycling Inhibitor Endosidin2

by

Michelle Ardella Brown

Doctor of Philosophy, Graduate Program in Genetics, Genomics, and Bioinformatics
University of California, Riverside, June 2011
Dr. Natasha V. Raikhel, Chairperson

Protein trafficking through the endomembrane system of plants is a highly dynamic and transient process. Protein trafficking plays a role in several signal transduction and developmental pathways such as gravitropic response, plant pathogen resistance, cell pattern formation and autophagy. One of the most dynamic compartments of the endomembrane system is the endosome. The endosome is comprised on proteins that transit to and from the plasma membrane, the *trans*-Golgi network, and the prevacuolar compartment. Given the highly dynamic nature of this compartment, chemical genomics is a useful approach to slow down trafficking of proteins or inhibit trafficking through specific compartments. Since high-throughput cellular phenotyping is not available in *Arabidopsis thaliana*, tobacco pollen was used as a model system for

membrane cycling to identify putative trafficking inhibitors for Arabidopsis. Tobacco pollen displays polarized growth that is dependent on the delivery and recycling of protein to the apical tip, and tobacco pollen is highly amenable to high-throughput screening. Utilizing tobacco pollen, over 46,000 natural and synthetic compounds were assayed and 378 were found to inhibit pollen germination and/or morphology. Of these 378, 365 were novel compounds. These 378 were then screened on three highly characterized Arabidopsis plasma membrane protein known to cycle through endosomal compartments, the brassinosteroid receptor BRI1:BRI1:GFP, and two PINFORMED auxin efflux proteins PIN2:PIN2:GFP and PIN2:PIN1:GFP. With just these three markers, 129 compounds from the pollen screen disrupted the normal localization patterns of at least one of these proteins. To examine a specific physiological response, these 129 were screened for effects on root gravitropic response. Sixteen compounds were identified as strongly disrupting gravity response. From these, the compound Endosidin2 (ES2) was characterized. ES2 disrupted several specific plasma membrane markers without affecting any internal protein markers, with the exception of the auxin homeostasis protein PINFORMED5, which localizes to the endoplasmic reticulum. ES2 specifically inhibits endosomal recycling to the plasma membrane without affecting endocytosis. ES2 allows for specific interrogation into the effects of endosomal recycling without affecting any other internal compartments as Brefeldin A does, the only other compound known to inhibit endosomal recycling.

Table of Contents

Chapter 1
From herbal remedies to cutting edge science: A historical perspective of plant chemical biology

1.1 Herbal remedies and pharmacology in the ancient world……..1

1.2 Alchemy, Chemistry, and the Isolation of the Bioactive Metabolites……...3

1.3 The Discovery of Phytohormones and the Foundation of Modern Plant Chemical Biology……6

1.4 The Dawn of Plant Synthetic Chemistry…….8

1.5 Serendipity versus Rational Design of Chemical Libraries……10

1.6 The Development of Combinatorial Chemistry……..11

1.7 Plant Chemical Biology in the –omics Era…….14

1.8 Introduction of Bioinformatics and Cheminformatics……16

1.9 Breaking the Bottleneck of Target Identification: The Future of Chemical Genomics in Plants……..…18

1.10 References……..21

Chapter 2
An Overview of Endomembrane Trafficking and Membrane Cycling

2.1 The Endomembrane System……27

2.2 Membrane Cycling Through Endosomal Compartments……29

2.3 A Chemical Genomics Approach to Endomembrane Trafficking……..31

2.4 References……33

Chapter 3
Utilizing Tobacco Pollen to Identify Compounds that Inhibit Membrane Cycling

3.1 Abstract……...35

3.2 Introduction……36

3.3 Materials and Methods……....39

3.4 Results & Discussion……...40

3.5 References……...49

Chapter 4
Identifying and Clustering Compounds that are Bioactive in Arabidopsis

4.1 Abstract…….....51

4.2 Introduction……..…51

4.3 Materials and Methods…….53

4.4 Results & Discussion……...55

4.5 References…….....64

Chapter 5
Identifying Compounds that Inhibit Gravity Response

5.1 Abstract……65

5.2 Introduction……..65

5.3 Materials and Methods……....67

5.4 Results & Discussion……...69

5.5 References……...91

Chapter 6
Conclusion and Future Directions

6.1 Membrane Cycling Inhibitors in Tobacco Pollen……95

6.2 Identification and Clustering of Cycling Inhibitors in Arabidopsis……96

6.3 Inhibition with the Gravitropic Response Pathway…….97

6.4 Characterization of Endosidin2……...98

6.5 References……..100

List of Figures

Figure 1.1 Plant metabolites and chemical compounds useful in biology research……...4

Figure 1.2 Number of publications featuring chemical biology in Arabidopsis…….....15

Figure 3.1 Phenotypic classes observed on compounds...……..43

Figure 3.2 Phenotypic classes observed with RAB2:GFP pollen……44

Figure 3.3 Two categories of phenotypes observed when RAB2:GFP is inhibited…....47

Figure 4.1 Phenotypic classes observed in Arabidopsis seedlings……..58

Figure 4.2 Frequency of observed phenotypic classes in Arabidopsis seedlings……59

Figure 4.3 Clustering by phenotypic fingerprinting…….....61

Figure 4.4 Common motifs are found within fingerprinting clusters……..62

Figure 5.1 Dynamic root gravity response for the gravity Endosidins……73

Figure 5.2 Dose response curve for Endosidin2……..74

Figure 5.3 Structure activity relationship for Endosidin2……76

Figure 5.4 Physiological effects of Endosidin2……77

Figure 5.5 Specific plasma membrane markers affected by Endosidin2…….79

Figure 5.6 No internal markers were affected by Endosidin2…….....80

Figure 5.7 Additional ER and Golgi markers were not affected by Endosidin2…….....81

Figure 5.8 PIN2 and PIN5 can co-localize upon Endosidin2 treatment……..83

Figure 5.9 Endosidin2 inhibits endosomal recycling……...84

Figure 5.10 Endosidin2 does not affect endocytosis……85

Figure 5.11 Endosidin2 increased trafficking to the vacuole in the dark…….87

Figure 5.12 Endosidin2 increased brassinosteroid signaling……....89

Figure 5.13 Endosidin2 affects auxin and brassinosteroid pathways…….90

List of Tables

Table 3.1 Compounds that disrupt tobacco pollen germination/morphology……41

Table 3.2 Phenotypes observed through dose response of PMRP library……..46

Table 4.1 PMRP compounds that disrupted localization in Arabidopsis……56

Chapter 1

From herbal remedies to cutting edge science: A historical perspective of plant chemical biology

1.1 Herbal Remedies and Pharmacology in the Ancient World

Herbal medicine is the oldest form of healthcare known to mankind and, dependent upon availability, different herbs have been used by emerging cultures throughout history. Much of the medicinal use of plants seems to have developed through observations of wild animals, and by trial and error. As time went on, each culture attempted to systematize and organize the acquired knowledge of the medicinal herbs to increase the health and productivity of those emerging civilizations (Saad and Said, 2011). As early as 2735 B.C., the Chinese emperor Shen Nong wrote an herbal treatise recommending the use of Ma Huang (ephedra, from which derives the ephedrin decongestant) against respiratory distress. The records of King Hammurabi of Babylon (1800 B.C.) prescribed the use of mint for digestive disorders, and multiple texts surviving from the ancient cultures of India, Mesopotamia, and Egypt illustrate the use of many medicinal plant products, including linseed oil, Castor oil, and white poppies for the treatment of multiple diseases (Alam et al., 2008).

Deriving knowledge from the medical treatises and methods of those ancient civilizations and other conquered cultures such as Greeks, Etruscans, and Persians,

allowed the Roman civilization to devise one of the best and most sophisticated medical systems of the ancient world. Roman contributions to the development of chemical biology include the cultivation of medicinal herbs for commercial purposes along with the introduction of a variety of painkillers and sedatives including extracts of opium poppies (morphine) and of henbane seeds (scopolamine) tested in battle by Roman physicians on wounded soldiers (http://www.crystalinks.com/romemedicine.html). The Roman physician Galen of Pergamo (130-200 A.C) outlined the basic principles of pharmacology, developed a methodical approach to drugs based on reasoning "logos", and defined a system of Galenic degrees of potency to treat varying levels of dysfunction which enabled physicians and pharmacists to gauge more precisely the effects of a medicinal substance in a dosage dependent manner (Debru 1997). For almost a thousand years the drugs and other methods of treatment popularized by Galen were used as standard protocol in European medicine (Porter et al., 1995).

With the advent and flourishing of the Islamic culture in Europe, the Persian text Qanun (Ibn Sina, also known as Avicenna, 1025), translated into Latin as *Canon medicinae*, displaced the works of Galen, and took a prominent position in the medical literature becoming the textbook for medical education in European Universities up to 1650 (Ashoor 1984). The *Canon* established the basis of modern clinical pharmacology by introducing concepts such as clinical trials, randomized controlled trials, and efficacy tests laying out rules and principles for testing the effectiveness of new drugs and medications. The *Canon* was also used for medieval medical practitioners as a drug encyclopedia listing no less than 700 preparations, including plant and mineral

substances, with comments on their pharmacological action (Jacquart 2008; Craig and Daly 2000a; Eldredge 2003; Craig and Daly 2000b; Langermann 2010).

1.2 Alchemy, Chemistry, and the Isolation of the Bioactive Metabolites.

By the 17th century, the knowledge of herbal medicine was widely disseminated throughout Europe and many natural remedies were dependent on plant-based substances. In that period, the study of alchemy and the quest for a "panacea," a remedy that was supposed to cure all diseases and prolong life indefinitely, was attempted by renowned scientists including Sir Isaac Newton, Roger Bacon and Tycho Brahe (Williams and Williams 2009). Although the final objective was never achieved, alchemy established the experimental basis of modern chemistry and was a very important milestone for the development of extractive techniques to isolating plant compounds for medicines, dyes and perfumes preparation (Newman and Principe 1998). Thus, as the science of chemistry matured, chemists were able to extract and purify the biologically active medicinal components from the natural substances used as herbal remedies for millennia. Among these extraction accomplishments were the isolation of the extract from foxglove (digitalis) commonly used as a treatment for oedema or dropsy (Withering 1785), the isolation of the addictive analgesic morphine from the seed capsules of poppy flowers (Sertürner 1806), and the isolation of the anti-inflammatory salicin from willow bark (Leroux 1830, Figure 1).

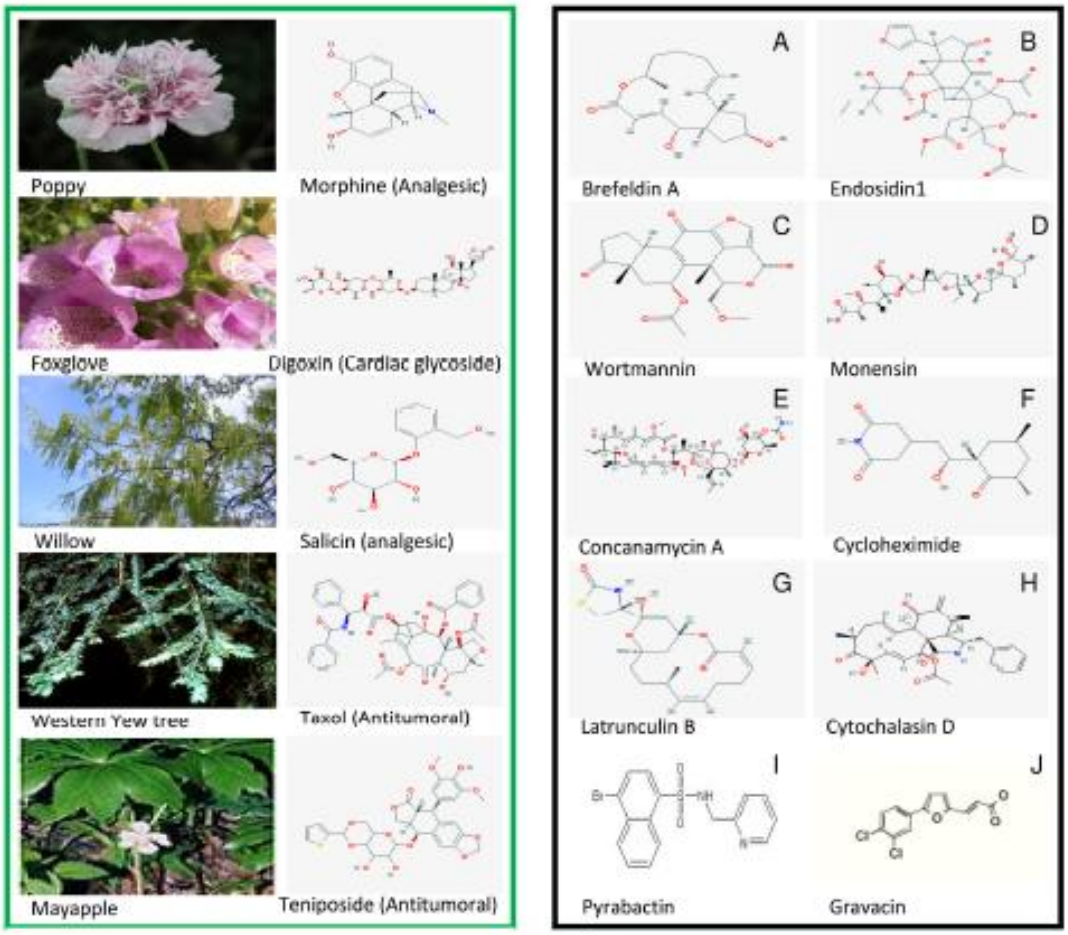


Figure 1.1 Left panel: Examples of bioactive molecules isolated from plant sources and their pharmacological action. Right panel: Example of natural and synthetic chemical tools utilized in plant research. The examples include protein trafficking and synthesis inhibitors (A-F), cytoskeleton modifiers (G-H), and transporter/receptor blockers (I-J).

With increased purity and knowledge of the active plant extracts and the advances in pharmacology, the first purely synthetic drugs based on natural products were elaborated in the middle of the 19th century. Thus, in 1839, the studies of the willow bark extract salicin identified the salicylic acid as the active ingredient providing its analgesic properties (Piria 1839). In 1853, chemist Charles Frédéric Gerhardt mixed acetyl chloride with sodium salicylate to produce synthetic acetylsalicylic acid for the first time (Gerhardt 1853). Academic chemists established the compound's chemical structure and devised more efficient methods of synthesis that finally led to Felix Hoffman's development of the acetylsalicylic acid formula named Aspirin®, the most widely used synthetic drug today and the origins of the synthetic pharmaceutical industry, in 1899 (Andermann 1996).

In parallel with the isolation and synthesis of bioactive metabolites derived from plants, the search for antibiotics began in the late 1800s, with the growing acceptance of the germ theory of disease, a theory that linked microbes to the causation of a variety of illnesses (Andry de Bois-Regard 1888). Initially, antibiotics were identified as natural substances released by moss and fungi into their environment, as a means of inhibiting growth of other organisms, and many extracts with antibiotic properties were reported in the literature from the late 1800s. Milestones in antibiotic research and in the development of the synthetic chemistry were the identification and isolation of the antibiotic penicillin from the *Penicillium* mold (Fleming 1929), together with the manufacturing process for Penicillin G Procaine developed by Sir Howard Florey in 1942, whose consequences have had the most profound impact on human health than any

other natural substances (Mailer and Mason 2001). The successes of isolating bioactive compounds and antibiotics from natural sources by the end of the 19th century allowed the development of multiple scientific disciplines in the plant field such as plant physiology with the identification of the hormonal requirements for plant growth, and chemical genetics or the interrogation of plant systems using chemical compounds that will be described more in depth in the following sections of this review.

1.3 The Discovery of the Phytohormones and the Foundation of Modern Plant Chemical Biology

Since antiquity, people have sought to explain plant physiological responses such as gravitropism and touch sensitivity via mechanical or passive physiochemical forces. In this context, Aristotle's conclusion that plants were insensitive and lack active motion against external stimuli pronounced in the third century B.C. was still prevalent and widely used by 18th century taxonomists to classify and differentiate plants from other organisms within the graduated scale of nature (Stafleau 1971). The publication in 1880 of *The Power of Movement in Plants* featuring Charles Darwin's research in the botany and plant physiology fields was a landmark attempt to integrate plant movements into a biological perspective of behavior by introducing the concepts of plant sensitivity and active perception of external stimuli (Darwin and Darwin 1880). The recognition that plants might respond to the environment in much the same way as animals was a paradigm shift for taxonomists and plant physiologists and became the seminal idea that

led to the discovery of the first plant growth factor, the auxin phytohormone indolacetic acid (IAA).

In 1881, Darwin's experiments on gravi- and phototropism showed that some internal signal or “influence” moved down from coleoptile tips of the annual canarygrass, *Phalaris canariensis*, to control cell elongation after stimulation (Darwin and Darwin 1880). The presence of these internal signals in plants lead Hans Fitting (1909) to introduce for the first time the term “hormone” in plant physiology referring to substances stimulating specific plant developmental processes (Fitting 1909). In 1913, Danish botanist P. Boysen Jensen suggested that the phototropic signal described by Darwin was indeed a chemical messenger moving down from the tip of the coleoptile (Boysen Jensen 1913). During the early 1920s several botanists provided circumstantial evidence of the presence of such a chemical in different plants, and finally in 1928, the Dutch physiologist Frits Went defined an experimental procedure for isolating this chemical and quantifying its physiological activity (Went 1928). A next important step concerned the purification of sufficient amounts of this chemical for analytical purposes. Five years of attempts made by K. Thimann, and J. Haagen-Smit among others, finally succeeded and the Darwinian “influence” was identified as indolacetic acid (IAA) (Thimann and Kipefli 1935; Haagen Smit and Went 1935).

The identification of IAA as the growth factor responsible for the gravitropic and phototropic responses crowned with success of a half century of research, delivered the concept of internal chemical signals in plants, and opened the door to the synthetic

chemistry of plant growth factors a milestone that developed modern agricultural techniques such as growth stimulation and weed control.

1.4 The Dawn of Plant Synthetic Chemistry

The implementation of synthetic and combinatorial chemistry in the agrochemical industry coincides with the development of the modern pharmaceutical industry and the availability of many organic compounds derived from coal tar, plants, and microorganisms. In this context, pharmacological studies by P. Ehrlich (1913) led to the receptor theory of small-molecule action and receptors were defined as specific body constituents where the active drugs selectively bind (Ehrlich 1913). After the identification of IAA as a plant growth factor, the 1940s witnessed the creation of multiple academic and industrial laboratories in which combinatorial chemistry and high-throughput screens were used to uncover new hormones and herbicides targeting the auxin receptors in plants. As a result, a wide array of IAA analogs and derivatives including 1-naphthalene acetic acid (1-NAA), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4-dichlorophenoxy acetic acid (2,4-D) were synthesized. Further research demonstrated that these derivatives were able to elicit the same type of plant responses as IAA such as elongation in aerial tissues and stimulated lateral root formation, but with a longer-lasting and stronger intensity of action owing to their higher stability in the plant (Cobb 1992; Sterling and Hall 1997; Fedtke and Duke 2005).

With increased concentration, stability, and activity in plant tissues, synthetic auxins gained considerable importance for probing auxin function in basic research as

well as for applied civil and military uses. Example of civil uses range from the commercialization of the synthetic auxin 1-NAA, marketed as "Hortomone A" to stimulate lateral rooting in crops (1939) (Taylor 1940) to the commercial elaboration in 1945 of the first systemic herbicide based on synthetic auxins marketed as "Weedone", a product that started a new era of weed control in modern agriculture (Troyer 2001). In parallel, the development of synthetic auxins for military purposes was fast-tracked during World War II and early investigations regarding the use of synthetic auxins were kept secret because these novel herbicides were deemed putative bioweapons (Martin et al., 2007). It was not until the Vietnam Conflict nearly twenty years later, when hormone based weedkillers would become infamous due to the extensive use of the defoliant Agent Orange, an equal mixture of 2,4D and 2,4,5-trichlorophenoxyacetic acid, as part of the US herbicidal warfare program. The use of Agent Orange in the Vietnam War caused the loss of millions of acres of upland and mangrove forests and crops and its side effects to humans and the environment were a crude reminder of our limited knowledge of the effects of synthetic chemicals to the natural environment (Kolko 1994).

In the post World War II era, the excitement caused by the synthesis of the novel antibiotic penicillin and its impact for human health, the increased crop production after use of synthetic herbicides, and the development of affordable synthetic chemicals led to a scientific frenzy to identify more natural and synthetic substances derived from plants and microorganisms. These advances had profound implications in the field of plant chemical biology because multitudes of small molecular probes were available to explore biological processes in cells and whole organisms without the need for a stringent

pharmacokinetic or safety profile. While not all of these substances led to use as commercial drugs, antibiotics or herbicides, many of these compounds, such as Cycloheximide, Brefeldin A, cytochalasins and latrunculins became useful probes of biological function in plants and animals, providing relevant information on protein targets and metabolic pathways, and developed into classic molecular tools to address basic and applied biological questions.

1.5 Serendipity versus Rational Design of Chemical Libraries

By the end of the 1940s the use of natural and synthetic compounds as probes to modulate biological pathways in plants was extended and synthetic organic chemistry had progressed to the point where the large scale preparation of synthetic analogs of bioactive molecules such as antibiotics and plant hormones was economically feasible. At the same time, the introduction of powerful commercial spectrometers (NMR, 1952) and separation techniques (HPLC, 1961) for determining the structures of minute quantities of biologically active natural products boosted the identification and refinement of natural drugs. Thus, the resulting "methyl, ethyl, isopropyl" analog model for drug discovery, although empirical, was quite successful for several decades. However, two important limitations derived from the use of collections of natural compound and their synthetic derivatives in mass screening started to emerge. First, the basis of drug discovery heavily relied on the serendipity of the natural products isolated, and second, those natural product collections contained a limited number of compounds representing

only a minute fraction of the structural diversity possibilities in the chemical space (UC Regents 2010).

With the development of computer tools and concurrent advances in structural biology, chemistry and protein crystallography in the 1980s a shift to a "rational" computational archetype for drug discovery emerged, and the computational drug design was born (Craig 1980). In this approach, the continuous stream of new protein structures and the identification of their active sites and binding pockets would provide the basis for the computational drug design. However, limitations in the crystallographic techniques, together with the requirement of experimental validation of the candidate molecules, the stability of the compound within plant cells, and the bioavailability of the compound to interact with the target protein, hastened the "rational drug design" supplantation rather quickly (but not completely) by a return to largely empirical methods of small molecule library synthesis and high-throughput screening. Technological advances in robotics and biological techniques for simultaneously assaying thousands of compounds in concert during the 1990s fueled this paradigm shift.

1.6 The Development of Combinatorial Chemistry

After the partial drawback of the rational drug design, chemists and biologists realized that large collections of structurally distinct molecules should be generated to perform efficient pharmacological studies and therefore the traditional synthesis and purification of one compound at a time for screening was no longer a feasible approach. An important step towards increasing the diversity and the speed in which structurally

related compounds were generated, was the application of combinatorial chemistry techniques involving systematic mixing and matching of various chemical building blocks to generate libraries of small molecules. The development of these techniques, running in parallel with advances in automation and robotics in the early 1990s, enabled the application of Merrifield's solid-phase synthesis of peptides described in the 1960s for the combinatorial synthesis of peptides on solid phase using mix and split reactions pioneered by the organic chemists Furka and Lam in the early 1990s (Merrifield 1963; Lam et al., 1991; Furka 1995). The success of combinatorial chemistry to generate peptide and peptidemimetic libraries had immediate impact for pharmacological studies due to the essentially iterative reactions, its amenability to automation, and the ease of removing detritus from the products by simple washing and filtration away from the resins.

Early combinatorial chemistry techniques successfully generated enormous numbers of compounds, still their 'diversity' was limited to variations in appendages attached to a small number of common skeletons and the compounds frequently lacked chirality. Since most of the small molecules known to disrupt protein-protein interactions were structurally complex natural products, the organic chemist Stuart Schreiber reasoned that maximizing the number of structures and scaffolds, by increasing the size and number of rigidifying elements (macrocycles, polycycles, olefins, etc.) in small molecules libraries would be the most efficient ways to populate the largest amount of chemical space. Hence, the creation of smaller diversity oriented library of compounds containing wider spectra of molecular shapes would provide both more hits and a wider

range of biological targets than the traditional combinatorial libraries (Schreiber 2000). Proof of principle for this technology was the use of diversity oriented synthesis of the known immunosuppressors molecules rapamycin and FK506 to identify the protein calcineurin as an important component of the immune signaling pathway (Schreiber 1991).

As the number and diversity of characterized bioactive synthetic compounds increased the biochemist Chris Lipinski was able to define general properties and structural features that make molecules more or less drug-like based on their molecule's size, lipophilicity and hydrogen bond acceptors and donors. Lipinski's guidelines known as the "Rule of Five" (Lipinski et al., 2001) were quickly adopted by the pharmaceutical industry acting as an early filter during the development of orally active drugs.

By the end of the millennia, an important limitation to expanding and defining the possibilities of combinatorial chemistry for basic plant research was the closed environment of the biotechnology and pharmaceutical industries in which most of the research projects were carried out. In this context, a milestone for the development of plant chemical biology as a subject for basic science was the creation of publicly available high-throughput screening facilities such as the Harvard's Institute of Chemistry and Cell Biology (1998) whose highly successful Investigator-Initiated Screening Program facilitated the development of small molecule screening projects for basic research and oriented the pursuit of Chemical Genetics as an academic discipline.

1.7 Plant Chemical Biology in the –omics era

As chemical biology was gaining prominence as academic discipline, plant biologists were also gaining a new host of molecular and genetic tools allowing plant genetics to undergo its own development into the age of genomic research. The release of the complete sequence of the model plant *Arabidopsis thaliana* (2000), together with many unique genetic/genomic tools developed in this plant system such as whole-genome microarrays, a large collection of knockout and activation-tagged mutations, a rich array of mutants, and the ease of cloning a gene by map-based cloning aided by DNA microarrays, allowed the integration of chemical screens in the Arabidopsis model at various biological levels ranging from subcellular to the whole organism.

The development sophisticated imaging techniques such as confocal laser scanning microscopy (CLSM) which compared with traditional optic microscopy allows for greater resolution of the image and is able to generate a three dimensional representation of the specimen, and the development of plant specific probes such as engineered green fluorescent protein (GFP), originally isolated from the jellyfish *Aequorea victoria*, fused to the now sequenced Arabidopsis genes, allowed researchers to view the *in vivo* subcellular localization of proteins instead of the static state of fixed specimens giving a greater understanding of the dynamics of the cell (Cutler et al., 2000). Thus, rather than have to wait for growth or gross morphological plant phenotypes, plant researchers were now able to observe immediate subcellular phenotypes based on the aberrant localization of proteins after chemical treatments.

The combination of chemical screens, Arabidopsis genomics/proteomics tools, advances in microscopy, and protein tagging allowed plant scientists to analyze and effectively dissect the global effects of chemicals of interest in complex gene networks, to rapidly identify their gene targets, and to efficiently investigate their mode of action in real time. Examples of invaluable contributions of chemical genomics approaches in basic and applied plant research include the identification of the receptors for brassinosteroids (Wang et al., 2001), ethylene (Schaller and Bleecker 1995) and ABA (Park et al., 2009) which boosted the number of publications applying chemical genomic analysis for plant research in the last decade (Figure 2).

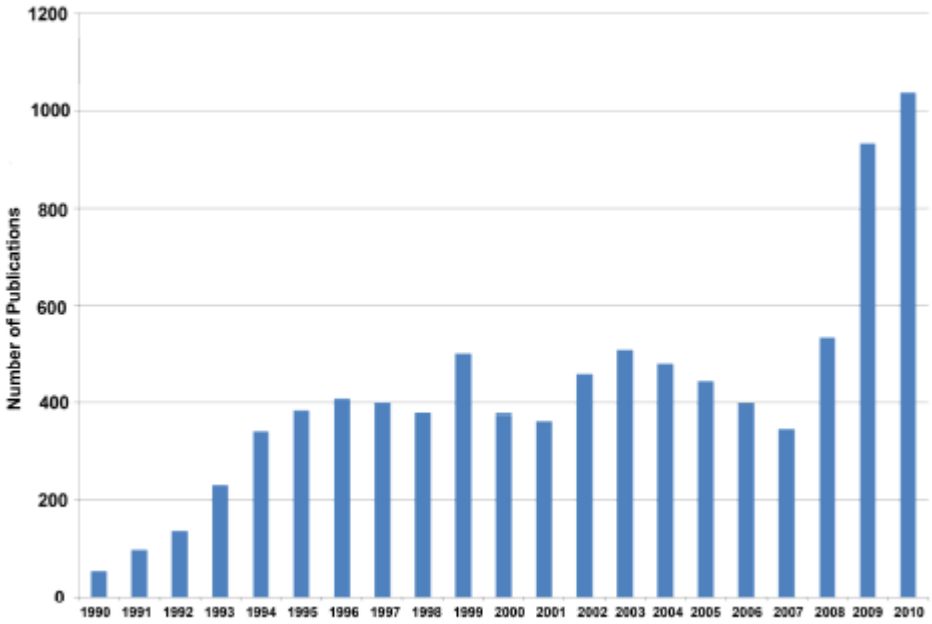


Figure 1.2 Evolution of the number of publications using chemical genomic approaches in the model system *Arabidopsis thaliana*. Source: National Center for Biotechnology Information (NCBI).

1.8 Introduction of Bioinformatics and Cheminformatics

The deluge of information generated by the –omics analyses could only be managed with the help of computers and their programmers, and thus, the field of bioinformatics was born. Facilitating the growth of bioinformatics were the internet-acessible public repositories, such as the National Center for Biotechnology Information (NCBI). At first, bioinformatics merely managed the wealth of sequences and structures. Algorithmns to analyze homology of gene sequences, such as Basic Local Alignment Search Tool (BLAST), were necessary to navigate the over 100 million sequence records contained within GenBank at NCBI. This helped to identify the members of large gene families and genes similar in sequence to ones with known function in other sequenced organisms, such as yeast. Bioinformatics has helped identify common promoter motifs among genes that promote both brassinosteroid and auxin pathways (Nemhauser et al., 2004). Now, with a wealth of analysis and modeling algorithmns and tools available, bioinformatics represents just a portion of the field of computational biology. Bioinformatics also provides a critical tool in the field of comparative genomics.

As high-throughput screens continued, the utility of bioinformatic techniques to manage both assay results and compound structures broadened the specialized field of cheminformatics. The formation of the NCBI's PubChem open-access database in 2004 was initially a cause of concern for the American Chemical Society Chemical Abstract Service (CAS) Registry which maintained a subscription-based comprehensive database of compounds dating back to the early 1900s. Since the inception of PubChem, however,

several publicly accessible compound databases have been established (e.g. ChemDB (Chen et al., 2005), DrugBank (Wishart et al., 2006), ChemmineR (Cao et al., 2008)). In these databases, three-dimensional compounds are described using the simplified molecular input line entry specification (SMILES). These descriptors along with physiochemical descriptors can be mined for structural and activity relationships among compounds.

While a little more than 10 million pure compounds are described in chemical databases, the potential chemical diversity (defined as the number of unique chemical structures) of compounds composed of carbon, hydrogen, nitrogen, oxygen, sulfur, phosphorous, and the halogens (the organic chemist's periodic table) of molecular weight <1,000 likely exceeds 10^{60} (Bohacek et al., 1996). However, in a time in which Moore's Law (1963) stating that computer processing speed doubles every two years still prevails, it is not utopist to think that in the near future the vastness of chemical spaces could be overcome with enough computing power. The virtual screening of compounds is extremely appealing because it saves the time and expense of physically screening vast numbers of compounds. *In silico* screening requires either the identification of the ligand to be bound or the specifications of the docking site for competitive inhibition. Docking programs that generate the possible conformations of interaction between ligand and docking site are of critical importance. Maintaining the open access example of the databases, many docking programs such as AutoDock (Goodsell et al., 1996) and ZINC (Irwin and Shoichet 2005) are publically accessible. *In silico* screening allows an enrichment of putatively active compounds based on the ligand or docking site. These

compounds then need to be actively assayed to determine actual activity. To demonstrate the utility of this approach, Doman et al. (2002) in a screen to identify novel inhibitors of tyrosine phosphatase 1B utilized both *in silico* chemical genomics and high throughput screening (HTS). The *in silico* program determined 365 putative hits and 127 of these were confirmed, for a hit rate of 34.8%. The HTS screen of over 400,000 compounds produced only 85 hits, for a hit rate of 0.021%. With *in silico* applications however, results need to be verified with wet lab experiments when the targets are known. Without a directed chemical screen, compound targets must be identified through mutational analysis and mapping or biochemical means.

1.9 Breaking the Bottleneck of Target Identification: The Future of Chemical Genomics in Plants

In efforts to speed up the time required to identify the molecular target of a compound, some libraries have been created with incorporated biochemical tools that allows the purification of the putative target in an easier manner than traditional libraries. One such compound library containing over 4,000 chemicals utilizes the copper catalyzed synthesis of triazoles from azides and acetylenes "click chemistry" (http://cutlerlab.blogspot.com/2008/05/clickables.html) and a two-step purification procedure was recently published utilizing click chemistry in plants by Kaschani et al. (2009). Photoaffinity tags have also been introduced to compounds to efforts to identify target proteins. With photoaffinity tags, the compound can interact with its target while the tag is unactivated. Upon irradiation, the tag covalently links the probe with the target

(Leslie and Hergenrother 2008). Photoaffinity tagged compounds have been recently used to interrogate the jasmonate pathway, using a coronatine photoaffinity probe (Gu et al., 2010). Interestingly, some tagged libraries have proven effective in some organisms yet have failed to yield results in plants. For example,,diversity oriented fluorescence libraries (DOFL) (Vendrell et al., 2010) in which compounds are tagged with fluorophores has been used to study cellular differentiation in mammalian cell lines (Wagner et al., 2008), but no study applying DOFL libraries in plants has not yet been published.

Besides modified compound libraries, other tools have now been made available in plants. One of these is the protein microarray chip that arrays purified, functional proteins on a chip platform instead of the cDNA segments used in traditional microarray chips (Popescu et al., 2007). Another tool is the yeast-three-hybrid (Y3H) assay that uses a compound tagged with dexamethosone or methotrexate that is applied to yeast cells where the tag fuses to the DNA-binding site of a transcription factor (Becker et al., 2004). The activation domain of this transcription factor is bound to a plant cDNA library and an interaction between the compound and the target protein turns on the reporter gene. Y3H has been recently been used to identify negative effectors of gravitropic bending in Arabidopsis (Knauer et al., 2011).

Although the different approaches and techniques enormously facilitate the target identification, there is an intrinsic property of the system that cannot be overcome by chemical genomic approaches. This limitation is the percentage of the plant genome that

is “druggable,” representing a viable drug target. While there have been no estimates of that percentage in plants, the human genome, which has a similar number of genes as Arabidopsis, was predicted to contain only 10-14% druggable genes (Hopkins and Groom 2002). Therefore chemical genomics must still rely on genetics and other disciplines to elucidate the function of non-druggable plant genes.

The future of chemical genomics will undoubtedly continue to incorporate and streamline novel technologies for increased utility and understanding. Biologists have long taken a reductionist view of organisms, reducing them to basic component parts and pathways. However, system biologists’ view of organisms is integrated into networked complex pathways, with the belief that unique properties can only be observed through the study of the whole system. An example of this is plant root biology, in which growth and development are regulated by a complex network of biotic and abiotic response pathways. The next natural step would be to incorporate chemical genomics into the systems biology paradigm. This type of chemical genomic systems biology is already being utilized in yeast studies (Hillenmeyer et al., 2011). Thus, chemical genomics would continue its interdisciplinary journey, joining various biological fields such as genomics, epigenomics, metabolomics, and phenomics, with chemistry and computer science.

1.10 REFERENCES

Alam A, Ali J, Saini N, Singla N, Mittal A, and Saini P (2008) Green Pharmacy: An Innovative Pharmaceutical Emergence. Medvarsity Online Limited. http://www.medvarsity.com/vmu1.2/st/lp/ejournals/18_5_08_article.html

Andermann AAJ (1996) Physicians, fads and pharmaceuticals: A history of aspirin. *McGill J Med* **2**:115–120.

Andry de Bois-Regard N (1888) The History of the Germ Theory. *The British Medical Journal* **1**:1415

Ashoor S. (1984) Islamic Medicine in the European Universities. The Bulletin of the 3rd International Conference on Islamic Medicine. Publication of Islamic Organization for Medical Sciences 77-104.

Becker F, Murthi K, Smith C, Come J, Costa-Roldan N, Kaufmann C, Hanke U, Degenhart C, Baumann S, Wallner W, Huber A, Dedier S, Dill S, Kinsman D, Hediger M, Bockovich N, Meier-Ewert S, Kluge AF, and Kley N (2004) A three-hybrid approach to scanning the proteome for targets of small molecule kinase inhibitors. *Chem Biol* **11**(2):211-223.

Bohacek RS, McMartin C, and Guida WC (1996) The art and practice of structure-based drug design: a molecular modelling perspective. *Med. Res. Rev.* **16:** 3–50.

Boysen Jensen P (1913) U¨ ber die Leitung des phototropischen Reizes in der Avenakoleoptile. Ber. Deutsch. *Bot. Ges.* **31**:559–566.

Cao Y, Charisi A, Cheng LC, Jiang T, and Girke T (2008) ChemmineR: a compound mining framework for R. *Bioinformatics* **24**(15):1733-1734.

Chen J, Swamidass SJ, Dou Y, Bruand J, and Baldi P (2005) ChemDB: a public database of small molecules and related chemoinformatics resources. *Bioinformatics* **21**(22):4133-4139.

Cobb AH (1992) Auxin-type herbicides. In Herbicides and Plant Physiology. London, UK: Chapman and Hall, 82–106.

Craig BD and Daly WJ (2000a) Clinical pharmacology in the Middle Ages: Principles that presage the 21st century. *Clinical Pharmacology & Therapeutics* **67** (5): 447–450.

Craig BD and Daly W (2000b) Medieval contributions to the search for truth in clinical medicine. *Perspectives in Biology and Medicine* **43** (4): 530–540.

Craig P (1980) Guidelines for drug and analog design. in Burger's Medicinal Chemistry; Part 1, The Basis of Medicinal Chemistry, 4th ed. edit. Wolff, M.E. New York, NY: Wiley.

Crystal E (2011) "Medicine and surgery in the ancient Rome." http://www.crystalinks.com/romemedicine.html

Cutler SR, Ehrhardt DW, Griffitts JS, and Somerville CR (2000) Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. *PNAS* **97**(7): 3718-3723.

Darwin C and Darwin F (1880). The power of movements in plants. London, England: John Murray Publishing.

Debru A (1997) Galen on Pharmacology: Philosophy, History, and Medicine: Proceedings of the Vth International Galen Colloquium, Lille, 16-18 March 1995. Boston, MA: Brill academic publishers.

Doman TN, McGovern SL, Withervee BJ, Kasten TP, Kurumbail R, Stallings WC, Connolly DT, and Schoichet BK (2002) Molecular docking and high-throughput screening for novel inhibitors of protein tyrosine phosphatase-1B. *J Med Chem* **45**(11):2213-2221.

Eldredge JD (2003) The Randomised Controlled Trial design: unrecognized opportunities for health sciences librarianship. *Health Information and Libraries Journal* **20**: 34–44.

Ehrlich P (1913) Address in Pathology, ON CHEMIOTHERAPY: Delivered before the Seventeenth International Congress of Medicine. Br Med J **2**(2746):353-359.

Fedtke C and Duke SO (2005) Herbicides. In Plant Toxicology. Ed. HockB and Elstner EF. New York, NY: Marcel Dekker, 247–330.

Fitting H (1909) Die Beeinflussung der Orchideenbluten durch die Besta¨ubing und durch andere Umsta¨nde. Ein entwicklunsphysiologische Studie aus den Tropen. *Zeit. Bot*. **1**:1–86.

Fleming A (1929) On the antibacterial action of cultures of a penicillium with special reference to their use in isolation of B. influenzae. *Br J Exper Pathol* **10**:226–36.

Furka A (1995) History of combinatorial chemistry. *Drug Development Research* **36**:1-12.

Gerhardt C (1853) Untersuchungen über die wasserfreien organischen Säuren. *Annalen der Chemie und Pharmacie* **87**: 149–179.

Goodsell DS, Morris GM, and Olson AJ (1996) Automated docking of flexible ligands: applications of AutoDock. *J Mole Recognit* **9**(1):1-5.

Gu M, Yan J, Bai Z, Chen YT, Lu W, Tang J, Duan L, Xie D, and Nan FJ (2010) Design and synthesis of biotin-tagged photoaffinity probes of jasmonates. *Bioorg Med Chem* **18**(9):3012-3019.

Haagen Smit AJ and Went FW (1935) A physiological analysis of the growth substance. *Proc. Kon. Akad. Wet. Amst.* **38**:852–857.

Hillenmeyer ME, Ericson E, Davis RW, Nislow C, Koller D, and Giaever G (2011) Systematic analysis of genome-wide fitness data in yeast reveals novel gene function and drug action. *Genome Biol* **11**(3):R30.

Hopkins AL and Groom CR (2002) The druggable genome. *Nature Reviews Drug Discovery* **1**(9):727-730.

Irwin JJ and Shoichet BK (2005) ZINC – A free database of commercially available compounds for virtual screening. *J Chem Inf Model* **45**:177-182.

Jacquart D (2008) Islamic Pharmacology in the Middle Ages: Theories and Substances. *European Review* **16** (2): 219–227.

Kaschani F, Verhelst SH, van Swieten PF, Verdoes M, Wong CS, Wang Z, Kaiser M, Overfleeft HS, Bogyo M, and van der Hoorn RA (2009) Minitags for small molecules: detecting targets of reactive small molecules in living plant tissues using 'click chemistry'. *Plant Journal* **57**(2):33-385.

Knauer T, Duemmer M, Landgraf F, and Forreiter C (2011) A negative effector of blue light-induced and gravitropic bending in Arabidopsis thaliana. *Plant Physiol* **156**(1):439-47.

Kolko G (1994) Anatomy of a War: Vietnam, the United States, and the Modern Historical Experience. New York: Pantheon Publishing.

Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, and Knapp RJ (1991) A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* 354:82-84.

Langermann YT (ed.) (2010) Avicenna and his Legacy. A Golden Age of Science and Philosophy. Belgium: Brepols Publishers.

Leroux H (1830) Découvert de la salicine. *J Chimie Médicale* **6**:341.

Leslie BJ and Hergenrother PJ (2008) Identification of the cellular targets of bioactive small organic molecules using affinity reagents. *Chem Soc Rev* **37**:1347-1360.

Lipinski CA, Lombardo F, Dominy BW, and Feeney PJ (2001) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* **46**(1-3): 3-26.

Mailer JS and Mason B (2001) "Penicillin : Medicine's Wartime Wonder Drug and Its Production at Peoria, Illinois". http://www.lib.niu.edu/2001/iht810139.html. Retrieved 2011-04-10

Martin JW, Christopher GW and Eitzen EM (2007) History of Biological Weapons: From Poisoned Darts to Intentional Epidemics. In Medical Aspects of Biological Warfare. Washington, DC: The Borden Institute.

Merrifield RB (1963) Solid phase peptide synthesis. 1. Synthesis of a tetrapeptide. *JACS* 85(14):2149-2154.

Nemhauser JL, Mockler TC, and Chory J (2004) Interdependency of brassinosteroid and auxin signaling in Arabidopsis. *PloS Biol* **2**(9):E258.

Newman WR and Principe LM (1998) Alchemy vs. Chemistry: The Etymological Origins of a Historiographic Mistake. *Early Science and Medicine* **3**:32-65.

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, and Cutler SR (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* **324**(5930):1068-1071.

Piria (1839) Untersuchungen über das Salicin und die daraus entstehenden Producte. *Annalen der Pharmacie* **29**: 300–306.

Popescu SC, Snyder M, and Dinesh-Kumar S (2007) Arabidopsis protein microarrays for the high-throughput identification of protein-protein interactions. *Plant Signal Behav* **2**(5):416-420.

Porter R, Wear A, Nutton V, Conrad LI, and Neve M (1995) The Western Medical Tradition: 800 Bc to Ad 1800 Cambridge UK: Cambridge University Press.

Saad B and Said O (2011) Greco-Arab and Islamic Herbal Medicine: Traditional System, Ethics, Safety, Efficacy, and Regulatory Issues. Hoboken NJ: John Wiley and Sons.

Schaller GE and Bleecker AB (1995) Ethylene-binding sites generated in yeast expressing the Arabidopsis ETR1 gene. *Science* **270**(5243):1809-1811.

Schreiber SL (2000) "Target-oriented and diversity-oriented organic synthesis in drug discovery". *Science* **287** (5460): 1964–9

Schreiber SL (1991) Chemistry and biology of the immunophilins and their immunosuppressive ligans. *Science* **251**(4991):283-287.

Sertürner (1806) Darstellung der reinen Mohnsäure (Opiumsäure) nebst einer Chemischen Untersuchung des Opiums mit vorzüglicher Hinsicht auf einen darin neu entdeckten Stoff und die dahin gehörigen Bemerkungen. *Journal der Pharmacie fuer Aerzte und Apotheker* **14**: 47–93.

Stafleau FA (1971) Linnaeus and the Linnaeans. The spreading of their ideas in systematic botany, 1753–1789. Utrecht: Oosthoek.

Sterling TM and Hall JC (1997) Mechanism of action of natural auxins and the auxinic herbicides. In Herbicide Activity: Toxicology, Biochemistry and Molecular Biology. Ed. Roe RM, Burton JD and Kuhr RJ. Netherlands: IOS Press, 111–141.

Taylor FS (1940) Science Front 1939. US: Ayers Publishing.

Thimann KV and Kipefli JB (1935) Identity of the growth-promoting and root-forming substances of plants. *Nature* **135**:101–102.

Troyer JR (2001) In the beginning: the multiple discovery of the first hormone herbicides. *Weed Sci.* **49**:290– 297.

UC Regents (2010) A short story of drug discovery. www.pharmsci.uci.edu/history.php. Retrieved 2011-04-10

Vendrell M, Lee JS, and Chang YT (2010) Diversity-oriented fluorescence library approaches for probe discovery and development. *Curr Opin Chem Biol* **14**(3):383-389.

Wagner BK, Carrinski HA, Ahn YG, Kim YK, Gilbert TJ, Fomina DA, Schreiber SL, Chang YT, and Clemons PA (2008) Small-molecule fluorophores to detect cell-state switching in the context of high-throughput screening. *JACS* **130**(13):4208-4209.

Wang ZY, Seto H, Fujioka S, Yoshida S, and Chory J (2001) BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature* **412**(6826):380-383.

Went FW (1928) Wuchstoff and Wachstum. *Rec. Trav. Bot. Neerl*. **15**:1-116.

Williams HS and Williams EH (2009) A History of Science (Volume 2) The Beginnings of Modern Science. Memphis, TN: General Books LLC Publishers.

Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, Chang Z, and Woolsey J (2006) DrugBank: a comprehensive resource in silico drug discovery and exploration. *Nucleic Acids Res* **34**:D668-672.

Withering W (1785) An Account of the Foxglove and Some of its Medical Uses: With Practical Remarks on Dropsy and Other Diseases. London: J and J Robinson.

Chapter 2

An Overview of Endomembrane Trafficking and Membrane Cycling

2.1 The Endomembrane System

The endomembrane system consists of subcellular, membrane-bound compartments that include the endoplasmic reticulum (ER), the Golgi apparatus, the trans-Golgi network (TGN), endosomal compartments, the prevacuolar compartment (PVC) or multi-vesicular body (MVB), and the vacuole. The endomembrane system is responsible for the synthesis, modification, trafficking, and storage of proteins within the cell.

Endomembrane trafficking of a newly synthesized protein begins in the ER. Proteins containing an ER retention signal are inserted co-translationally into the ER and then either insert into the ER membrane or translocate into the ER lumen (Vitale and Denecke 1999). Some proteins remain within ER-derived compartments that join to protein storage vacuoles (Chrispeels and Herman 2000), but usually, proteins are transported to the Golgi apparatus for further processing and packaging.

The Golgi apparatus consists of numerous stacks of distinct membrane cisternae from the *cis* to the *trans* side. Golgi stacks are the site of synthesis of cell wall polysaccharides as well as various membrane lipids, and also function in glycosylation of proteins synthesized in the ER (Hawes, 2005). Every Golgi cisternae consists of several functionally discrete domains that are demarcated by budding membranes or protein aggregates (Otegui et al., 2006; Hinz et al., 2007). Modified

proteins then packaged into transport vesicles for their delivery to the correct destination in the endomembrane system or secretory pathway, either downstream to the vacuole or plasma membrane, or back to the ER for recycling.

The *trans*-Golgi network (TGN) was originally thought to be located within the matrix of the Golgi apparatus but is now known to exist separately. Evidence for differentiation of TGN compartments into early and late subcompartments has been suggested using electron tomography (Staehelin and Kang 2008). The TGN compartment participates in protein trafficking through the secretory and endocytotic pathways, as indicated by the rapid labeling of the endocytotic, lipophilic styryl dye, FM4-64 (Dettmer et al., 2006). It remains unknown if additional compartments exist between the TGN and the plasma membrane (PM).

The prevacuolar compartment (PVC) or multi-vesciular body (MVB) is comprised of microvesicles containing proteins to be delivered to the vacuole or the lysosome (Tse et al., 2004). The PVC participates in the vacuolar and endocytotic protein trafficking (Bethke and Jones 2000). In the vacuolar trafficking pathways, only proteins with the C-terminal propeptide (CTPP) signal appear to transit through the PVC, indicated by an inhibition of the trafficking of the CTPP proteins to the vacuole in the presence of the phosphotidylinostol-3 kinase inhibitor, wortmannin (Matsuoka et al., 1995). In later studies, wortmannin was found to cause swelling of the PVC, determined by confocal laser scanning microscopy (Tse et al., 2004).

The central vacuole, the most prominent compartment of the cell, occupies up to 90% of the volume of a mature cell (De 2000) and serves as both the storage site and the

degradation site for numerous organic molecules, as well as protons and ions, which create the osmotic gradient that produces osmotic pressure against the cell wall (De 2000). This osmostic pressure or turgor pressure is essential for structural support and cell elongation (Cosgrove 1993). Studies have confirmed that at least two types of vacuoles exist, and sometimes coexist within a cell (Frigerio et al., 2008). Historically defined by pH or antibodies, lytic vacuoles are acidic, lysosome-like compartments while protein storage vacuoles are less acidic with the capacity to store materials for retrieval. The vacuole is involved in several cellular processes, such as gravitropic response, autophagy, and pathogen response (Surpin and Raikhel 2004).

2.2 Membrane Cycling Through Endosomal Compartments

Certain types of plasma membrane proteins have been found to cycle between internal compartments and back to the PM or to the vacuole for degradation. One of the first of these proteins to be identified was the auxin efflux transporter PINFORMED1 (PIN1), which agglomerated in an intracellular compartment (endosome) when treated with Brefeldin A, known at the time as an auxin inhibitor (Geldner et al., 2001). Since then, many proteins have been found to cycle to endosome compartments. Endosomes are highly dynamic and transient compartments that are characterized by their cargoes, however, little is known about their sequential trafficking (Hicks and Raikhel 2010).

There are three main types of endosome compartments that have been identified. The early endosome (EE) was identified as the TGN compartment through the use of the

endocytic dye FM4-64 (Dettmer et al., 2006). The early endosome serves as the sorting point between the process of recycling back to the PM and trafficking to the vacuole for degradation. The putative retromer complex, which can retrieve proteins from the late endosome compartment (LE) and traffic proteins back to the early endosome/TGN, exists between the early endosome and the late endosome compartments in plants (Kleine-Vehn et al., 2008; Jaillais et al., 2006). The late endosome (LE) consists of multivesicular bodies (MVBs) or prevacuolar compartments (PVCs) and it is the last compartment before a protein is delivered to the vacuole for degradation, but can also participate in retrograde trafficking via the retromer complex(Oliviusson et al., 2006).

Endosomes participate in a variety of functions including physiological responses and hormone signaling. These physiological responses include shoot and root gravitropic response, in which a plant's ability to respond to a change in gravity vector is dependent upon an asymmetric distribution of the hormone auxin (Abas et al., 2006). This auxin gradient restricts root growth and increases hypocotyl growth so that the roots can continue to grow down into the soil and the hypocotyls can grow up into the light (Vanneste and Friml 2009). In examining the redistribution of the auxin efflux transporter PINFORMED2 (PIN2), Abas et al., found that intracellular trafficking of proteins and proteolysis are both required for proper response (2006). In that study, authors used the Brefeldin A (BFA) as an inhibitor of endosome trafficking.

Receptor-like kinases (RLKs), one of the largest protein families in Arabidopsis, are known to be affected by endosomal accumulation (Geldner et al., 2007; Robatzek et al., 2006). The RLK BRI1, the brassinosteroid receptor, is constitutively endocytosed and when it

accumulates in early endosome BFA bodies, brassinosteroid signaling increases (Geldner et al., 2007). This indicates that the endosomal fraction of BRI1 proteins is responsible for brassinosteroid signaling (Geldner et al., 2007). In addition, the RLK FLS2, the receptor that recognizes bacterial flagellin22 (fls22), also undergoes ligand-mediated endocytosis (Robatzek et al., 2006). The late endosome indicator, wortmannin inhibits FLS2's ability to internalize, but BFA did not affect it. Thus, endosomal signaling as well as endosomal trafficking plays an important role in hormone and defense pathways.

2.3 A Chemical Genomics Approach to Endomembrane Trafficking

While classical genetics has yielded a lot of information about the steady state of trafficking proteins, utilizing a chemical genomics approach allows us to dissect the dynamic changes occurring during protein trafficking. Chemical genomics also circumvents the common problems of lethality and redundancy faced in classical genetics (Rojo *et al.,* 2001; Sanderfoot *et al.,* 2001). Chemical genomics utilizes natural and/or synthetic molecules that can be applied at any developmental stage in a tunable manner to affect protein function (Hicks and Raikhel 2010). Such bioactive compounds can be useful reagents to discover new compartments and dissect pathways involved in endomembrane trafficking.

Given the highly transient nature of most endosomal compartments, the chemical compounds like BFA and wortmannin have become critical in studying endosome compartments. BFA causes the *trans*-Golgi network (TGN) and Golgi compartments to fuse into one large body, making it an early endosome indicator (Ritzenthaler et al.,

2002). BFA inhibits recycling of proteins to the PM but does not affect the process of endocytosis (Baluska et al., 2002). Wortmannin identifies late endosome compartments because it causes prevacuolar swelling by inhibiting trafficking to the vacuole (Jaillais et al., 2006). These two compounds help separate endosome populations into two populations but do little to determine finer definitions of sequential endosome trafficking.

Additional compounds have been identified that specifically inhibit endocytosis at the TGN compartment. These include Endosidin1 (ES1) and Concanamycin A (ConcA) (Robert et al., 2008; Dettmer et al., 2006). ES1, a natural complex liminoid, inhibits endocytosis at the early endosomal TGN compartments (Robert et al., 2008). ConcA is a natural product and antibiotic that inhibits a subunit of the V-ATPase (Huss et al., 2002). ConcA affects Golgi and TGN morphology and inhibits FM4-64, a lipophilic styryl dye, from trafficking from the TGN to the vacuole (Dettmer et al., 2006).

Chemical genomics clearly represents an advantageous methodology to dissect the dynamic and transient nature of endosomal trafficking. Endosome cycling has come to the forefront of many research programs in recent years with implications in hormone signaling and defense pathways. This Dissertation aims to identify additional membrane cycling disruptors, specifically those that affect the specific physiological process of gravitropic response and to determine how endosomal cycling triggers physiological processes.

2.4 References

Abas L, Benjamins R, Malenica N, Paciorek T, Wisniewska J, Moulinier-Anzola J, Sieberer, T., Friml, J., and Luschnig, C (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nature* **8**(3): 249-256

Baluska F, Hlavacka A, Samaj J, Palme K, Robinson DG, Matoh T, McCurdy DW, Menzel D, and Volkmann D (2002) F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. *Plant Physiology* **130**(1):442-431

Bethke PC and Jones RL (2000) Vacuoles and prevacuolar compartments. *Curr Opin Plant Biology* **3**:469–475

Chrispeels MJ and Herman EM (2000) Endoplasmic reticulum-derived compartments function in storage and as mediators of vacuolar remodeling via a new type of organelle, precursor protease vesicles. *Plant Physiology* **123:** 1227-1234

Cosgrove DJ (1993) Water uptake by growing cells: an assessment of the controlling roles of wall relaxation, solute uptake, and hydraulic conductance. *Int. J. Plant Sci*. **154:** 10-21

De DN (2000) Plant cell vacuoles: an introduction. CSIRO Publishing: Collingwood, Australia

Dettmer J, Hong-Hermesdorf A, Stierhof YD, and Schumacher K (2006) Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. *Plant Cell* **18:**715-730

Frigerio L, Hinz G, and Robinson DG (2008) Multiple Vacuoles in Plant Cells: Rule or Exception? *Traffic* **9**(10):1564-1570

Geldner N, Hyman DL, Wang X, Schumacher K, and Chory J (2007) Endosomal signaling of plant steroid receptor kinase BRI1. *Genes and Development* **21**(13):1598-1602

Geldner N, Friml J, Stierhof Y, Jurgens G, and Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**: 425-428

Hawes C (2005) Cell biology of the plant Golgi apparatus. *New Phytologist* **165:** 29-44

Hicks GR and Raikhel NV (2010) Advances in dissecting endomembrane trafficking with small molecules. *Curr Opin Plant Biology* **13**(6):706-713

Hinz G, Colanesi S, Hillmer S, Rogers JC, and Robinson DG (2007) Localization of vacuolar transport receptors and cargo proteins in the Golgi apparatus of developing Arabidopsis embryos. *Traffic* **8:** 1452-1464

Jaillais Y, Fobis-Loisy I, Miege C, Rollin C, and Gaude T (2006) AtSNX1 defines an endosome for auxin-carrier trafficking in Arabidopsis. *Nature* **443**(7107): 106-109

Kleine-Vehn J, Leitner J, Zweiwka M, Sauer M, Abas L, Luschnig C, and Friml J (2008) Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. *PNAS* **105**(46): 17812-17817

Matsuoka K, Bassham DC, Raikhel NV, and Nakamura K (1995) Different sensitivity to wortmannin of two vacuolar sortings signals indicates the presence of distinct sorting machineries in tobacco cells. *J Cell Biology* **130**(6):1307-1318

Oliviusson P, Heinzerling O, Hillmer S, Hinz G, Tse YC, Jiang L, and Robinson DG (2006) Plant retromer, localized to the prevacuolar compartment and microvesicles in Arabidopsis, may interact with vacuolar sorting receptors. *Plant Cell* **18**(5):1239-1252

Otegui MS, Herder R, Schulze J, Jung R, and Staehelin LA (2006) The proteolytic processing of seed storage proteins in Arabidopsis embryo cells starts in the multivesicular bodies. *Plant Cell* **18:** 2567-2581

Ritzenthaler C, Nebenfuhr A, Movafeghi A, Stussi-Garaud C, Behnia L, Pimpl P, Staehelin LA, and Robinson DG (2002) Reevaluation of the effects of brefeldin A on plant cells using tobacco bright yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. *Plant Cell* **14:** 237–261

Robatzek S, Chinchilla D, and Boller T (2006) Ligand-induced endocytosis of the pattern recognition receptor FLS2 in Arabidopsis. *Genes and Development* **20**(5):537-542

Rojo E, Gillmor C, Kovaleva V, Somerville C, and Raikhel NV (2001) VACUOLELESS1 is an essential gene required for vacuole formation and morphogenesis in Arabidopsis. *Developmental Cell* **1**:303-310

Sanderfoot A, Kovaleva V, Bassham DC, and Raikhel NV (2001) Interactions between syntaxins identify at least five SNARE complexes within the Golgi/Prevacuolar system of the Arabidopsis cell. *Molecular Biology of the Cell* **12**:3733-3743

Staehelin LA and Kang BH (2008). Nanoscale architecture of endoplasmic reticulum export sites and of Golgi membranes as determined by electron tomography. *Plant Physiol*ogy **147**: 1454–1468

Surpin M and Raikhel NV (2004) Traffic jams affect plant development and signal transduction. *Nature Rev Mol Cell Biology* **5**(2):100-109

Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, Robinson DG and Jiang L (2004) Identification of multivesicular bodies as prevacuolar compartments in Nicotiana tabacum BY-2 cells. *Plant Cell* **16**(3):672-693

Vanneste S and Friml J (2009) Auxin: a trigger for change in plant development. *Cell* **136**(6):1005-1016

Vitale A and Denecke J (1999) The endoplasmic reticulum-gateway of the secretory pathway. *Plant Cell* **11:** 615-62

Chapter 3

Utilizing Tobacco Pollen as a Model System to Identify Compounds that Inhibit Membrane Cycling

3.1 Abstract

To identify chemical compounds that inhibit membrane cycling, tobacco pollen was assayed in a high-throughput chemical screen, where compounds that inhibited pollen germination or disrupted pollen tube morphology were selected for a sub-library named the Plasma Membrane Recycling inhibitors in Pollen (PMRP). This sub-library maintained a high degree of bioactivity at low concentrations and inhibition of pollen germination was the prevalent phenotype observed. The PMRP sub-library was screened with the ER to Golgi trafficking protein RAB2:GFP transgenic line to identify compounds that would specifically inhibit trafficking in the early secretory pathway. Nineteen compounds were identified that disrupted RAB2 movement or localization. Interestingly, wortmannin, which is known to affect the prevacuolar compartment, disrupted the localization of RAB2. Thus, the PMRP sub-library consists of 360 compounds that disrupt membrane cycling as well as trafficking through the secretory pathway.

3.2 Introduction

While the focus of this dissertation was to focus on the dissection of endosomal trafficking via chemical genomics, truly high-throughput sub-cellular chemical screening

techniques have yet to be developed in Arabidopsis, and to manually screen the subcellular effects of thousands of compounds would be both, cost and time prohibitive. Thus, a different model system needed to be identified to utilize in a primary screen that would identify compounds that inhibit membrane cycling. Tobacco pollen was chosen because pollen tubes have continuous membrane cycling at the apical tip, germinate *in vitro,* making pollen amenable to high-throughput screening and are easily imaged at a 20X magnification (Robert et al., 2008).

In higher plants, pollen germination and pollen tube growth is an excellent model system to study polar growth, cell-to-cell interaction and cell differentiation as well as an essential component of sexual reproduction (Johnson-Brousseau and McCormick 2004; Cheung and Wu 2008; Lee et al., 2008). Apical growth at the pollen tip is established by post-Golgi vesicles being directed to a specific area of the plasma membrane, allowing the vegetative cell to grow in a directional manner to create the pollen tube (Krichevsky et al., 2007). Pollen tube growth is subject to spatial and temporal controls as a result of endocytosis, exocytosis, and cellular oscillations involving calcium gradients and actin organization (Zonia and Munik, 2008; Feijo et al., 2001; Lancelle and Hepler 1992; Cole and Fowler 2006). A complex network of signaling pathways including GTPases, reactive oxygen species (ROS), protein kinases and phospholipids help coordinate this intricate dance of cellular oscillations with constitutively active endocytosis and exocytosis (Cole and Fowler 2006).

A transcriptome analysis using the Affymetrix ATH1 GeneChip array, found over 6,500 genes expressed in pollen with only 11% of those genes pollen-selective (Becker

and Feijo 2007; Pina et al., 2005). The remaining 89% represent genes that are found throughout various tissues and cell types. This signifies the value of using pollen germination and pollen tube growth as a model system to investigate membrane cycling, actin organization and multiple signaling pathways.

A pilot screen of 2,000 bioactive compounds was performed in tobacco pollen to identify putative membrane cycling inhibitors based on morphological phenotypes of the pollen tubes/grains that eventually led to the characterization of Endosidin1, which defined an overlapping pathway between the auxin transporters AUX1 and PIN2 and the brassinosteroid receptor BRI (Robert et al., 2008). In this thesis, the primary screening which was expanded to assay the other 46,000 compounds housed at the UCR Institute for Integrative Genome Biology and was also based on morphological phenotypes. A secondary screening in tobacco pollen utilized the RAB2:GFP trangenic tobacco line to examine endomembrane trafficking effects as well.

A member of the Ras family of GTPases, RAB2 is expressed in tobacco and Arabidopsis. Arabidopsis polle has a high degree of expression of RAB2 while in *Nicotiana tabacum* RAB2 is expressed almost exclusively in pollen (Moore et al., 1997; Cheung et al., 2002). RAB2 plays an important role in ER to Golgi trafficking, especially in the secretory trafficking pathway. Utilizing a RAB2:GFP transgenic line, ER to Golgi secretory protein trafficking was interrogated with the chemical compounds that caused pollen germination inhibition or pollen tube morphology defects. By examining both morphological and trafficking defects, general membrane inhibitors can be compared to specific pathway inhibitors.

3.3 Materials and Methods

Screen for bioactive chemicals. The screen was conducted as described in Robert *et al.,* (2008). Wildtype *Nicotiana tabacum* anthers were collected and placed in a germination media, and then vortexed to release the pollen into solution. The pollen-containing media (20 μL) was then pipetted into multi-well plates (ThermoFisher 12-566-280 or ThermoFisher 12-566-607) containing germination media and chemical compound (20 μL) for 40 μL total assay volume. Library compounds were distributed to the multi-well plate utilizing the BioMek and the 0.2 uL pin tool attachment. The final concentration for each compound was between 50-100 μM. The multi-well plate was then placed under a black cloth on an orbital shaker (~70 rpm) for three hours. The plates were then visualized using the BD Pathway HT automated microscope. Images were then manually screened for tobacco pollen phenotype using ImageJ (Abramoff et al 2004).

Chemical libraries Five chemical libraries containing 46,418 compounds in total were screened. The Chembridge Diverset library contained 20,000 drug-like low molecular mass molecules designed to maximize structural diversity (http://www.chembridge.com/collected-screening -libraries.html). The Chembridge Novacore library contained 10,000 low molecular mass compounds based on medicinally relevant templates (http://www.chembridge.com/combinatoriallibraries-diversity.html). The Sigma TimTec Myria library contained 10,000 diverse drug-like compounds (http://www.sigmaaldrich.com/chemistry/drugdiscovery/screening-compounds.html?cm_mmc=affiliate-_-Bioscreening-_-myria-

_-text). The LATCA library, collected by Sean Cutler of University of California Riverside, contained 3,650 compounds, selected for bioactive in *Arabidopsis* (http://cutlerlab.blogspot.com/2008/05/latca.html). The CLICKables library, also collected by Sean Cutler, contained 2,768 compounds with a terminal acetylene available for undergoing CLICK chemistry (http://cutlerlab.blogspot.com/2008/05/clickables.html).

Germination media. Germination media was comprised of 18% sucrose, 0.01% boric acid, 5 μM $CaCl_2$, 5 μM $Ca(NO_3)_2$, and 1 mM $MgSO_4$. The solution was then modified to a pH of 6.5-7.5 using 1N KOH and filtered sterilized. Solution was stored at 4° C.

3.4 Results & Discussion

To identify compounds that disrupt membrane cycling, five chemical libraries housed within the Institute of Integrative Genome Biology which totaled 46,418 compounds were assayed with tobacco pollen looking for compounds that would inhibit pollen germination or disrupt pollen tube morphology. Three hundred and sixty five novel trafficking compounds and thirteen known compounds were identified, for an overall hit rate of 0.78% (Table 3.1). The most active chemical library was Dr. Sean Cutler's LATCA bioactive library with a hit rate of 2.4%, followed by the Chembridge Diverset library which had a hit rate of 1.56% and the least active library was the Chembridge Novacore library. Several of the LATCA compounds had known membrane cycling inhibition effects and served as good internal controls for the screen. These

compounds included Brefeldin A which inhibits recycling to the plasma membrane (Baluska et al 2002), wortmannin which inhibits trafficking to the vacuole (Oliviusson et al., 2006), tyrphostin which inhibits internalization at the plasma membrane (Ortiz-Zapater et al., 2006), and cantharidin, a protein phosphatase inhibitor, that has been shown to phenocopy the *root curling in the presence of NPA (rcn1)* mutant (Shin et al., 2005). Only 360 of the total 378 compounds identified were re-orderable to populate a sub-library of membrane cycling inhibitors named Plasma Membrane Recycling inhibitors in Pollen (PMRP).

Library	Compounds	Primary hits	Percentage
Diverset	20,000	223	1.56%
Novacore	10,000	18	0.18%
TimTec	10,000	33	0.33%
CLICK	2,768	15	0.54%
LATCA	3,650	89 (76)	2.40%
TOTAL	46,418	365	0.78%

Table 3.1: Assaying over 46,000 compounds from combinatorial and bioactive chemical libraries, 365 novel compounds were identified that inhibited pollen germination or disrupted pollen tube morphology for a hit rate of less than 1%, indicating a high degree of specifity in the screening technique. The LATCA bioactive library had the highest hit rate followed by the Chembridge Diverset library. The least bioactive library in this screen was the Chembridge Novacore library.

Seven morphological phenotypes were observed after a three hour germination time in the assay compounds (Figure 3.1). The most prevalent phenotype was a complete inhibition of pollen germination (32%), followed by a collapsed pollen tube phenotype (15%). A rough pollen grain phenotype accounted for 13% of the phenotypes observed while delayed pollen tube growth occurred with 10% of the hit compounds. The final three morphological phenotypes accounted for 11% of the phenotypes observed; swollen pollen tubes (1%), vesiculated pollen tubes (2%), and a complex phenotype containing at least two of the other phenotypes (8%). Nineteen percent of the 360 total compounds in the sub-library failed to repeat after compounds were re-ordered from their respective companies. This lack of activity could have been due to the degradation of the original library compounds or contamination between wells.

With a large percentage of the PMRP sub-library inhibiting pollen germination, dilution assays were performed with tobacco pollen expressing RAB2:GFP to determine morphological phenotypes, bioactivity range and putative ER to Golgi trafficking inhibitors. The same conditions for the germination assay were used for the RAB2:GFP as the wildtype tobacco pollen. Six morphological phenotypes were observed with the RAB2:GFP pollen (Figure 3.2). Most of the morphology phenotypes were similar to the wildtype pollen, including germination inhibition, collapsed pollen tubes, delayed growth, swollen tubes, vesiculated tubes and a complex phenotype, containing two of more additional phenotypes. However, no rough pollen grain phenotype was observed with the RAB2:GFP pollen, however, an increased fluorescence signal was observed with some of the germination inhibitors.

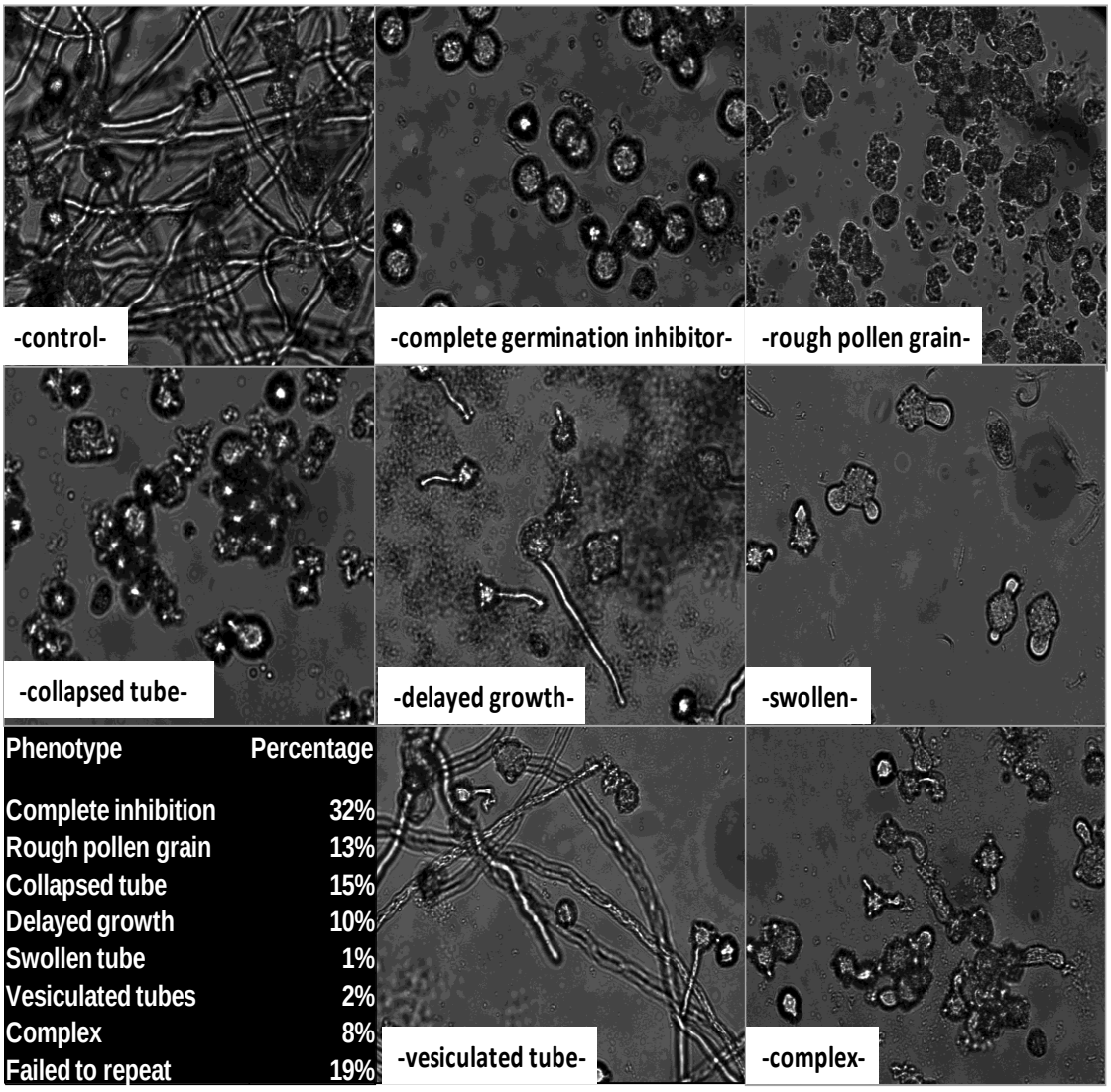


Figure 3.1. Seven morphologial phenotypes were observed after germination of wildtype tobacco pollen in compound containing media for three hours. The most prevalent phenotype was complete inhibition of pollen germination. Images were obtained with the BD Pathway HT confocal microscope using 20X magnification.

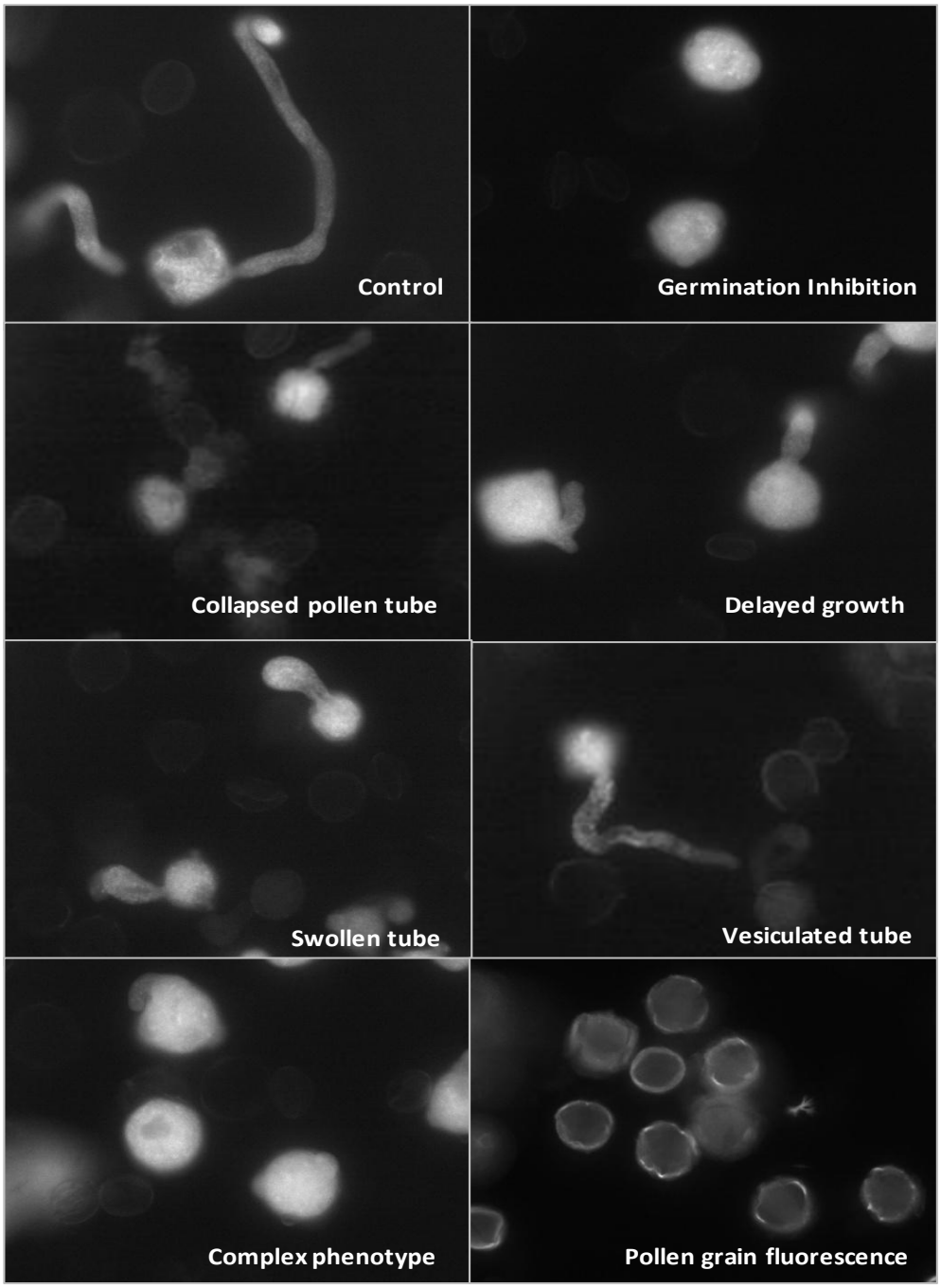


Figure 3.2 Six morphological phenotypes were observed with the RAB2:GFP tobacco pollen and an increased fluorescence in the pollen grain was observed with two compounds that also inhibited germination. Images were taken with a BD Pathway HT confocal microscope at 40X magnification.

To determine bioactivity range of the PMRP sub-library, dilution assays were performed. The original assay was at a concentration of 50-100 μM depending on the compound structure. In the dilution assays, two additional concentrations were examined: 25-50 μM and 10-20 μM (Table 3.2). Overall the PMRP library maintained a relatively high degree of bioactivity with 34% of compounds still active at a concentration of 10-20 μM. Inhibition of pollen germination was the prevalent phenotype in all concentrations assayed. The number of compounds causing a collapsed tube phenotype was consistently at roughly 20% for each dilution while the number of compounds causing the delayed pollen tube growth increased from 13% in the highest concentration to 23% in the lowest concentration assayed. The number of compounds causing the swollen pollen tube phenotype experienced an increase from ~1% at the highest concentration to ~5% at the two lower concentrations. This increase in the moderate phenotypes at lower concentrations demonstrates the varying range of bioactivity for compounds, e.g. high concentrations inhibit germination while lower concentrations causing pollen tube swelling.

RAB2 is known to participate in ER to Golgi trafficking of proteins (Cheung et al., 2002). Twenty compounds affecting RAB2 localization or movement were identified in our screen; nine of these compounds affected RAB2 localization causing agglomerations of RAB2 protein, nine affected RAB2 movement visualized through a time series of images, and one affected both localization and movement dependent upon concentration (Figure 3.3).

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

Table 3.2 The PMRP sublibrary maintains a relatively high degree of bioactivity even at the 10-20 µM concentration with a third of the compounds still have an effect. The predominant phenotype with compound treatment at all concentrations was an inhibition of pollen germination. The rarer phenotypes of delayed growth, swollen pollen tubes, and vesiculated pollen tubes increased in percentage at the lower concentrations.

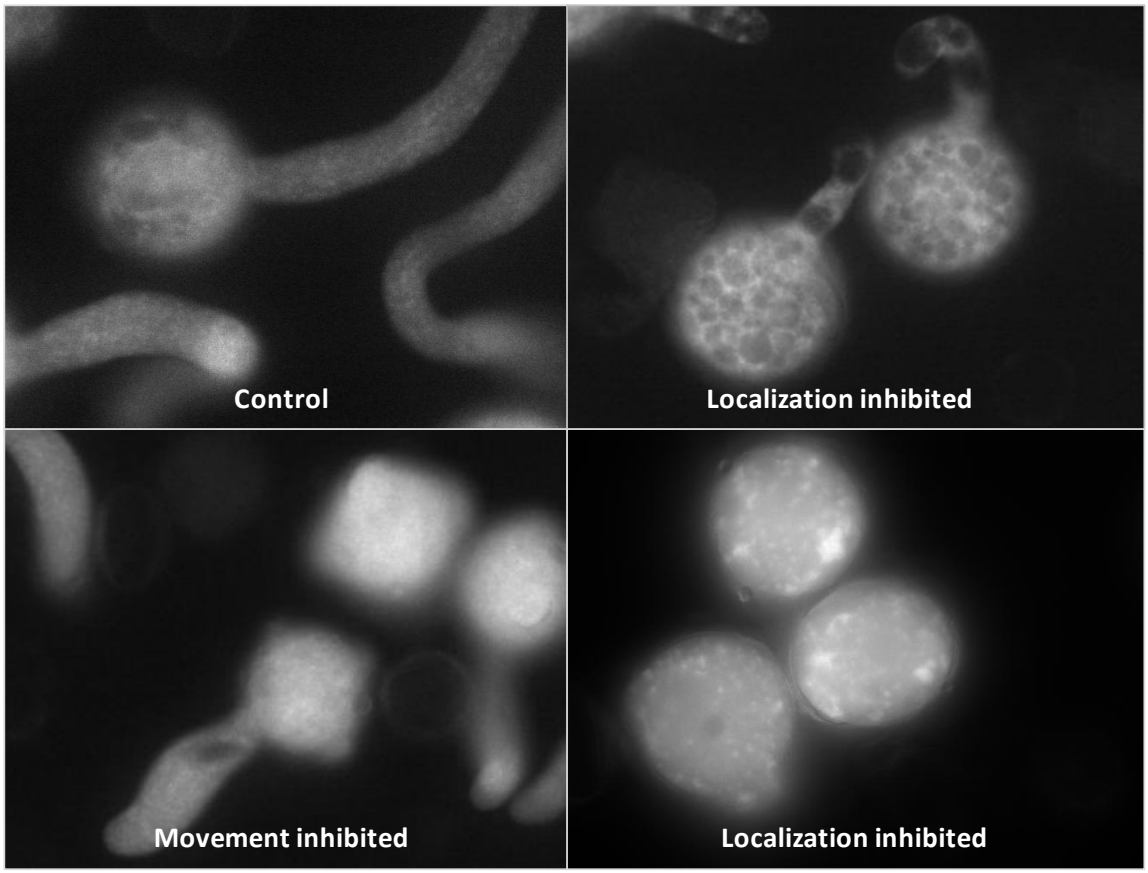


Figure 3.3 Two categories of phenotypes were observed with specific inhibitors of the ER to Golgi trafficking protein RAB2; those that inhibited movement of the protein and those that caused localization problems with either a web-like pattern or with agglomerations.

In summary, over 46,000 compounds were screened using tobacco pollen as a high-throughput model system for membrane cycling. This screening identified 378 compounds, of them 365 are novel, which affected tobacco pollen germination or pollen tube morphology. From these compounds, a specialized sub-library (PMRP) was created and assayed using a pollen expressing RAB2:GFP marker. From the PMRP sub-library, nineteen compounds were found to specifically affect the ER to Golgi trafficking of RAB2 protein. Potentially, further studies with these compounds could help elucidate the specific inhibitory effects on the trafficking of RAB2 protein.

The PMRP compounds could be used to undertake a chemical screen to identify membrane cycling inhibitors bioactive in both tobacco pollen and Arabidopsis plants. These Arabidopsis membrane cycling inhibitors could then be used to dissect endosomal trafficking.

3.5 References

Abramoff, MD, Magelhaes, PJ and Ram, SJ. (2004) Image Processing with ImageJ. *Biophotonics International* **11**(7):36-42.

BeckerJD and Feijo JA. (2007) How Many Genes are Needed to Make a Pollen Tube? Lessons from Transcriptomics. *Annals of Botany* **100**:1117-1123.

Cheung A and Wu H. (2008) Structural and Signaling Networks for the Polar Cell Growth Machinery in Pollen Tubes. *Annual Review of Plant Biology* **59**:547-572.

Cheung A, Chen CY, Glaven RH, de Graaf BH, Vidali L, Hepler PK, and Wu HM (2002) Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. *Plant Cell* **14**(4):945-962.

Cole R and Fowler J. (2006) Polarized growth: maintaining focus on the tip. *Current Opinion in Plant Biology* **9:**579-588.

Feijo J, Sainhas J, Holdaway-Clarke T, Cordeiro M, Kunkel J, and Hepler P. (2001) Cellular oscillations and the regulation of growth: the pollen tube paradigm. *BioEssays* **23**: 86-94.

Johnson-Brousseu S and McCormick S. (2004) A compendium of methods useful for characterizing Arabidopsis pollen mutants and gametophytically-expressed genes. *The Plant Journal* **39**:761-775.

Krichevsky A, Kozlovsky S, Tian GW, Chen MH, Zaltsman A, and Citovsky V. (2007) How pollen tubes grow. *Developmental Biology* **303**: 405-420.

Lancelle SA and Hepler PK. (1992) Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum. Protoplasma* **167**:215-230.

Lee YJ, Szumlanski A, Nielsen E, and Yang Z. (2008) Rho-GTPase-dependent filamentous actin dynamics coordinate vesicle targeting and exocytosis during tip growth. *The Journal of Cell Biology* **181**(7): 1155-1168.

Moore I, Diefenthal T, Zarsky V, Schell J, and Palme K (1997) A homolog of the mammalian GTPase Rab2 is present in Arabidopsis and is expressed predominantly in pollen grains and seedlings. *PNAS* **94**(2):762-767.

Oliviusson P, Heinzerling O, Hillmer S, Hinz G, Tse YC, Jiang L, and Robinson DG (2006) Plant retromer, localized to the prevacuolar compartment and microvesicles in Arabidopsis, may interact with vacuolar sorting receptors. *Plant Cell* **18**(5):1239-1252.

Ortiz-Zapater E, Soriano-Ortega E, Marcote MJ, Ortiz-Masia D, and Aniento F (2006) Trafficking of the human transferrin receptor in plant cells: effects of tyrphostin A23 and brefeldin A. *Plant Journal* **48**(5):757-770.

Pina C, Pinto F, Feijo JA, and Becker JD (2005) Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division, control, and gene expression regulation. *Plant Physiology* **138**: 744-756.

Potocky M, Jones M, Bezvoda R, Smirnoff N, and Zarsky V (2007) Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. *New Phytologist* **174**: 742-751.

Robert S, Chary S, Drakakaki G, Li S, Yang Z, Raikhel NV, and Hicks GR. (2008) Endosidin1 Defines a Compartment Involved in Endocytosis of the Brassinosteroid Receptor BRI1 and the Auxin Transporters PIN2 and AUX1. *PNAS* **105**(24): 8464-8469.

Shin H, Guo Z, Blancaflor EB, Masson PH, and Chen R (2005) Complex regulation of Arabidopsis AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. *Plant Journal* **42**: 188-200.

Waisel Y, Borger GA, and Kozlowski TT (1969) Effects of Phenylmercuric acetate on stomatal movement and transpiration of excised Retula papyrifera Marsh. Leaves. *Plant Phys* **44**(5): 685-690.

Zonia L and Munik T (2008) Vesicle trafficking dynamics and visualization of zones of exocytosis and endocytosis in tobacco pollen tubes. *Journal of Experimental Botany* **59**(4):861-73.

Chapter 4

Identifying and Clustering Compounds that are Bioactive in Arabidopsis

4.1 Abstract

Having isolated the membrane cycling inhibitors in tobacco pollen (PMRP library), the translatability of these compounds to Arabidopsis needed to be determined. In total, 129 compounds were identified as disrupting the localization of three plasma membrane proteins, two of which were polarly localized PINs and one of which was the non-polar brassinosteroid receptor BRI1. From these 129 compounds, 16 phenotypes were observed with the highest density of compounds creating a small agglomeration phenotype. To identify a potential relationship between the phenotypes and the physio-chemical properties of the compounds, a "fingerprinting" cluster strategy was used to cluster compounds that caused similar phenotypes with the marker lines assayed. Using this strategy, clusters of compounds were found to contain both similar and diverse chemical moieties, indicating potentially similar targets for similar compound moieties and diverse targets for diverse chemical moieties.

4.2 Introduction

After identifying a sub-set of membrane cycling inhibitors from the 46,418 compounds housed at UCR's Integrative Institute for Genome Biology utilizing tobacco pollen as a model system, interrogation of membrane cycling in *Arabidopsis thaliana* could be achieved *in vivo* at the cellular level using fluorescently tagged transgenic lines

and laser scanning confocal microscopy (LSCM). Three plasma membrane proteins known to cycle through endosomal compartments were chosen to be assayed on the chemical membrane cycling inhibitors from pollen.

These were the polar auxin efflux transporter plasma membrane proteins PIN2::PIN1:GFP (Wisniewska et al.,2006), PIN2::PIN2:GFP (Muller et al., 1998) and the non-polar plasma membrane brassinosteroid receptor BRI1::BRI1:GFP (Friedrichsen et al., 2000). One of the advantages in using the PIN2::PIN1:GFP line was that the PIN1 protein could be observed in the epidermal layers, instead of its usual location deep within the stele, while still maintaining its normal apical cellular localization (Wisniewska et al., 2006). This allowed for the potential identification of compounds that could shift polarity from apical to basal, while the PIN2::PIN2:GFP could be used to identify compounds that could shift polarity from basal to apical. However, a shift in polarity would require a longer assay time of at least 24 hours to be observed, while most other cellular phenotypes would be visible after a three hour incubation time. BRI1:BRI1:GFP was chosen to interrogate non-polar localization effects because BRI1 is known to constitutively endocytose and endosomal accumulation has been shown to alter brassinosteroid signaling (Geldner et al., 2007).

Once the translatability of the compounds from tobacco pollen to Arabidopsis was determined, two additional markers were chosen to interrogate the bioactive compounds, the non-polar plasma membrane protein UB:NPSN12:YFP (Geldner et al 2009; Sanderfoot and Raikel 2001) and the early endosome trans-Golgi network (TGN) protein VHAa1::VHAa1:GFP (Dettmer et al., 2006). The Qc SNARE protein provided another

non-polar plasma membrane protein while the VHAa1 protein provided a measure to indicate which compounds disrupted an endosome compartment rather than a plasma membrane protein as it cycled back to the plasma membrane.

The next key step was to determine the number of cellular phenotypes and the diversity in which they occurred with the bioactive compounds. A lesser number of phenotypes could indicate a relatively narrow range of protein targets, while a lesser degree of diversity among the occurrence of the phenotypes could indicate that multiple protein targets could produce similar phenotypes. Depending on the number and diversity of occurrence of the phenotypes, it will be important to determine if a relationship exists between the cellular phenotype and the physio-chemical properties of the compound(s).

4.3 Materials and Methods

Plant Material

Seeds were sterilized and sown on 0.5X MS medium (Invitrogen) containing 0.8% Phytoagar (Invitrogen) and vernalized for 48 hours at 4°C, then grown in an 18 hour light cycle at 22°C.

Chemical Treatments and Confocal Microscopy

Four-day-old light-grown seedlings were treated with chemicals in a liquid 0.5X MS liquid media corresponding to a final concentration of 50 to 100 μM based on the molecular mass of each compound. Images were obtained using a TCS SP2 (Leica

Microsystems) with fluorescence detection at manufacturer settings of 492-557 nm for GFP signals and 518-557 nm for YFP.

Bioactive Clustering

The bioactive molecules were clustered by phenotype profiles generated in the screens with different subcellular markers. For this, a phenotype matrix was produced where rows represented compounds and columns the marker types. Each field in this matrix was populated by the numeric labels (0-17) corresponding to the classes of chemicals. In cases of multiple phenotypes, all observed phenotypes were rescored in the corresponding matrix field. To cluster the compounds by phenotype profiles, the Hamming distance was used to compute a distance matrix. In this case, the Hamming distance was the number of vector positions for each compound pair where the corresponding phenotype labels were different. If any of the multiple phenotype labels in a position of a vector pair were identical then they were counted as a match. In addition, missing data points (with numeric value 17) were allowed to match themselves, any single or multiple phenotype situations. The distance matrix obtained by this method was used to perform agglomerative hierarchical complete linkage clustering with the hclust function from R (Murtagh, 1985). The resulting dendrogram was used to sort the rows of the phenotype matrix. A custom color scheme was used for this map where a distinct color is assigned to each phenotype label. Because a meaningful quantitative relationship among the different phenotypes does not exist, it is important to focus the interpretation of the map exclusively on identical color patterns rather than a gradient of

similar colors as is typical for quantitative data sets such as maps for gene expression profiles.

4.4 Results & Discussion

Utilizing both polar and non-polar plasma membrane fluorescently tagged proteins that are known to cycle through endosomal compartments, an assessment was conducted of the translatability of the compounds that inhibited membrane cycling in tobacco to a membrane cycling system in Arabidopsis. Of the total tobacco pollen germination inhibitors/morphological disruptors, 129 also disrupted localization of at least one Arabidopsis protein (Table 4.1), a translatability percentage of 35.34%. This is an incredibly impressive translability between tobacco and Arabidopsis, considering only three cycling proteins in Arabidopsis were assayed. This high rate of translability indicates that the PMRP library could be used to interrogate the trafficking of a variety of endomembrane proteins. As in the tobacco pollen screen, the LATCA library was the most bioactive in Arabidopsis with 50% translability of the compounds. The next active was the Chembridge Diverset that had an almost 30% translability between the two model organisms. Unfortunately, none of the Clickable library compounds, which are amenable to use of click chemistry in target identification, disrupted the Arabidopsis proteins assayed for membrane cycling.

For the 129 compounds that affected localization of PIN2:PIN1:GFP, PIN2:PIN2:GFP or BRI1:BRI1:GFP, sixteen distinct phenotypes were observed

Library	Primary hits from pollen	Seedling Secondary hits	Percentage of primary
Diverset	223	69	30.94%
Novacore	18	3	16.67%
TimTec	33	12	36.36%
CLICK	15	0	0.00%
LATCA	89 (76)	45	50.56%
TOTALS	365	129	35.34%

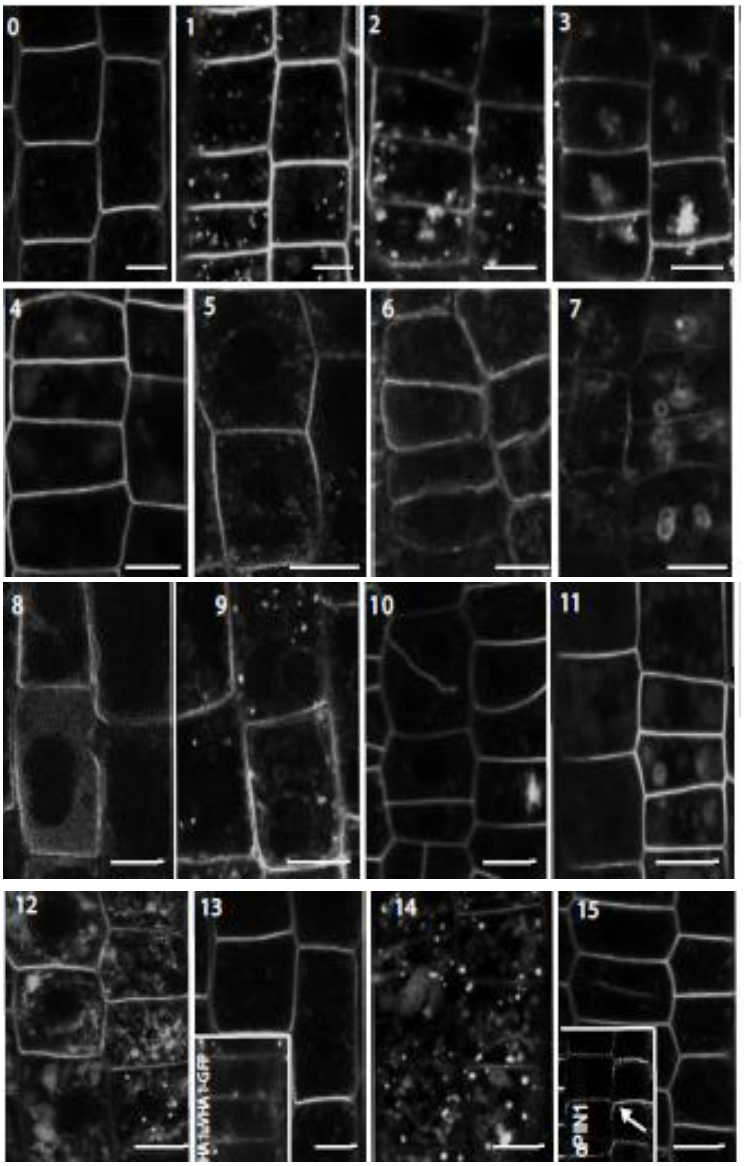
Table 4.1. The total translability of membrane cycling inhibitors from tobacco pollen to Arabidopsis cycling proteins was 35%. The LATCA library compounds were the most translatable and the Clickable library compounds were the least. The terminal acetylene that all Clickable compounds contains may have permeability problems in Arabidopsis.

(Figure4.1). These phenotypes included protein agglomeration phenotypes, ranging from small to large agglomerations (Figure 4.1). The only compound to cause the large

agglomeration phenotype was the internal control compound Brefeldin A. It's interesting that no other compounds mimicked BFA and that the effects of BFA appear to be unique. The medium agglomeration phenotype was similar to the agglomerations observed with Endosidin1 treatment. In addition to agglomeration phenotypes, phenotypes with increased or reduced localization to the plasma membrane were also observed. Specialized phenotypes in which cell plate defects or increased trafficking to the vacuole were also observed.

From the 129 compounds, 121 were commercially available and were used to populate a sub-library named the Plasma Membrane Recycling inhibitors in Arabidopsis (PMRA). As all phenotypes were being confirmed with the PMRA library, two additional markers were also assayed. These additional markers were the Qc SNARE plasma membrane protein UB::NPSN12:YFP (Geldner et al 2009; Sanderfoot and Raikel 2001) and the tran-Golgi network (TGN) protein VHAa1::VHAa1:GFP (Dettmer et al 2006). The frequency of the observed phenotypes was calculated for all five Arabidopsis protein marker lines (Figure 4.2), and the small agglomeration phenotypes was the most prevalent phenotype observed. Phenotype 16 represents compounds that were autofluorescent under laser scanning confocal microscopy (LSCM) and Phenotype 17 represents the compounds that were not assayed on the varying lines or assay times. Phenotype 17 is largely represented among the NPSN and VHA marker lines as only the

.



Phenotype number	Phenotype description
0	Control
1	Small agglomeration
2	Medium agglomeration
3	Large agglomeration
4	Diffuse agglomeration
5	Faint punctate structures
6	Cell vitality affected
7	Cell vitality affected with increased internal agglomeration
8	Cytosol localization
9	Tonoplast localization with agglomeration
10	Cell plate defective
11	Vacuolar localization
12	Lytic body localization
13	Enhanced PM localization
14	Reduced PM localization
15	Polarity change

Figure 4.1 Sixteen distinct phenotypes were identified with the 129 compounds that disrupted Arabidopsis protein localization

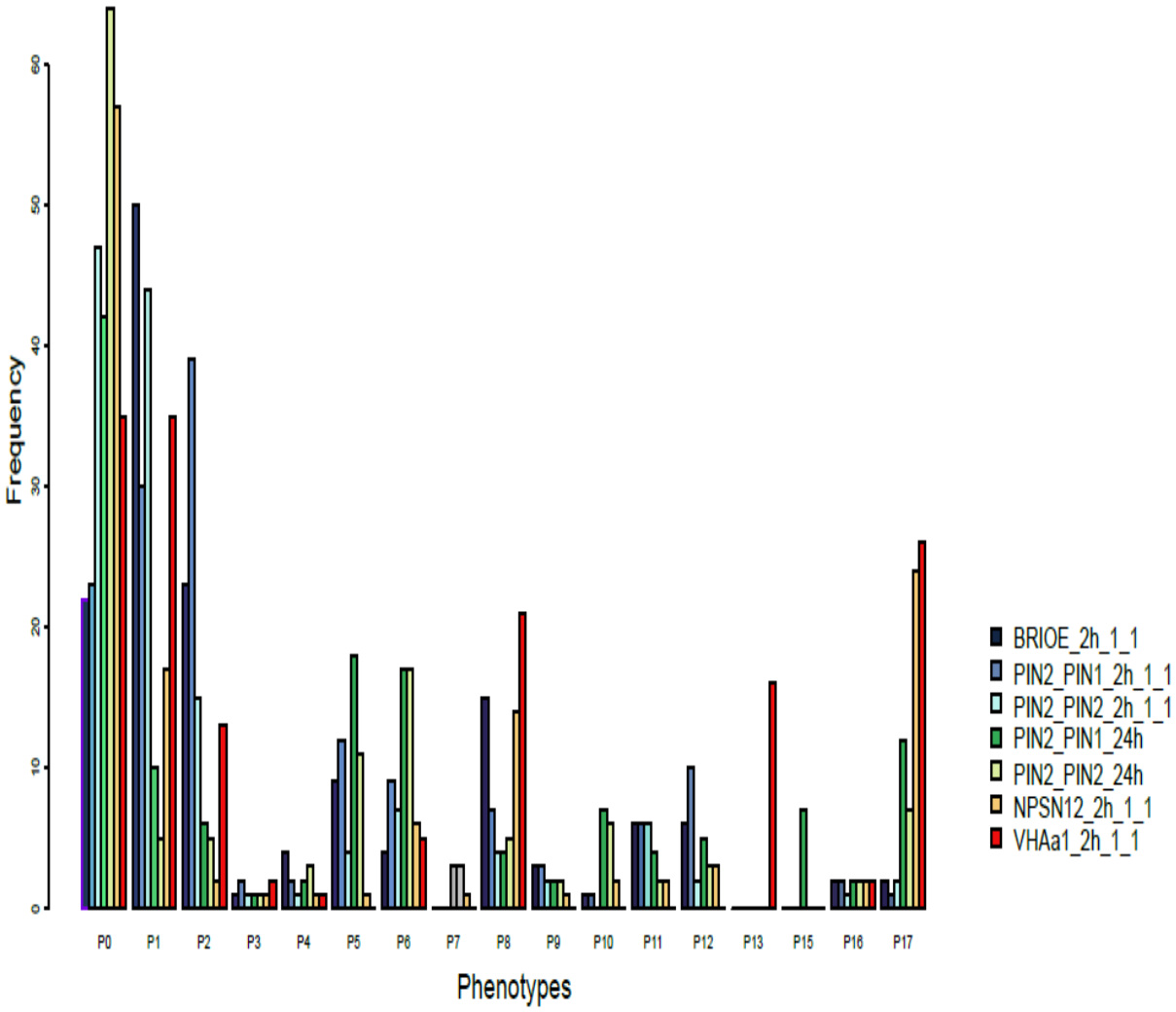


Figure 4.2 The frequency of the phenotypes by marker line and assay time displayed the most prevalent phenotype was the small agglomeration phenotype (P1). The more specialized phenotypes such as the cell plate defective (P10) are caused by fewer compounds.

129 Arabidopsis active compounds were assayed on these lines, meaning that 239 were not assayed on these two lines.

Of key importance at this point was to determine if the compounds that caused similar phenotypes also had physio-chemical similarities. To accomplish this, the PMRP compounds, color coded with the phenotype in Arabidopsis were clustered by physio-chemical properties (Figure 4.3). Unfortunately, the clustering of compounds based upon their properties did not yield clustering of phenotypes. An alternative approach was then undertaken in a process of transgenic marker "fingerprinting" for each compound. With five fluorescently tagged fusion lines used altogether, the phenotype created by each compound on the five different marker lines provided a specific identifier. Using this "fingerprinting" method based on a distance matrix algorithmn, compounds that caused the most similar phenotypes on the five marker lines could be clustered together (Figure 4.3).

Taking a closer look at individual clusters, one can observe similar and diverse compound moieties (Figure 4.4). In viewing the compounds that cause a change in polarity, two different base moieities can be found amoung these compounds, indicating potential similarities of protein targets for these two groups. While this does not give information about the necessity or sufficiency of the moieties in these compounds, it does indicate that there are likely several protein targets that cause similar phenotypes. In each of the phenotypic clusters based upon "fingerprinting," similarities and differences of base moieties can be observed.

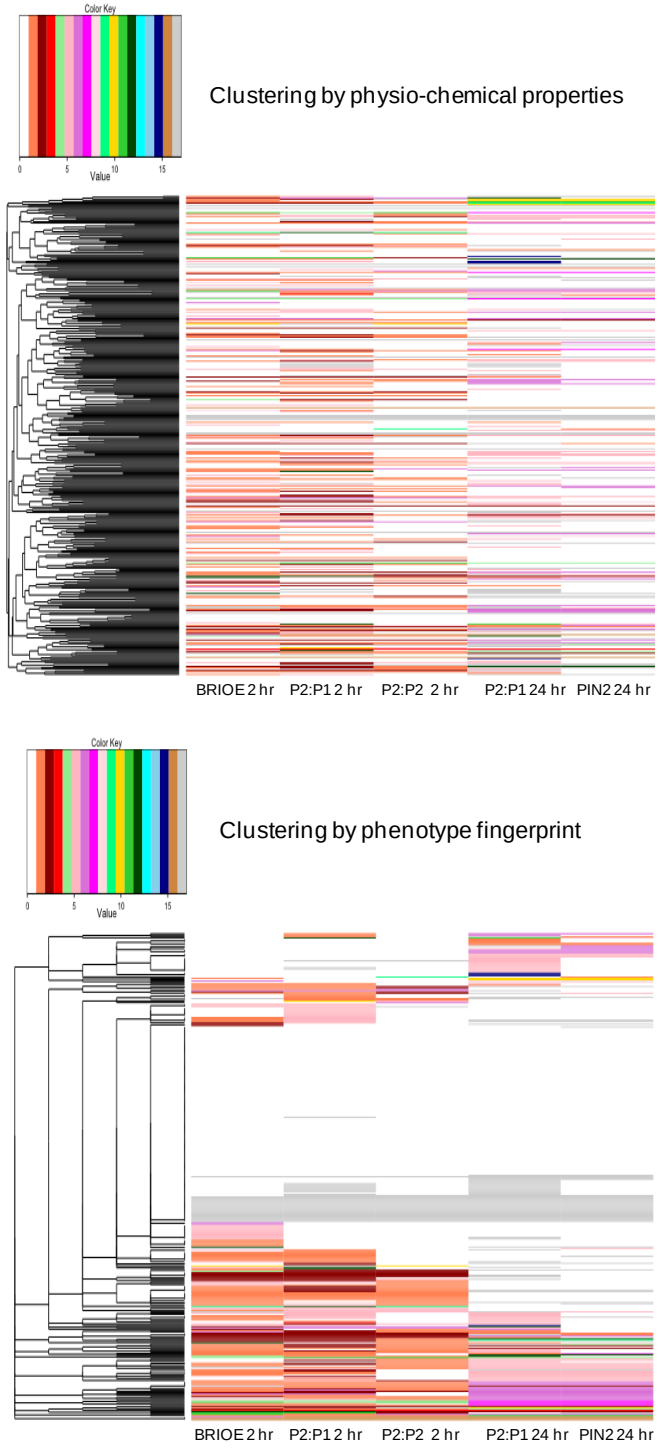


Figure 4.3 Clustering compounds by physio-chemical properties did not lead to clustering of phenotypes. However, a "fingerprinting" method across the five transgenic marker lines allowed for clustering of compounds that caused similar phenotypes on different marker lines.

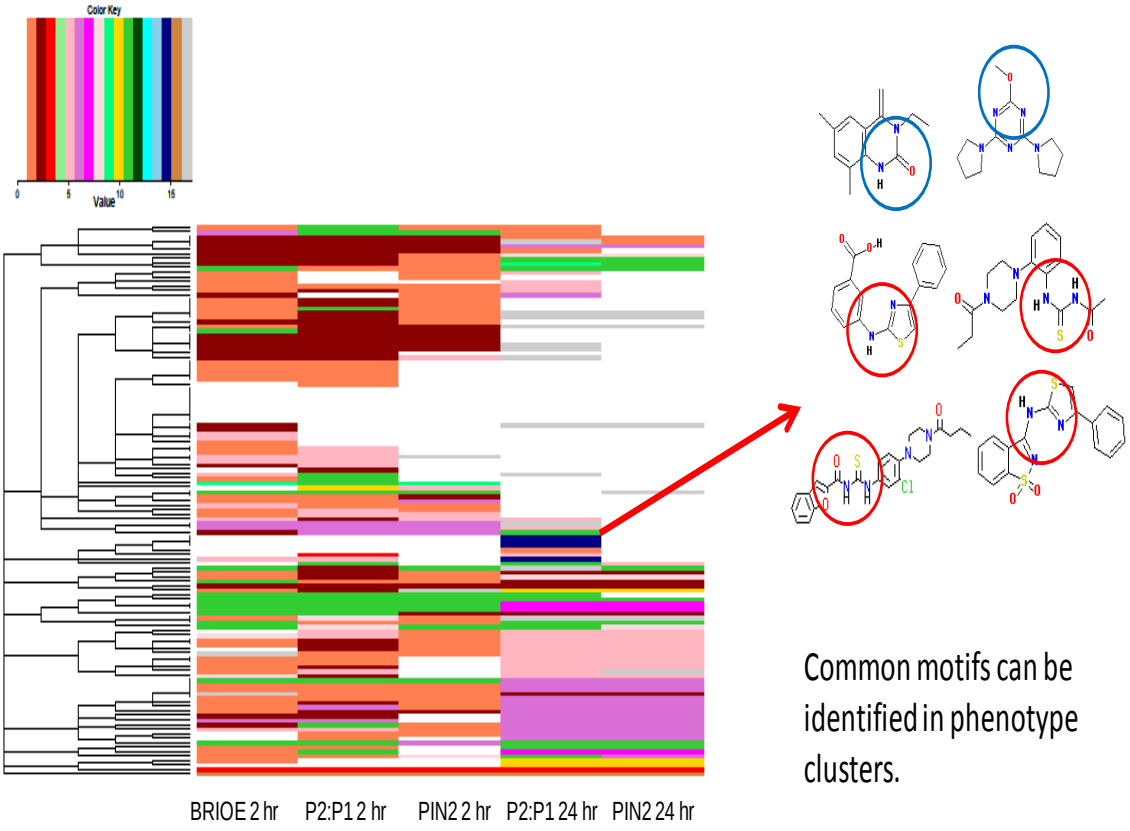


Figure 4.4 By focusing in on the compounds that cause a cause of polarity in the PIN2:PIN1 marker, different and similar chemical moieties can be found, indicating a potentially similar target for each of these compounds while also indicating that there are several protein targets that cause a similar cellular phenotype

.

Overall, a high degree of translability was observed between tobacco pollen and Arabidopsis cycling proteins, a sub-library of Arabidopsis membrane cycling inhibitors was created and sixteen different cellular phenotypes were observed in Arabidopsis. Utilizing a "fingerprinting" method to cluster compounds, compounds that caused similar phenotypes among the different markers were found to have both similar and diverse chemical moieties, indicating the potential to have putatively similar targets for some groups while having different targets that also cause the same cellular phenotype. The PMRA library could now be used to interrogate a specific physiological response known to rely upon endomembrane trafficking.

4.5 References

Dettmer J, Hong-Hermesdorf A, Stierhof YD, and Schumacher K (2006) Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. *Plant Cell* **18**:715-730.

Friedrichsen DM, Joazeiro CA, Li J, Hunter T, and Chory J (2000) Brassinosteroidinsensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. *Plant Physiol* **123**(4): 1247-1256.

Geldner N, Denervaud-Tendon V, Hyman DL, Mayer U, Stierhof YD, and Chory J (2009) Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. Plant Journal **59:**169-178.

Geldner N, Hyman DL, Wang X, Schumacher K, and Chory J (2007) Endosomal signaling of plant steroid receptor kinase BRI1. *Genes and Development* **21**(13):1598-1602

Muller A, Guan C, Galweiler L, Tanzler P, Huijser P, Marchant A, Parry G, Bennett M, Wisman E, and Palme K (1998) AtPIN2 defines a locus of Arabidopsis for root gravitropism control. Embo Journal **17**(23): 6903-6911.

Murtagh, F (1985) Multidimensional Clustering Algorithms. In COMPSTAT Lectures 4 Wuerzburg: Physica-Verlag (for algorithmic details of algorithms used)

Sanderfoot AA and Raikhel NV (2001) Arabidopsis could shed light on human genome. *Nature 410*: 299.

Wisniewska J, Xu J, Seifertova D, Brewer PB, Ruzicka K, Blilou I, Rouquie D, Benkova E, Scheres B, and Friml J (2006) Polar PIN localization directs auxin flow in plants. *Science* **312**(5775): 883.

Chapter 5

Identifying Compounds that Inhibit Gravity Response

5.1 Abstract

Sixteen compounds of the PMRA sub-library disrupted root gravitropic response. Eight of these compounds also disrupted the localization of PIN2, which is known to play a significant role in gravitropic response. Compounds that also disrupted cytoskeleton proteins were eliminated from future study because the link between cytoskeleton function and gravitropic response is well known. From these eight compounds, Endosidin2 was chosen for further characterization. ES2 affected specific plasma membrane proteins and the ER-localized protein PIN5, but no other internal compartments. ES2 specifically inhibited endosomal recycling and caused increased auxin transport and increased brassinosteroid signaling. The only other compound known to inhibit endosomal recycling is Brefeldin A. In contrast to ES2, BFA also causes morphological changes to the Golgi apparatus and causes the collapse of TGN compartments. Therefore, ES2 represents a more precise tool to dissect the effects of endosomal recycling.

5.2 Introduction

With the identification of over 120 compounds that disrupt the localization of proteins that cycle through endosomal compartments in Arabidopsis and the knowledge that the endomembrane system participates in several response pathways, it was

important to isolate a specific physiological pathway. Since the marker proteins were primarily localized to the roots, root gravitropic response was chosen. Gravitropic response is dependent upon the formation of an asymmetric auxin gradient. This gradient allows for differential growth to occur because a high gradient of auxin in the root inhibits growth (Abas et al., 2006). This asymmetric gradient is dependent upon proteolysis and intracellular trafficking.

Two of the proteins selected for the Arabidopsis bioactive assay (PIN2 and PIN2:PIN1) direct the flow of auxin throughout the seedling. Auxin from the shoot apical meristem is directed rootward through the stele and cortex tissues (Feraru and Friml, 2008). Upon reaching the quiescent center of the root, the auxin is transported laterally to the epidermal cells where it is then transported shootward, in a reverse fountain formation. Root gravitropic resopnse was specifically chosen because there is a differential auxin effect between roots and shoots, meaning that increased auxin concentration will inhibit root growth but stimulate shoot growth (Friml 2003).

Using a gross gravitropic response assay, degree of gravitropic response was determined for seedlings on compound after 24 hours of reorientation. This assay allowed the identification of Arabidopsis bioactive compounds that disrupt the gravitropic response pathway. These compounds were then assayed on cytoskeletal protein markers to determine if the cytoskeleton was affected. It is known that inhibiting the cytoskeleton will disrupt gravitropic response (Baluska et al., 1996). Any compound inhibiting cytoskeletal proteins was eliminated from future study. Then a dynamic gravity response assay was used to quantify real-time orientation every three minutes for 8 hours. The

dynamic gravity response allowed for a distinction between compounds that slow gravitropic response as oppposed to those that inhibit it.

At this stage, one compound was selected, Endosidin2 (ES2), and fully characterized. Characterization of Endosidin2 included determining which endomembrane compartments were affected, whether ES2 was disrupting endosomal recycling, endocytosis, or both, and determining physiological effects of the compound.

5.3 Materials and Methods

Chemical Treatments and Confocal Microscopy

Four to six-day old light grown seedlings grown on 0.5X MS medium containing 0.8% phytagar were transferred to 0.5X MS liquid media containing indicated compound. Fluorescence was visualized using the laser scanning confocal microscopy with a TCS SP2 (Leica Microsystems, Wetzlar, Germany). Fluorescence emission using manufacturer settings was detected at 492-557nm for GFP, 518-557nm for YFP, 505-600nm for CFP, and 600-700 nm for FM4-64. For BFA washout experiments, seedlings are treated with 40 µM BFA and then are transferred to either normal media or ES2 containing media for two hours. To determine co-localization for PIN2:GFP and FM4-64 in ES2 bodies, the signal for multiple endosomes per seedling were quantified using the TCS SP2 software.

For growth assays, seedlings were germinated on indicated compounds for five days or fourteen days as described.

Gravitropic response assays

The gross gravitropic assays were performed as in Surpin et al (2005). Seeds were plated on compound containing media, stratisfied for two days and then light-treated for 8 hours. Plates were then grown vertically in the dark. After three days of growth, plates were rotated 90°. After another two days of growth, seedlings were visualized using an Epson scanner (Model 2450, Long Beach, California) and angles were quantified using the free image analysis software Image J (Abramoff et al 2004).

The dynamic gravitropic assays were performed as in Miller et al (2007). Seedlings were germinated on solid media and after seven days of growth, were transferred to ES2 containing solid media or DMSO containing solid media and treated for two hours. The plates containing the seedlings were then rotated 90° and images of seedlings were taken every three minutes for eight hours. These images were analyzed by the algorithmn created by Miller et al (2007).

qPCR assay

Quantitative PCR was performed as in Robert et al (2008). Four day old seedlings were treated in liquid media with ES2, hormone, or both as indicated. RNA was purified (Qiagen), and first-strand cDNA was synthesized (Invitrogen). For quantitative PCRs, SYBRGreen Supermix (BioRad) was used in reaction volume of 20-l. Reactions were run on an CFX96 thermocycler (BioRad).Primers for gene-specific detection of BL-responsive NAC transcription factor
(At5g46590) were 5-GTT TAC CTC CAG GGT TCC GGT T-3 (forward) and

5-GCA CTG AGA TGC GAC ATC TTG G-3 (reverse). Ubiquitin primers were 5-GAT CTT TGC CGG AAA ACA ATT GGA GGA TGG T-3- (forward) and 5-CGA CTT GTC ATT AGA AAG AAA GAG ATA ACA GG-3- (reverse). For relative quantification, ratios were calculated according to the method of Pfaffl (46). All *Ct* values were the average of three replicates and all treatments were subject to at least three biological replicates.

Auxin transport assay

Seedlings were grown on solid media for seven days and then treated with ES2 or DMSO for two hours. After treatment, radiolabeled auxin was applied in droplets at the root tip or at the root-shoot junction (Lewis and Muday 2009). The radiolabeled auxin was allowed to transport through the seedlings for five hours. After six hours, rootward transport was determined using the entire root after a 2 mm distance from the auxin droplet. Shootward transport was determined using a portion of the root 2 mm from the root tip and 5 mm long.

5.4 Results & Discussion

Using root gravitropic response as a specific endomembrane pathway, seedlings were assayed for gross gravitropic response (measured response after 24 hours of re-orientation) with the compounds from the PMRA sub-library that were bioactive in Arabidopsis. From this sub-library, sixteen compounds strongly disrupted root gravitropic response, twenty-two had a mild effect on gravity response, five were

hypergravitropic and fifteen compounds did not permit any root growth on compound (Table 5.1). These results were compared with the localization effects of these compounds on the PIN2::PIN2:GFP marker since PIN2 is known to play a role in gravitropic response (Abas et al., 2006). Of the compounds that strongly effect gravitropic response, half (eight compounds) also disrupted PIN2 localization. PIN2 localization was disrupted on approximately 50% of the slightly agravitropic and hypergravitropic effect compounds and over seventy percent of the compounds that inhibited root growth disrupted PIN2 localization.

Compounds that disrupt cytoskeletal activity are also known to disrupt gravitopic response. Therefore, all compounds were assayed with the 35S::MAP4:GFP (Van Damme et al., 2004) to determine if tubulin proteins were disrupted. Eight of the sixteen compounds that strongly affected root gravitropic response also disrupted tubulin localization (Table 5.2). Compound 2-A04 phenocopied the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) and was not pursued. The remaining compounds were then reordered and given Endosidin designations: 1-A11 (ES2), 1-B10 (ES5), 1-D11 (ES10), 1-F03 (ES11) and 2-C03 (ES12). The compound 2-C06 was not reorderable and the compound 2-D07 was not bioactive upon reorder. ES5 demonstrated non-specific endomembrane effects and was not pursued as a gravity Endosidin.

Category	Affect gravity response	Affect gravity and PIN2 localization
Agravitropic	16	8
Slightly agravitropic	22	12
Hypergravitropic	5	3
Inhibited growth	15	11

Table 5.1 Forty-three compounds from the PMRA sub-library affected root gravitropic response. Fifteen compounds could not be effectively assayed because the seedlings did not produce root systems when exposed to these compounds.

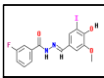
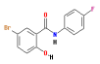
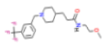
	1-A11	PIN2 – medium agglomeration		1-C06	Affects cytoskeleton
	1-B02	Affects cytoskeleton		1-C07	Affects cytoskeleton
	1-B10	Non-specific endomembrane effects		1-D02	Affects cytoskeleton
	1-D11	PIN2 – small agglomeration		1-F03	Affects PIN2:PIN1 – potential polarity shift
	1-F11	Affects cytoskeleton		2-C03	Affects PIN2:PIN1 – small agglomeration
	2-A03	Affects cytoskeleton		2-D06	Affects PIN2:PIN1 – potential polarity shift
	2-A04	PIN2 – small agglomeration		2-D07	Affects PIN2:PIN1 – potential polarity shift
	2-E02	Affects cytoskeleton		2-D09	Affects cytoskeleton
Affect PIN2 localization			Do not affect PIN2 localization		

Table 5.2 Eight of the sixteen compounds that strongly disrupt root gravitropic response disrupted localization of actin or tubulin fluorescently labeled protein lines. The corresponding localization effect for each compound is listed beside the non-cytoskeletal affecting compounds.

To gain a better understanding of the response to the gravity by Endosidins, dynamic gravity response measurements were taken using automatic image collection and a specialized algorithmn developed by Miller et al. (2007). This was done in conjunction with determining the auxin gradient using the DR5rev::DR5:GFP (Friml et al., 2003) after reorientation while on compound. The dynamic gravity response measurements indicated that ES2 and ES10 would most probably fully reorient if given more time while ES11 and ES12 measurements indicated that the seedlings on these compounds would probably never fully reorient (Figure 5.1) All compounds failed to form an asymmetric auxin gradient required for gravitropic response as seen in the control (Figure 5.1) and maintained auxin distribution on the upper side of seedling root.

Based upon its similar phenotype of initial proteins markers affected to Endosidin1, the founding member of the Endosidin family (Robert et al., 2008), Endosidin2 (ES2) was chosen to be fully characterized. The first steps of this characterization included determination of a dose response curve and determination that ES2 was not a novel auxin (Figure 5.2). ES2 did affect primary root growth so an appropriate concentration had to be determined in which root gravity response was impaired but primary root growth was within normal range. The IC_{50} for root gravitropic response for ES2 was determined to be ~15 μM. Using the DR5rev:DR5:GFP line, ES2 did not demonstrate an auxin gradient along the epidermal cells as the exogenous auxins did, indicating that ES2 was not acting as a known auxin.

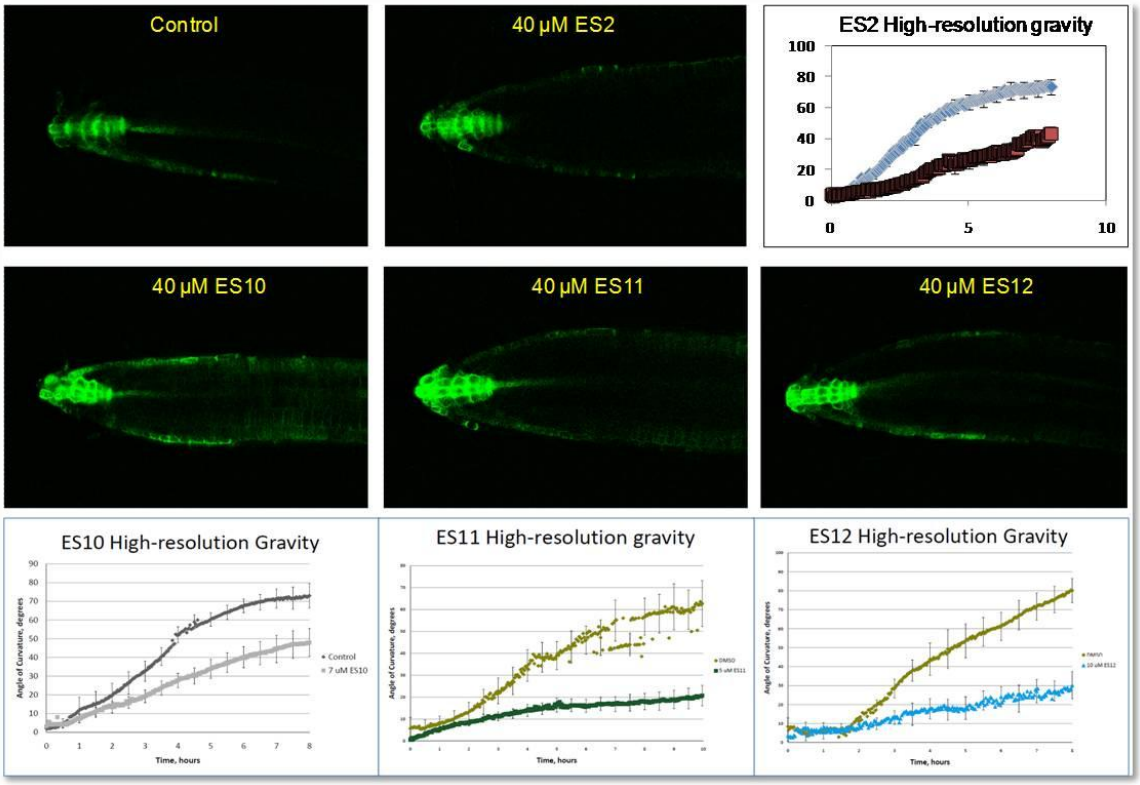


Figure 5.1. Dynamic root gravitropic response assayed determined that ES2 and ES10 would likely fully reorient given enough time, while ES11 and ES12 were likely not to ever reorient. All four compounds failed to develop an asymmetric auxin gradient required for gravitropic response.

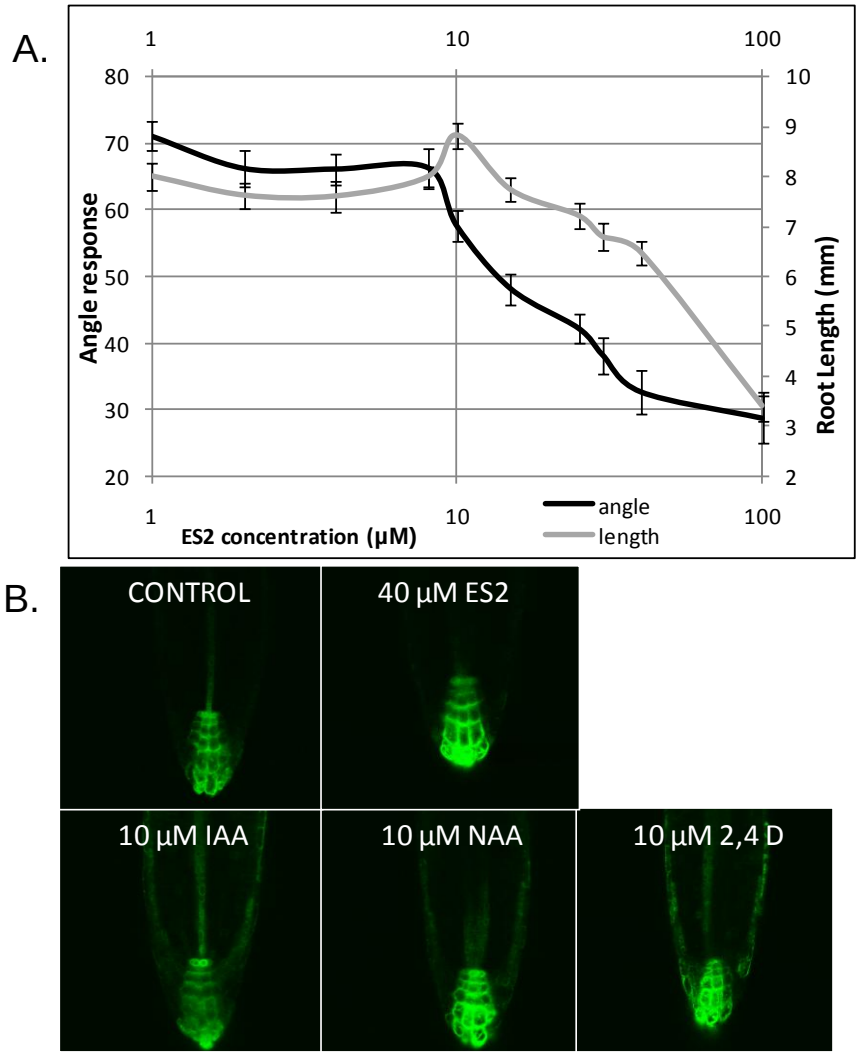


Figure 5.2. The dose response in comparison to the angle and root length determined an IC50 for root gravitropic response to be ~15 µM (A). ES2 did not phenocopy an exogenous auxin on the DR5rev:DR5:GFP auxin marker line.

The next step was to look at structure activity relationship of Endosidin2. By assaying compounds with similar chemical moieties, essential bioactive regions of the compound could be identified. Based upon the structures of Analog 1, 2, 3 5 and 6, the bioactivity of ES2 appeared to depend upon having a halogen at both ends of the entire molecule (Figure 5.3). Analogs 4, 7 and 8 demonstrated that neither end of the ES2 molecule had the same bioactivity as ES2. Thus, the entire backbone of the ES2 molecule and the halogens at both ends are required for bioactivity in Arabidopsis.

The physiological effects of ES2 on Arabidopsis seedlings included inhibited primary root growth, inhibited lateral root growth, and slightly increased hypocotyl growth (Figure 5.4). These types of inhibited root growth have been associated with auxin deficient mutants (Quint et al., 2009; Rashotte et al., 2001), so auxin transport assays were conducted to determine if ES2 inhibited auxin transport. However, auxin transport was increased both rootward and shootward (Figure 5.4). Increased auxin transport has been associated with both inhibited gravity response and inhibited lateral root formation (Rashotte et al., 2001). With this increased auxin transport, ES2 treated seedlings were hypersensitive to nanomolar concentrations of the auxins indole acetic acid (IAA) and 2,4-(dichlorophenoxy) acetic acid (2,4D) (Figure 5.4). Treatment with 1-naphthaleneacetic acid in combination with ES2 demonstrated a slightly increased sensitivity but was not as dramatic as the increased sensitivity with IAA and 2,4D. Interestingly, seedlings treated with ES2 and the auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA) demonstrated neither increased nor decreased sensitivity

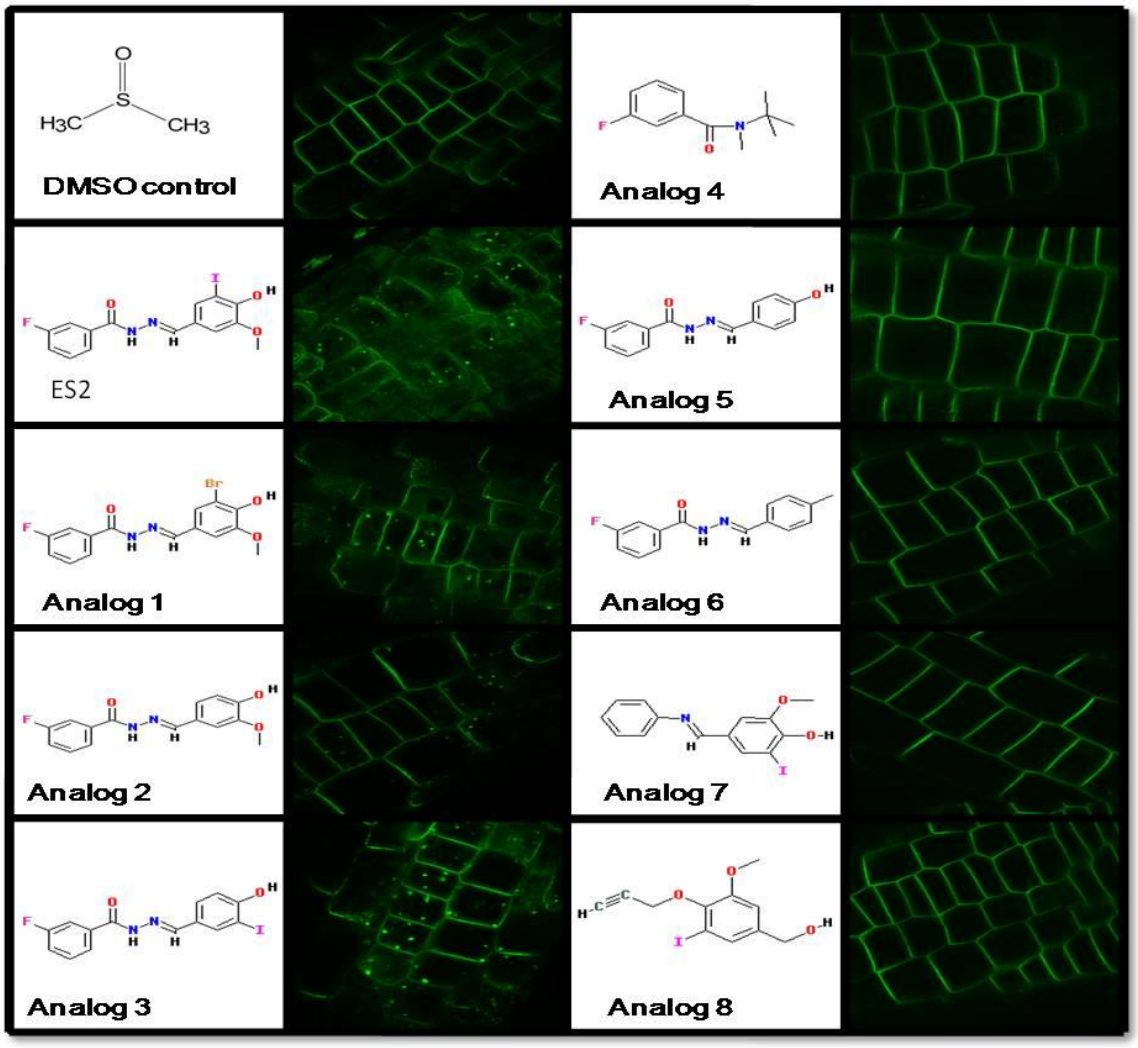


Figure 5.3 A structural activity relationship (SAR) was performed with analogs similar to the entire structure of ES2 and to pieces of ES2. Through this SAR, it became evident that the entire backbone and the dual halogens at opposite ends of the molecule are necessary for bioactivity.

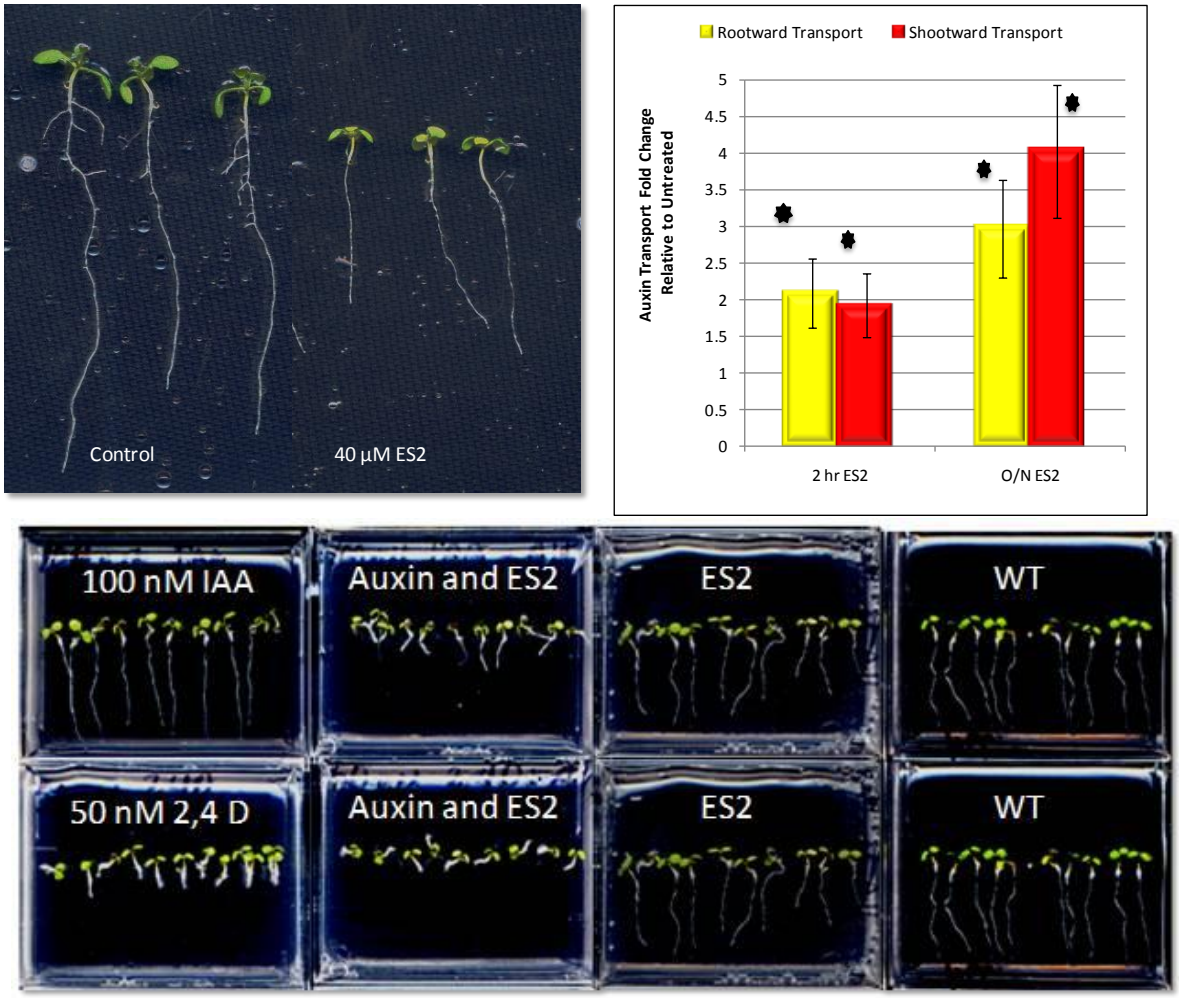


Figure 5.4 ES2 inhibited primary root elongation, lateral root formation, and increased hypocotyl growth. ES2 actually increased auxin transport both rootward and shootward by roughly two-fold after only a two hour treatment. An overnight ES2 treatment increased shootward transport by four-fold. Seedlings were hypersensitive to ES2 treatment in combination with nanomolar concentrations of IAA and 2,4D.

in terms of root elongation. This may have been because NPA blocks shootward transport but does not affect rootward transport.

Endosidin2 caused medium agglomeration phenotypes in specific plasma membrane proteins, BRI1, PIN2, PIN1, PIN7 (Friml et al., 2003), and AUX1 (Pickett et al., 1990), and in the endoplasmic reticulum (ER) localized PIN5 (Mravec et al., 2009) protein (Figure 5.5). Plasma membrane proteins not affected included PHOT (Kasahara et al., 2002), PIP2a (Cutler et al., 2000), PGP4 (Terasaka et al., 2005) and PGP19 (Geisler et al., 2003). The fact that PGPs, which play a role in auxin transport, were not affected was of interest because PINFORMED and AUX1 which also transport auxin were affected. Examination of the effects of ES2 was performed on additional endomembrane compartments: HDEL (Boston et al., 1994) – ER, Got1p (Geldner et al., 2009) – Golgi, SYP61 (Sanderfoot et al., 2001a) – TGN, and SYP21 (Sanderfoot et al., 2001b) – PVC. None of these internal compartments were affected (Figure 5.6). Since ER-localized PIN5 was affected, it was imperative to check additional ER and Golgi proteins. For the ER, AXR4 (Hobbie and Estelle 1995), which affects gravitropic response and lateral root initiation, and SEC12 (Bar-Peled and Raikhel 1997) were also not affected by ES2 treatment. Given the intimate relationship between the ER and Golgi, several different Golgi proteins were assayed from the WAVE multicolored lines (Geldner et al., 2009): Memb12, RAB2DA, RAB2DB, and SYP32. None of the additional Golgi proteins were affected by ES2 treatment.

This led to the question of whether ES2 bodies were forming in a novel endomembrane compartment. In a transgenic line expressing PIN2::PIN2:GFP

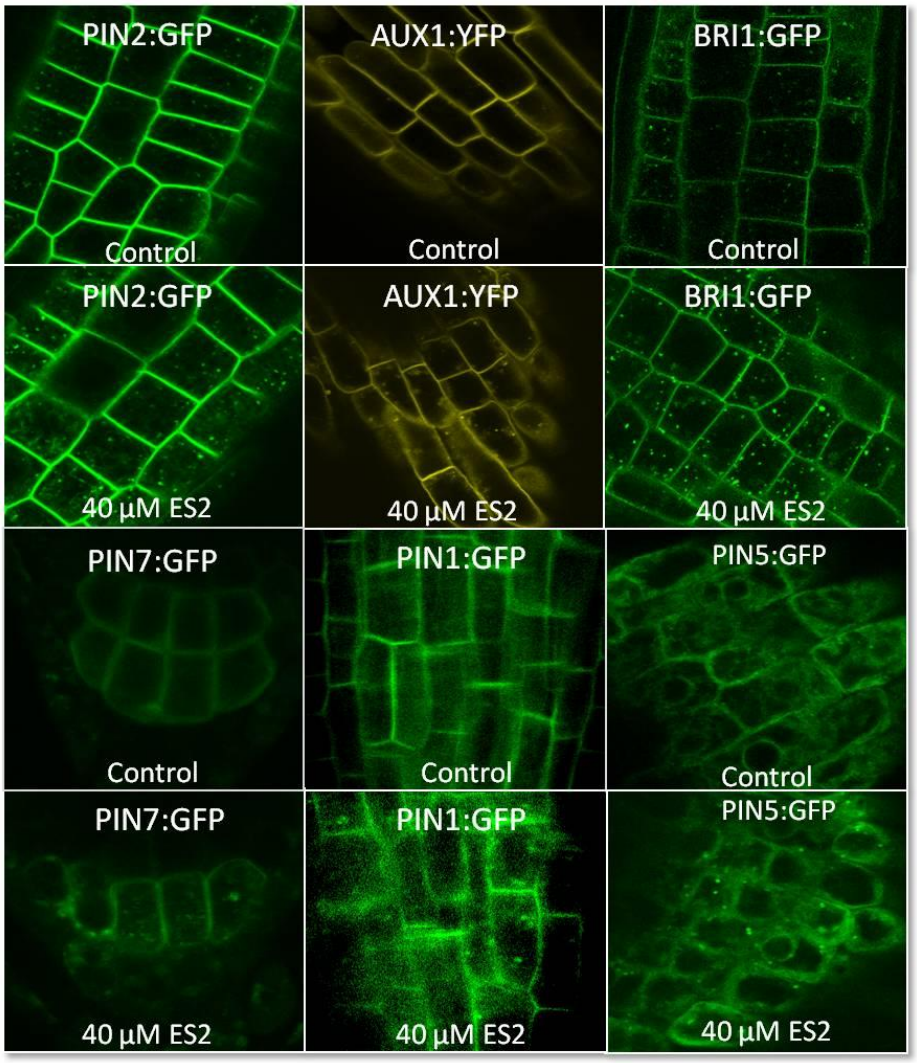


Figure 5.5. Specific plasma membrane proteins experienced medium sized agglomerations when treated with ES2. The only non-PM protein affected was the ER-localized PIN5 protein.

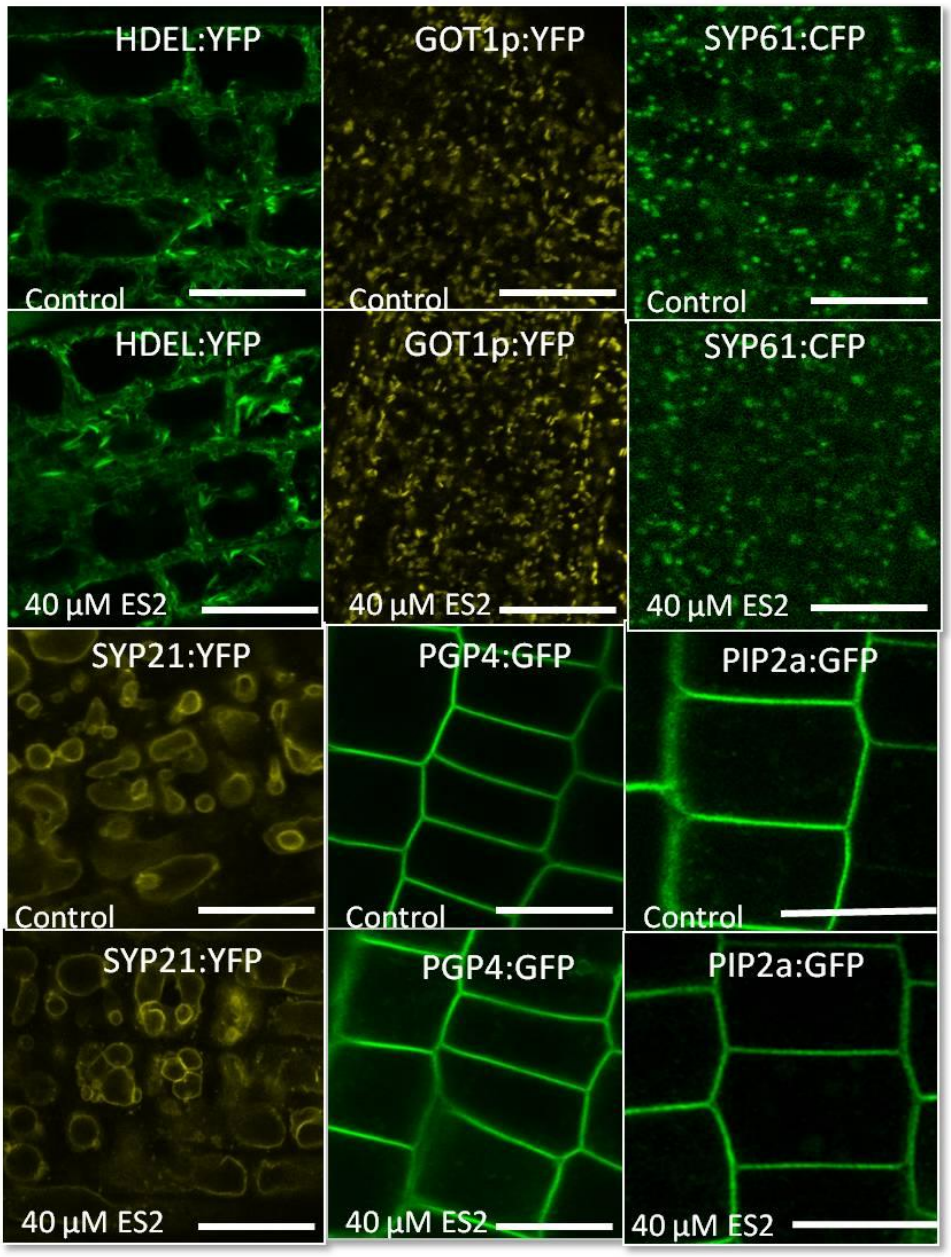
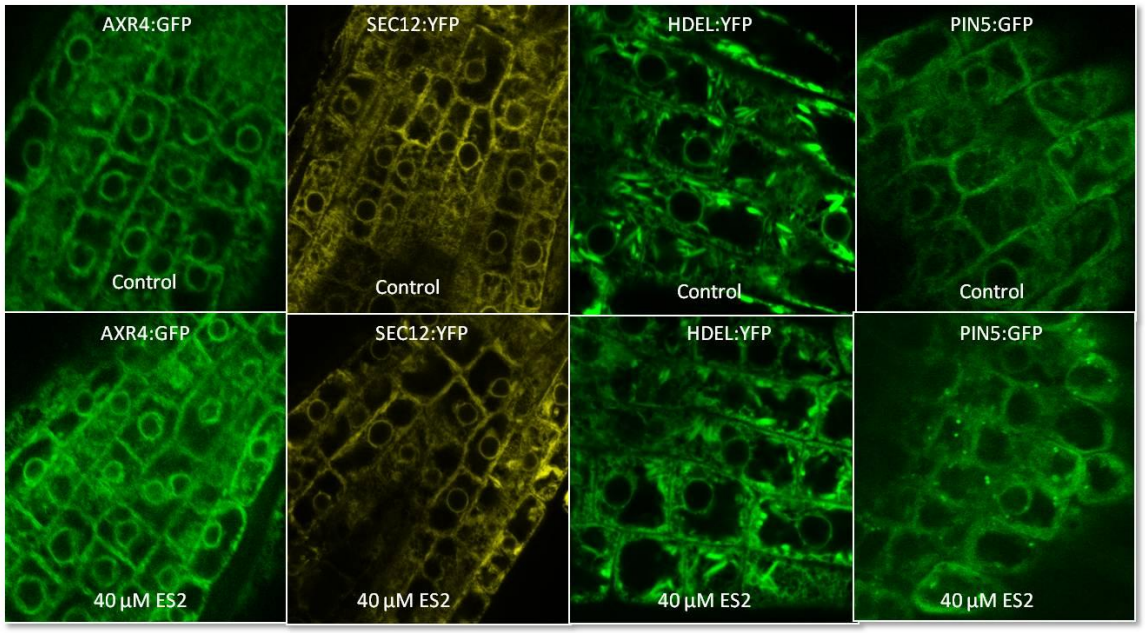


Figure 5.6 No internal endomembrane compartments were disrupted by ES2 treatment. This included other ER compartments (HDEL), Golgi (GOT1p), TGN (SYP61), and PVC (SYP21).



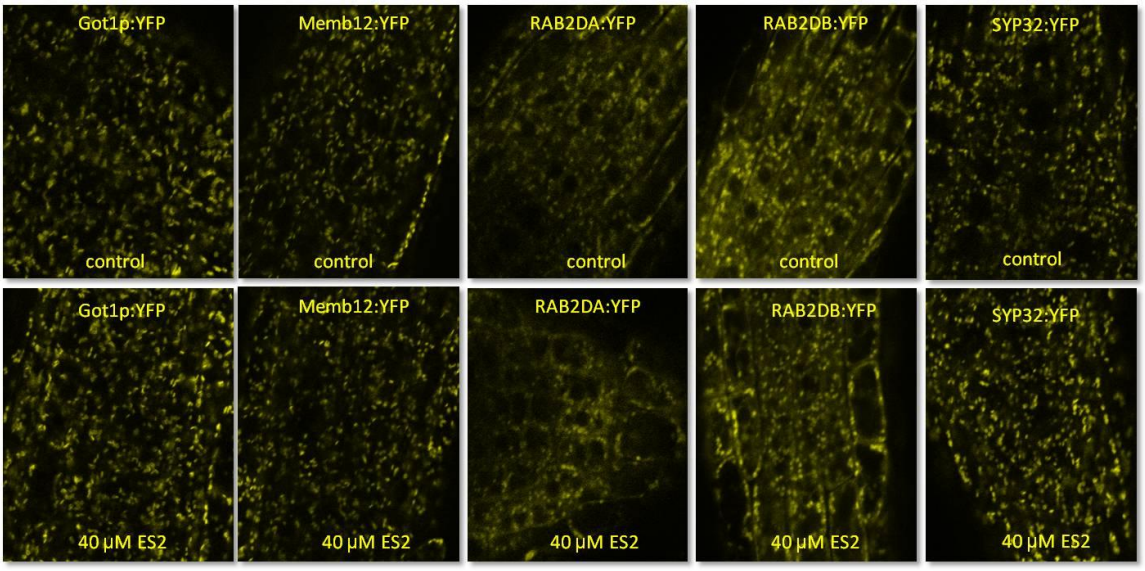
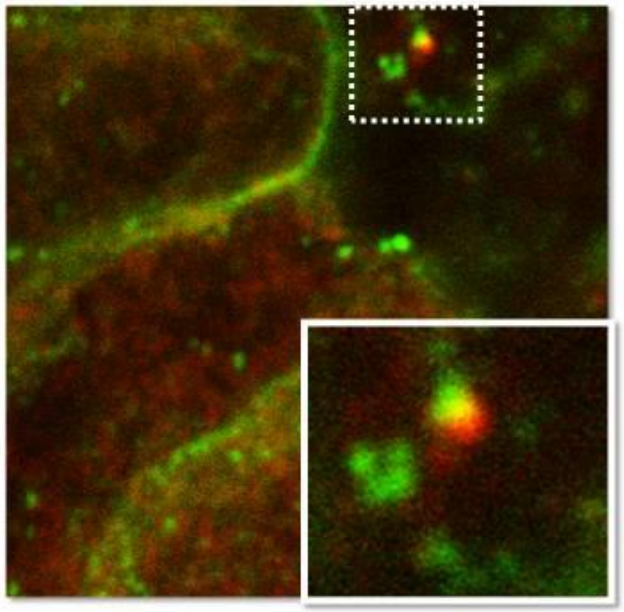


Figure 5.7 In testing additional ER and Golgi proteins, none were disrupted by ES2 treatment.

and PIN5::PIN5:myc, co-localization between the two proteins can sometimes be observed (Figure 5.8). Since co-localization is not always observed in seedlings, this may be indicative of a highly transient nature of the ES2 body. To further examine the possibility of a novel compartment, electron micrograph images of ES2 treated PIN5::PIN5:GFP seedlings treated with GFP antibody were taken. PIN5 in the control demonstrated a normal localization pattern to the ER. However, when treated with ES2, PIN5 can then be observed associating with parts of the Golgi apparatus, the PVC compartment and some independent bodies (Figure 5.8). Work is continuing to elucidate whether PIN2 and PIN5 truly co-localize and if the ES2 body is a novel compartment or the specific region of a known compartment.

The next thing to be determined was whether ES2 was inhibiting recycling, endocytosis or both. To address the question of recycling, a BFA washout experiment was performed. Plasma membrane markers known to be affected by ES2 experienced delayed recycling back to the plasma membrane when BFA was washed out in the presence of ES2 (Figure 5.9). Plasma membrane markers not affected by ES2 treatment did not experience this same delay. This indicated that the ES2 bodies observed were at least partially due to delayed recycling to the plasma membrane. However, delayed recycling did not exclude the possibility that endocytosis was also affected. To determine this, experiments with the endocytotic dye FM4-64 (Dettmer et al., 2006) and with the formation of BFA bodies with the pre-treatment of ES2 were performed. FM4-64 only partially co-localized with ES2 bodies consistent with the idea of ES2 inhibiting the recycling/secretory pathway (Figure 5.10) (Dettmer et al., 2006). BFA is known to inhibit



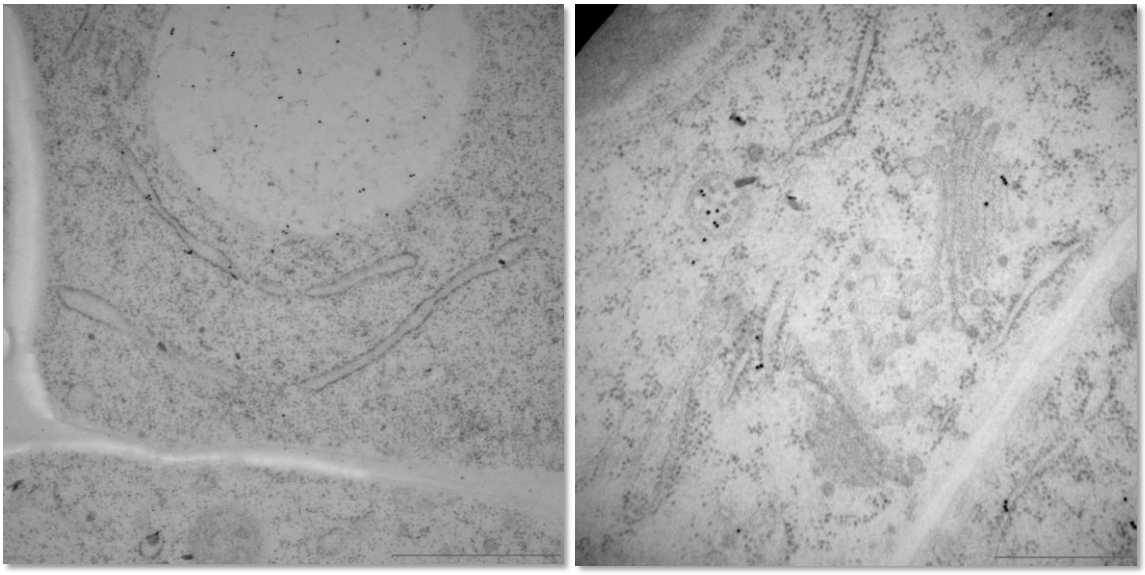


Figure 5.8 The top-most image demonstrates the partial co-localization than can be observed between PIN2 and PIN5 upon ES2 treatment. The bottom left image is a control PIN5:GFP with GFP antibodies showing a normal ER localization for PIN5. The bottom right image is PIN5:GFP with GFP antibodies treated with ES2. Abnormal localization to regions of the Golgi apparatus, the PVC compartment, and independent bodies are apparent when treated with ES2.

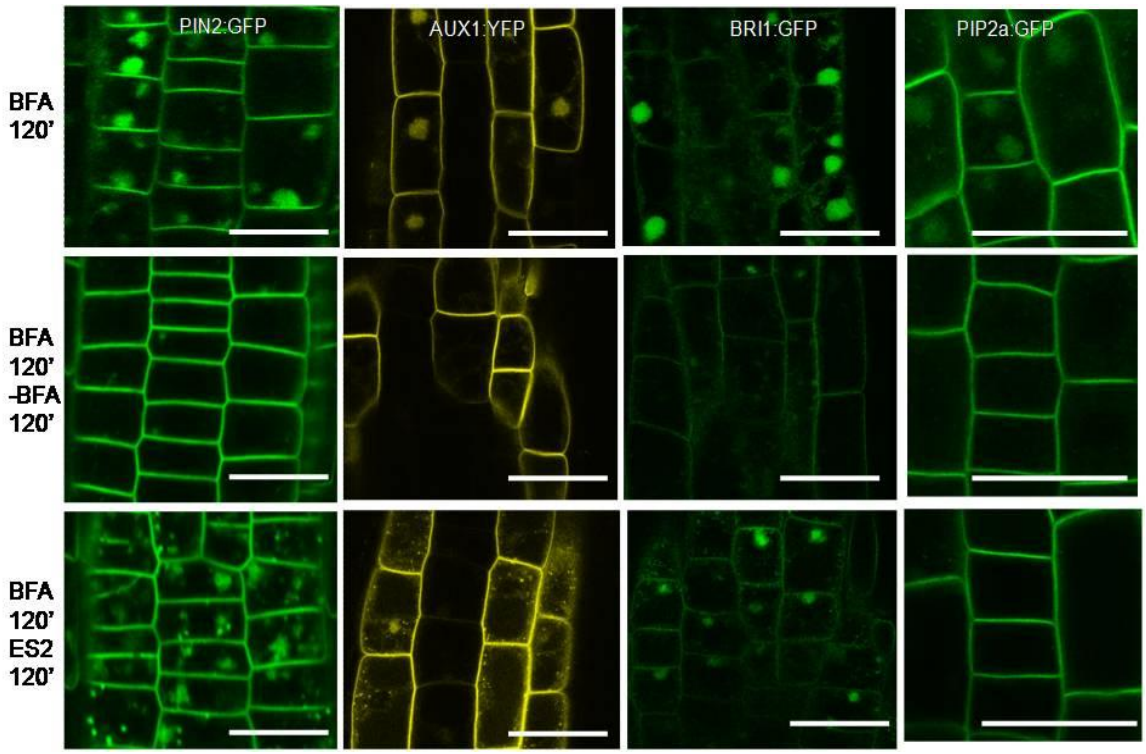


Figure 5.9 In BFA washout experiments, in the presence of ES2, the BFA bodies fail to cycle back to the plasma membrane. PIP2a was not affected by ES2 and served as a negative control.

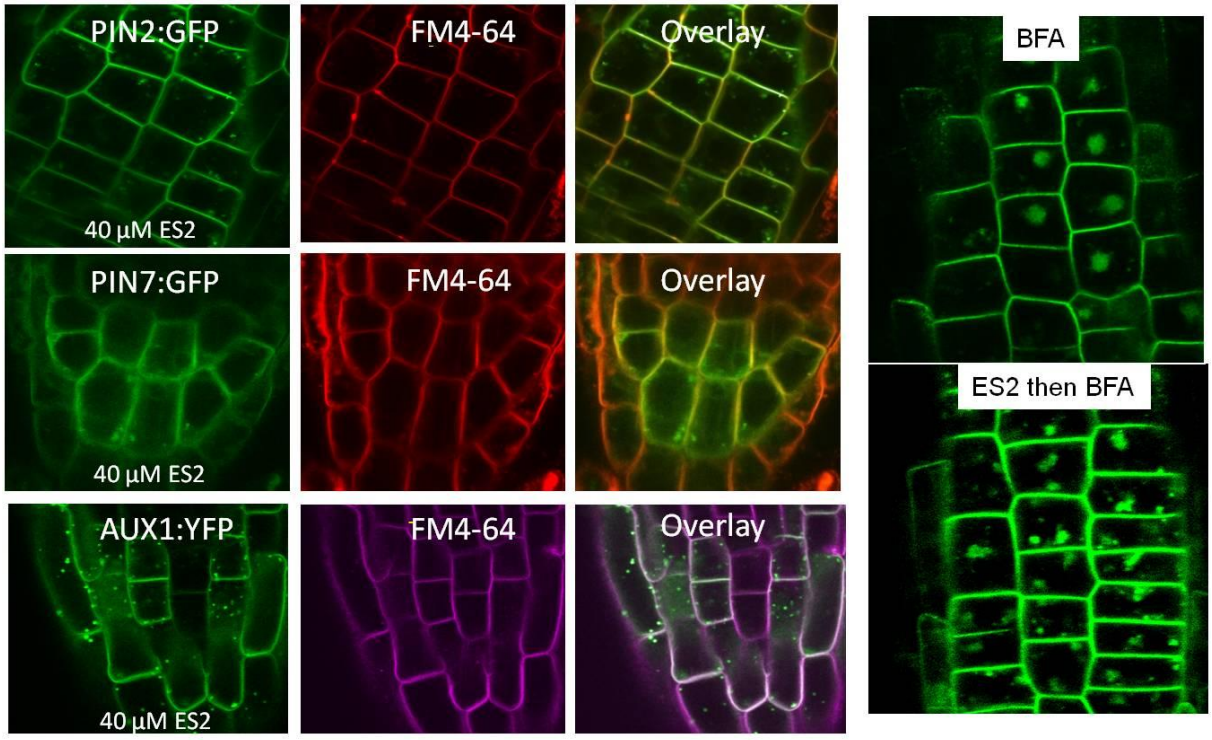


Figure 5.10 With FM4-64 and BFA experiments, ES2 did not demonstrate the ability to inhibit endocytosis. FM4-64 only partially co-localized with ES2 bodies indicating that the bodies are within the recycling/secretory pathway. BFA is known to inhibit endosomal recycling. In treating seedlings with ES2 and then with BFA, if endocytosis was inhibited with ES2 treatment, there would be a lack of BFA bodies. However, this does not occur.

recycling while allows endocytosis to occur (Grebe et al., 2003). Therefore, if seedlings are treated with ES2 and then treated with BFA and BFA body fails to form, this would indicate that ES2 was also blocking endocytosis. However, BFA bodies were still able to form after pre-treatment with ES2 (Figure 5.10). Between the FM4-64 experiments and the BFA body formation, it would be very reasonable to conclude that ES2 does not affect endocytosis and affects recycling/secretion to the plasma membrane. This is extremely significant because the only compound now known to inhibit recycling to the plasma membrane is BFA. Unfortunately, BFA also causes the collapse and agglomeration of TGN and Golgi compartments (Nebenfuhr e al., 2002), making it difficult to ascertain whether the effects of BFA are due to inhibiton of recycling or the morphological changes to the TGN and Golgi compartments. ES2 has the advantage over BFA in that ES2 is more specific in the proteins affected and specifically affects recycling/secretion to the plasma membrane without affecting other internal compartments. ES2 offers a novel way to precisely interogate the effects of an inhibition of recycling to the plasma membrane.

PIN2 proteins are known to increase trafficking to the vacuole in dark conditions (Kleine-Vehn et al., 2008) and that this process is dependent upon the putative retromer complex protein SNX1 (Jaillais et al., 2006), that retrieves proteins from the PVC and shuttles them back to the TGN. The snx1 mutant experiences increased vacuolar trafficking of PIN2 in dark conditions (Kleine-Vehn et al., 2008). By inhibiting endosomal recycling with ES2, would vacuolar trafficking of PIN2 in dark conditions also increase? After four hours of dark treatment on ES2, PIN2 accumulation in the

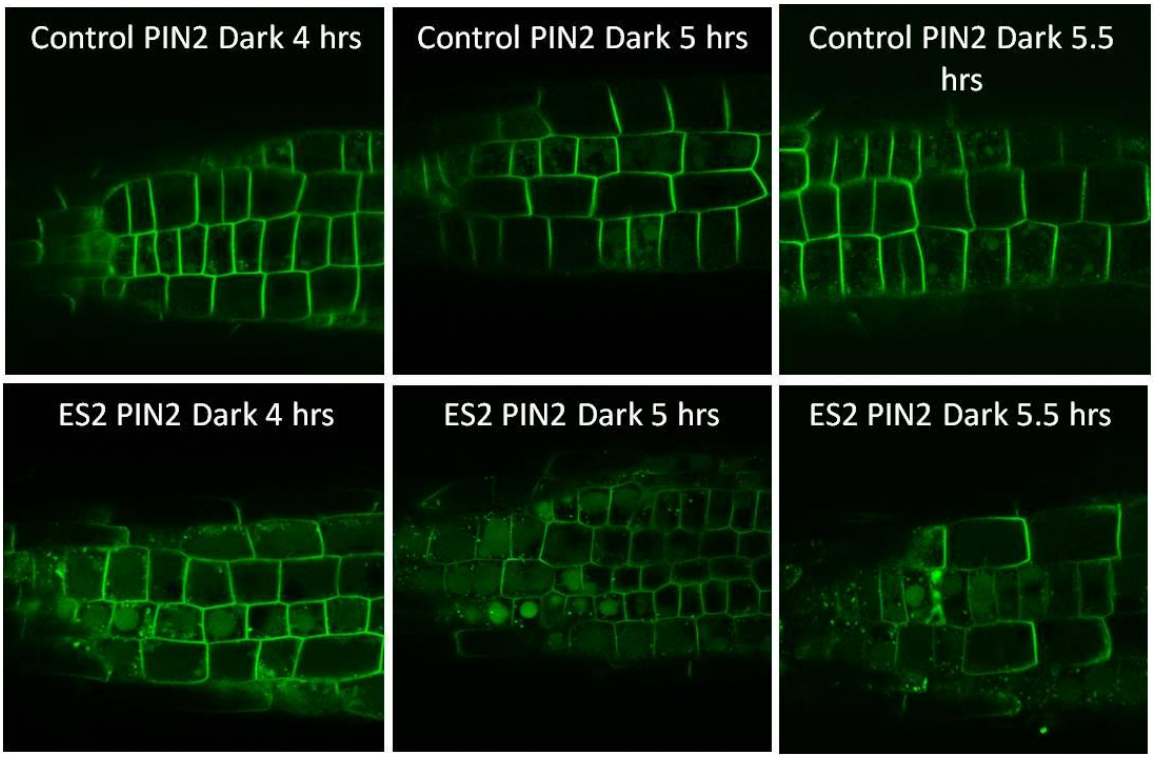


Figure 5.11 ES2 increased PIN2 trafficking to the vacuole in the dark. A similar phenomenon occurs when proteins of the putative retromer complex are inhibited, indicating that when normal recycling pathways are inhibited, there is an increased accumulation in the vacuole.

was significantly more abundant than the control. So by inhibiting recycling to the plasma membrane, the default trafficking pathway to the vacuole was increased.

Auxin is not the only hormone known to be affected by endosomal recycling. The brassinosteroid pathway has been shown to be inhibited by endocytosis inhibitors (Endosidin1 and Concanamycin A) and upregulated by the recycling inhibitor BFA (Robert et al., 2008; Kleinath et al., 2010; Geldner et al., 2007). To determine whether ES2 also increased brassinosteroid signaling, qPCR assays were performed using the brassinosteroid specific NAC transcription factor (Nemhauser et al., 2006). Indeed, ES2 upregulated the brassinosteroid specific NAC nearly as much as brassinolide did and a small additive effect was also observed (Figure 5.12). The NAA treatment was used as a control indicating that the NAC was not affected by exogenous auxin and the combination of NAA and ES2 would prevent internalization of proteins, so the ES2 effect was no longer observed.

Overall ES2 has been demonstrated to be a highly specific endosomal recycling inhibitor. By inhibiting endosomal recycling, root gravitropic response is disrupted, PIN2 increases trafficking to the vacuole in the dark, and auxin transport is increased (Figure 5.13). The auxin pathway was not the only hormone pathway affected. The brassinosteroid pathway experienced an increase in signaling in response to ES2 as experienced with BFA.

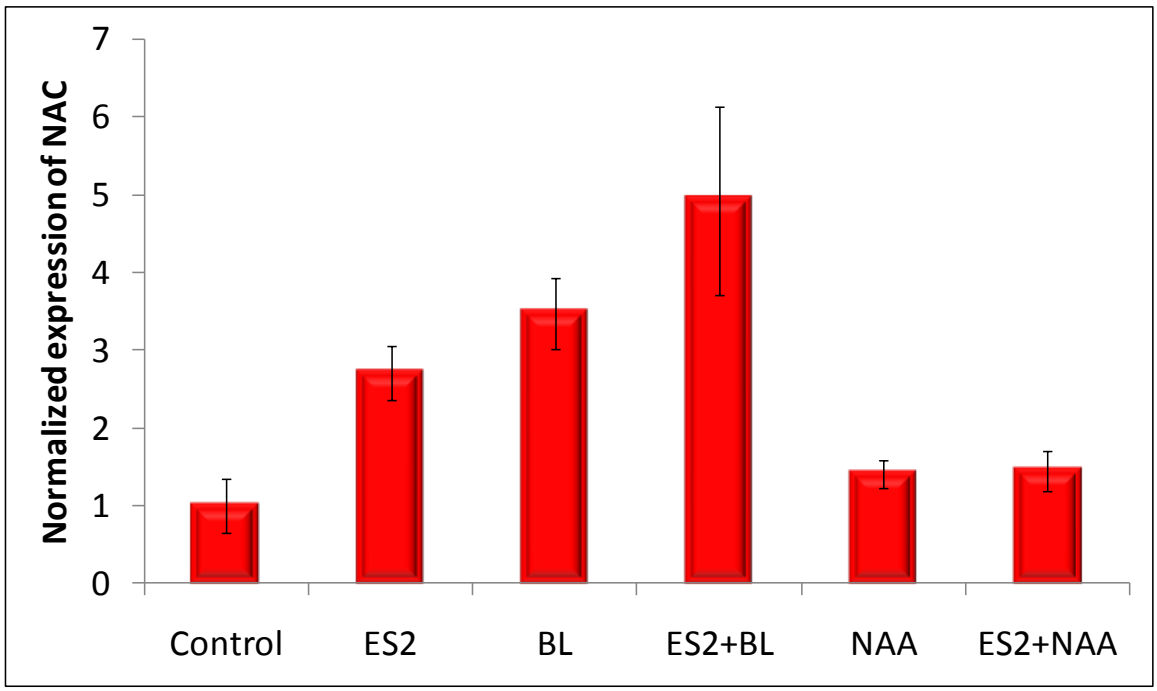


Figure 5.12 qPCR assays of the brassinosteroid specific NAC demonstrated an increase of brassinosteroid signaling in the presence of ES2 and a slightly additive effect in combination with brassinolide.

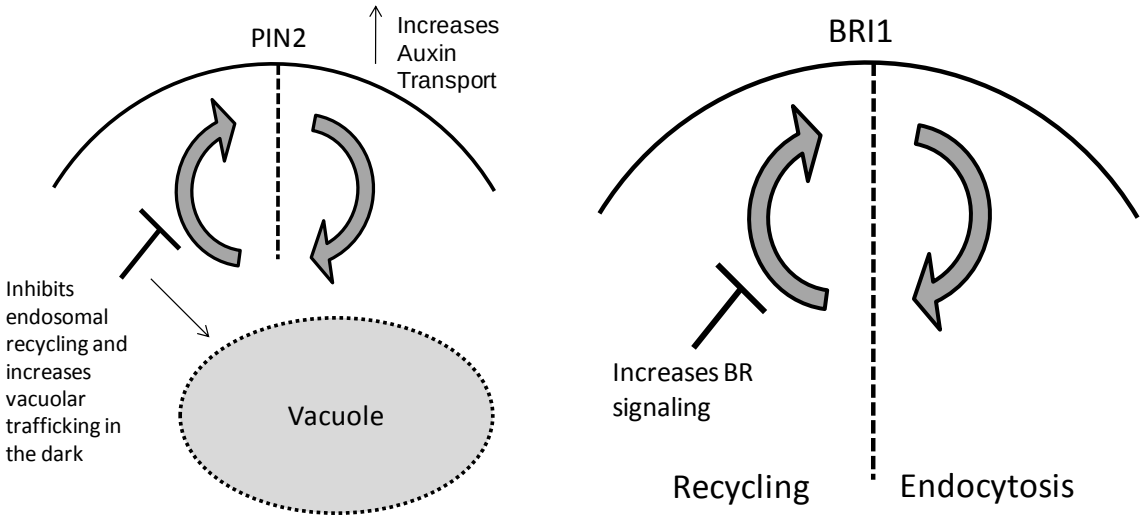


Figure 5.13 ES2 affects both the auxin and brassinosteroid pathways. ES2 increased auxin transport and increased PIN2 trafficking to the vacuole in the dark. By inhibiting endosomal recycling, ES2 increased brassinosteroid signaling of the BR specific NAC transcription factor.

5.5 References

Abramoff MD, Magelhaes PJ and Ram SJ. (2004) Image Processing with ImageJ. *Biophotonics International* **11**(7):36-42.

Abas L, Benjamins R, Malenica N, Paciorek T, Wisniewska J, Moulinier-Anzola J, Sieberer T, Friml J, and Luschnig C. (2006) Intracellular trafficking and proteolysis of the Arabidopsis auxin-efflux facilitator PIN2 are involved in root gravitropism. *Nature* **8**(3): 249-256.

Baluska F, Hauskrecht M, Barlow PW, and Sievers A. (1996) Gravitropism of the primary root ofmaize: a complex pattern of differential cellular growth in the cortex independent of the microtubular cytoskeleton. *Planta* **198**(2):310-318.

Bar-Peled M and Raikhel NV. (1997) Characterization of AtSEC12 and AtSAR1. Proteins likely involved in endoplasmic reticulum and Golgi transport. *Plant Physiol* **114**(1):315-324.

Boston RS, Fontes EB, Shank BB, and Wrobel RL. (1991) Increased expression of the maize immunoglobulin binding protein homolog b-70 in three zein regulatory mutants. *Plant Cell* **3**(5):497-505.

Cutler SR, Ehrhardt DW, Griffitts JS, and Somerville CR. (2000). Random GFP::cDNA fusions enable visualization of subcellular structures in cells of Arabidopsis at a high frequency. Proc Natl Acad Sci U S A **97**(7)**:** 3718-3723.

Dettmer J, Hong-Hermesdorf A, Stierhof YD, and Schumacher K. (2006). Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. *Plant Cell* **18:**715-730.

Feraru E and Friml J. (2008) PIN polar targeting. *Plant Physiol* **147**(4):1553-1559.

Friml J. (2003) Auxin transport – shaping the plant. *Curr Opin Plant Biol* **6**(1):7-12.

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R and Jurgens S. (2003) Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature 426(6963):147-153.

Geisler M, Kolukisaoglu HU, Bouchard R, Billion K, Berger J, Saal B, Frangne N, Koncz-Kalman Z, Koncz C, Dudler R, Blakeslee JJ, Murphy AS, Martinoia E, and Schulz B. (2003) TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. *Mol Biol Cell* **14**(10):4238-4249.

Geldner N, Denervaud-Tendon V, Hyman DL, Mayer U, Stierhof YD, and Chory J. (2009) Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. *Plant Journal* **59**(1):169-178.

Geldner N, Hyman DL, Wang X, Schumacher K, and Chory J. (2007) Endosomal signaling of plant steroid receptor kinase BRI1. *Genes and Development* **21**(13):1598-1602.

Grebe M, Xu J, Mobius W, Ueda T, Nakano A, Geuze HJ, Rook MB, and Scheres B. (2003) Arabidopsis sterol endocytosis involves actin mediated trafficking via ARA6-positive early endosomes. *Curr Biol* **13**:1378–1387.

Hobbie L and Estelle M. (1995) The axr4 auxin-resistant mutants of Arabidopsis thaliana define a gene important for root gravitropism and lateral root initiation. *Plant Journal* **7**(2):211-220.

Jaillais Y, Fobis-Loisy I, Miege C, Rollin C, and Gaude T. (2006) AtSNX1 defines an endosome for auxin-carrier trafficking in Arabidopsis. *Nature* **443**(7107): 106-109.

Kasahara M, Swartz TE, Olney MA, Onodera A, Mochizuki N, Fukuzawa H, Asamizu E, Tabata S, Kanegae H, Takano M, Christie JM, Nagatani A, Briggs WR. (2002) Photochemical properties of the flavin mononucleotide-binding domains of the phototropins from Arabidopsis, rice, and Chlamydomonas reinhardtii. *Plant Physiol* **129**(2): 762-773.

Keinath NF, Kierszniowska S, Lorek J, Bourdais G, Kessler SA, Shimosato-Asano H, Grossniklaus U, Schulze WX, Robatzek S, and Panstruga R. (2010) PAMP (pathogen-associated molecular pattern)-induced changes in plasma membrane compartmentalization reveal novel components of plant immunity. *Journal of Biological Chemistry* **285**(50):39140-39149.

Kleine-Vehn J, Leitner J, Zweiwka M, Sauer M, Abas L, Luschnig C, and Friml J. (2008) Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. *PNAS* **105**(46): 17812-17817.

Lewis DR and Muday GK (2009) Measurement of auxin transport in Arabidopsis thaliana. *Nature Protoc* **4**(4):437-451.

Miller ND, Parks BM, and Spalding EP. (2007) Computer-vision analysis of seedling responses to light and gravity. *Plant Journal* 52(2):374-381.

Mravec J, Skupa P, Bailly A, Hoyerova K, Krecek P, Bielach A, Petrasek J, Zhang J, Gaykova V, Stierhof YD, Dobrev PI, Schwarzerova K, Rolcik J, Seifertova D,

Luschnig C, Benkova E, Zazimalova E, Geisler M, and Friml J. (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* **459**(7250):1136-1140.

Nebenfuhr A, Ritzenthaler C, and Robinson DG. (2002) Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiology* **130**(3):1102-1108.

Nemhauser JL, Hong F, and Chory J. (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* **126**(3):467-475.

Pickett FB, Wilson AK, and Estelle M. (1990) The aux1 Mutations of Arabidopsis Confers Both Auxin and Ethylene Resistance. *Plant Physiology* **94**(3):1462-1466.

Pfaffl MW. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

Quint M, Barkawi LS, Fan KT, Cohen JD, and Gray WM. (2009) Arabidopsis IAR4 modulates auxin response by regulating auxin homeostasis. *Plant Physiol* **150**(2):748-758.

Rashotte AM, DeLong A, and Muday GK. (2001) Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. *Plant Cell* **13**(7):1683-1697.

Robert S, Chary S, Drakakaki G, Li S, Yang Z, Raikhel NV, and Hicks GR. (2008) Endosidin1 Defines a Compartment Involved in Endocytosis of the Brassinosteroid Receptor BRI1 and the Auxin Transporters PIN2 and AUX1. *PNAS* **105**(24): 8464-8469.

Sanderfoot AA, Kovaleva V, Bassham DC, and Raikhel NV. (2001a) Interactions between syntaxins identify at least five SNARE complexes within the Golgi/prevacuolar system of the Arabidopsis cell. *Mol Biol Cell*. **12**(12):3733-3743.

Sanderfoot AA, Pilgrim M, Adam L, and Raikhel NV. (2001b) Disruption of individual members of Arabidopsis syntaxin gene families indicates each has essential functions. *Plant Cell* **13**(3):659-666.

Surpin M, Rojas-Pierce M, Carter C, Hicks GR, Vasquez J, and Raikhel NV. (2005) The power of chemical genomcis to study the link between endomembrane system components and the gravitropic response. *PNAS* **102**(13):4902-4907.

Terasaka K, Blakelee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam SN, Lee OR, Richards EL, Murphy AS, Sato F, and Yazaki K. (2005) PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. *Plant Cell* **17**(11):2922-2939.

Van Damme D, Van Poucke K, Boutant E, Ritzenthaler C, Inze D, and Geelen D (2004). In vivo dynamics and differential microtubule-binding activities of MAP65proteins. *Plant Physiol* **136**: 3956-3967.

Chapter 6

Conclusions and Future Directions

6.1 Identification of Compounds that Inhibit Membrane Cycling in Tobacco Pollen

In an expanded screen from what was described in Robert et al. (2008), over 46,000 natural and synthetic compounds were assayed to determine those that would disrupt tobacco pollen germination/morphology. In this high-throughput screen, 378 compounds were identified with 360 of them being novel compounds as the LATCA library contained several known bioactive compounds in Arabidopsis. Seven phenotypic classes were observed among these 378 compounds including: complete germination inhibition, rough pollen grains, collapsed pollen tubes, delay of growth, swollen tubes, vesiculated tubes, and a complex phenotype containing two or more phenotypic classes. Dilution assays with these 378 demonstrated that one-third of the compounds remained bioactive at relatively low concentrations. Of these 378 compounds, those that were still commercially available were purchased to populate a sub-library named the Plasma Membrane Recycling inhibitors in Pollen (PMRP).

To specifically examine the effects of these compounds on protein trafficking, RAB2:GFP tobacco pollen was screened with these compounds. RAB2 is a member of the Ras family of GTPases is important in ER to Golgi trafficking (Cheung et al., 2002). Twenty compounds were identified as inhibiting localization or movement of the RAB2 protein. The compounds that disrupted the localization of RAB2 caused medium size protein agglomerations and the compounds that disrupted the movement of RAB2

significantly slowed and completely inhibited RAB2 cycling within the pollen tubes. Additional studies are currently being performed to quantitatively examine the disrupted movement of RAB2.

It's clear this library could be used to examine any number of trafficking pathways in tobacco pollen or Arabidopsis as with even the very specific pathway of ER to Golgi, twenty inhibitors were identified.

6.2 Identification and Clustering of Bioactive Compounds in Arabidopsis

Three highly characterized plasma membrane markers in Arabidopsis were selected to determine the translatability of the tobacco pollen inhibitors to Arabidopsis. These protein markers were the auxin efflux transporters PIN2:PIN2:GFP, PIN2:PIN1:GFP, and the brassinosteroid receptor BRI1:GFP. From these three markers, 129 compounds or over one-third of the compounds bioactive in tobacco pollen also disrupted the localization pattern in at least one of these markers. In the confirmation assays, two additional markers were added to the screen: the Qc SNARE NPSN12:YFP and a TGN-localized marker VHAa1:VHAa1:GFP. Of these 129, the re-orderable compounds were used to populate the sub-library Plasma Membrane Recycling inhibitors in Arabidopsis (PMRA).

Sixteen phenotypic classes were observed with the Arabidopsis bioactive compounds. These compounds included agglomeration phenotypes, ranging from small to large agglomerations. Large agglomerations are only observed with the known bioactive compound Brefeldin A. Medium agglomerations are similar to the

agglomerations observed with the compound Endosidin1. Additional phenotypes included inhibition of cell plate formation, increased localization to the vacuole, decreased plasma membrane localization and reversed cell polarity. The most frequently observed phenotype was the small agglomeration phenotype.

In an effort to prioritize these compounds, phenotypic fingerprinting was conducted to group compounds that caused similar phenotypes with several different markers together. Within these clusters, families of compounds with similar motifs can be defined. This type of clustering allows the researcher to focus on any specific phenotype for a grouping of compounds and a way to prioritize these compounds for future use.

6.3 Identification of Compounds that Inhibit Trafficking Within the Gravity Response Pathway

Since the endomembrane system plays an important role in several physiological responses, it was important to determine pathway in which trafficking was inhibited. From the PMRA sub-library, sixteen compounds strongly disrupted root gravity response. Eight of these compounds also disrupted PIN2, an important known protein in gravity response. From the total sixteen, any compound that inhibited cytoskeleton localization or had non-specific endomembrane effects was eliminated from the screening process. Four compounds were pursued and given Endosidin designations. These gravity Endosidins were assayed for the dynamic gravity response, taking images every three minutes for eight hours upon gravistimulation, to determine the compounds that had

inhibited versus delayed response. Endosidins 2 and 10 created a delayed gravity response while Endosidins 11 and 12 inhibited gravity response. From these four, Endosidin2 was chosen for greater characterization in this dissertation work. However, characterization work with the other gravity Endosidins continues with our collaborator Dr. Gloria Muday at Wake Forest University.

6.4 Endosidin2 Inhibits Endosomal Recycling of Specific Auxin Transporters

ES2 partially inhibited primary root growth and inhibited lateral root formation. This inhibition of lateral roots suggested that auxin transport was inhibited but auxin transport assays indicated that auxin transport was actually increased instead of decreased. This phenomenon has also been seen in the *ROOTS CURLING IN RESPONSE TO NPA (RCN1)* mutant. ES2 acted syngergistically with exogenously added nanomolar concentration of auxins. Given the phenotypic similarities between ES2 and the rcn1 mutant, *in vitro* phosphorylation assays are planned to determine if ES2 is inhibiting the phosphorylation pathway as rcn1 does.

Endosidin2 disrupted the localization patterns of several auxin transporters (PINs and AUX1 but not PGPs) and the brassinosteroid receptor BRI1. The only internal marker compartment affected was the ER-localized PIN5. Examination of other markers localization to the ER and Golgi confirmed that only PIN5 was affected by ES2 and not the ER or Golgi in general. Using a PIN5-myc/PIN2:GFP double marker, PIN5 and PIN2 can co-localize into ES2 bodies. Electron micrographs of ES2-treated PIN5:GFP

seedlings revealed that ES2 altered the expression pattern of PIN5 but it remains unclear if ES2 bodies are part of a specific region of the ER or whether they are a separate body. Experiments are continuing to examine the relationship between PIN2 and PIN5 upon ES2 treatment and to determine the nature of the ES2 body.

ES2 was found to inhibit endosomal recycling/secretion without affecting endocytosis. This is of interest because the only compound currently known to inhibit endosomal recycling is Brefeldin A, which also disrupted TGN/Golgi morphology. More directed comparison experiments of ES2 to BFA are currently being performed. It will be of great interest to determine if BFA-resistant GNOM mutants are also resistant to ES2 which could indicate a similar protein target. However, if the GNOM mutants are sensitive to ES2 this could indicate either a separate recycling pathway or a later step within the recycling/secretory pathway is being inhibited. ES2 stands to be a potentially significant compound, providing a way to more specifically examine the recycling/secretory pathway as opposed to the current use of BFA which also disrupts internal compartments.

Target identification screens with both homozygous T-DNA lines and EMS-mutagenized PIN2:GFP lines have already begun. Utilizing the primary root inhibition experienced by ES2-treated seedlings, ES2-resistant mutants will have longer primary roots. The target of ES2 will provide greater understanding of how the pathways of auxin transporters and the brassinosteroid receptor are inhibited with ES2 treatment, as well as where in the endomembrane system this inhibition is occurring.

6.6 References

Cheung A, Chen CY, Glaven RH, de Graaf BH, Vidali L, Hepler PK, and Wu HM (2002) Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. *Plant Cell* **14**(4):945-962.

Robert S, Chary S, Drakakaki G, Li S, Yang Z, Raikhel NV, and Hicks GR. (2008) Endosidin1 Defines a Compartment Involved in Endocytosis of the Brassinosteroid Receptor BRI1 and the Auxin Transporters PIN2 and AUX1. *PNAS* **105**(24): 8464-8469.