EXOGENOUS STEROLS ARE TAKEN UP BY A NOVEL PATHWAY BY THE ELONGATING CELLS OF ROOTS

A DISSERTATION

by

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ABSTRACT

A major plants vital mechanism relying on sterol, however how it transport from its site of synthesis to site of demand is not well known. So far, endocytic pathway has been studied in plants, which contributes to the transport of sterols. In this study by using fluorescent sterol analogues, we analyze the short- and long-term uptake of sterols into plant cells. The sterols traffic initially to the nuclear envelope and then, over the longer-term, to oil bodies and other membranes. The fluorescent sterols do not initially label endosomes. Inhibiting endocytosis with dominant negative AUX1 overexpression does not inhibit uptake of sterols. We propose a primarily non-endocytic pathway for uptake of sterols in plants that differs somewhat in different cell types. The other part of this study has revealed the role of sterols in the plant growth and development. To understand the importance of sterol for the growth, we applied exogenous sterol mixed with MβCD, which act as a carrier for sterol. Sterols at a higher concentration has shown a significant reduction in the growth of plant, however, it does not affect the growth of already growing plants, which suggest us the use of sterol as potential pre-emergent herbicide. The growth defected plants at a higher concentration of sterol had a phenocopy similar to many of the mutants such as; hyd1, smt1, smt2 and hyd2/fackel of sterol biosynthetic pathway. Our collected data may suggest that supplied sterols act by negative feedback inhibition on sterol biosynthesis and uptake but only when applied prior to and during germination. Further to test the pre-emerget quality of sterol we grew different seeds like; corn, pea, weed seeds and Arabidospis not only in basic agar media but also in hydroponic system, which we have developed in our lab. Initial experiments show that crop plants and weeds are inhibited in germination and growth when the sterol is included during imbibition.

DEDICATION

I would like to dedicate my Phd study to of course almighty god, my entire family and especially my brothers and my wonderful wife who have been always my source of support, strength and motivation when I thought of giving up.

I also dedicate my study to my advisor, Dr. Lawrence Griffing and my committee members who always shared their words of advice, guide and encouragement to finish my study.

I would also like to dedicate this thesis to my close friends who always supported and wished well for me and helped me in my all situations.

And lastly, I would like to dedicate this thesis to my newly born son, Parth Sharma who inspired me to work hard and finish this study.

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Contributors

This work was supervised by a thesis committee consisting of Dr. Lawrence Griffing (Advisor) of the Deaprtment of Biology and also advised by committee members, Dr. Kendal Hirschi (VFIC, TAMU), Dr. Kathy Ryan (Dapartment of Biology) and Dr. Libo Shan (Department of Plant Pathology).

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Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the Biology department.

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CHAPTER I

INTRODUCTION AND OVERVIEW OF RESULTS

Sterols are one of the important components of eukaryotic cell membranes and play an essential role in many biological processes in plants. Besides a basic function in the physical composition of membranes, where they influence membrane permeability and fluidity (Hartmann, 1998), sterols assemble, along with other proteins and lipids, into membrane nanodomains that influence receptor complex function, transporter efficiency, channel regulation, protein trafficking, and plant– bacterial interactions (Tapken and Murphy, 2015). The most common plant sterols, i.e., b-sitosterol, stigmasterol, campesterol, and cholesterol, profoundly influence embryonic development, root development, and vascular development (Andrade et al., 2017; Carland et al., 2010; Clouse, 2002; Fischer et al., 2004; He et al., 2003; Lindsey et al., 2003; Schaller, 2003; Schrick et al., 2002; Short et al., 2018). This is separate from the effect(s) of the hormonal end-products of the sterol biosynthetic pathway, the brassinosteroids (BR) (Clouse, 2002; He et al., 2003; Schaller, 2003).

BRs act as hormone signals that, when absent (or their receptor is defective), produce extreme dwarfism and interfere with etiolation, producing phenotypes in the dark that show constitutive photomorphogenesis (Planas-Riverola et al., 2019). This dissertation is divided into three separate, but related, hypotheses, each one being the subject of the following chapters: 1) Exogenous sterols are taken up by a novel pathway by the elongating cells of roots, Chapter 2. 2) Addition of exogenous sterols negatively feeds back on sterol biosynthesis with consequent effects on development, Chapter 3. 3) Exogenous sterols are an effective pre-emergent herbicide with a novel mode of action,

Chapter 4. This introduction serves as a background to the development of these hypotheses.

Sterol biosynthesis and diversity in *Arabidopsis thaliana*

The basic structure of sterols, Figure 1, is a four ring system (the 1,2-cyclopentanoperhydrophenanthrene ring skeleton), with the rings denoted A-D. The 10-linked alkyl side chain on carbon 17 of the D ring is the source of the difference in the major higher plant sterols, Figure 2. Cholesterol (4% of total free sterols in Arabidopsis) has a saturated 8-carbon alkyl side chain, while the others have 10-carbon alkyl side chains. β-sitosterol (64% of free sterol in Arabidopsis) has no double bonds in its alkyl chain, while campesterol (11% of total free sterols in Arabidopsis) and stigmasterol (8% total free sterols in Arabidopsis) have double bonds between carbon 24 and 25 and between carbon 22 and 23, respectively (Benveniste, 2004; Holmberg et al., 2002; Schaeffer et al., 2001). The mix of sterols differs with different plant species and the stage of development. On average for a particular plant species, the amount of cellular sterol remains constant and constitutes 2-3 mg per gram dry weight (Holmberg et al., 2002; Schaeffer et al., 2001).

However, the biosynthetic pathway of sterols is complicated, Figure 3, and the usage of different enzymes for different stages in the biosynthesis of sterols varies between plant families and between plants, animals, and fungi. For example, the biosynthesis of cholesterol is different in plants and animals, Figure 3. Orthologous matching of the genes encoding key enzymatic steps reveals the differences between plant families, Figure 4. Exactly which genes are responsible for which steps in sterol biosynthesis in

Arabidopsis is not completely clear, but the gene network involved in cholesterol, β-sitosterol and stigmasterol biosynthesis in solanaceous plants has been recently worked out (Sonawane et al., 2016) and is the basis for the pathway shown in Figure 3.

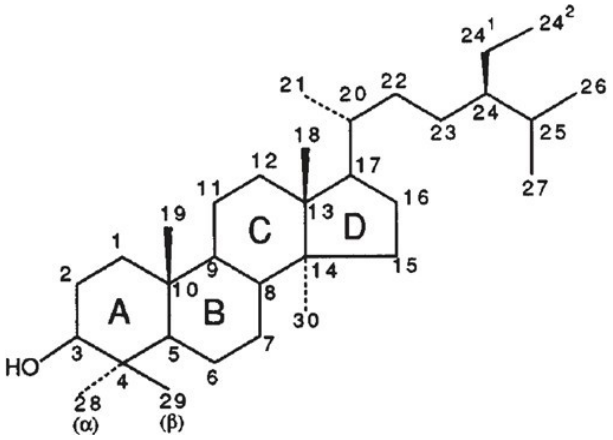

Figure1.Basicstructureofasterolwithstandardcarbonnumberingaccordingtothe1989 IUPAC-IUBrecommendations4. The carbonsC241 and C242 come from S-adenosyl-L-methi- onineand were previouslydesignatedasC28andC29.Thestereochemistryofthealkyl group is alpha.

According to the former nomenclature system, the two methyl groups located at C24 and the methyl group at C14 were numbered C30, C31 and C32, respective- ly. (**Hartmann et al; 2004**)

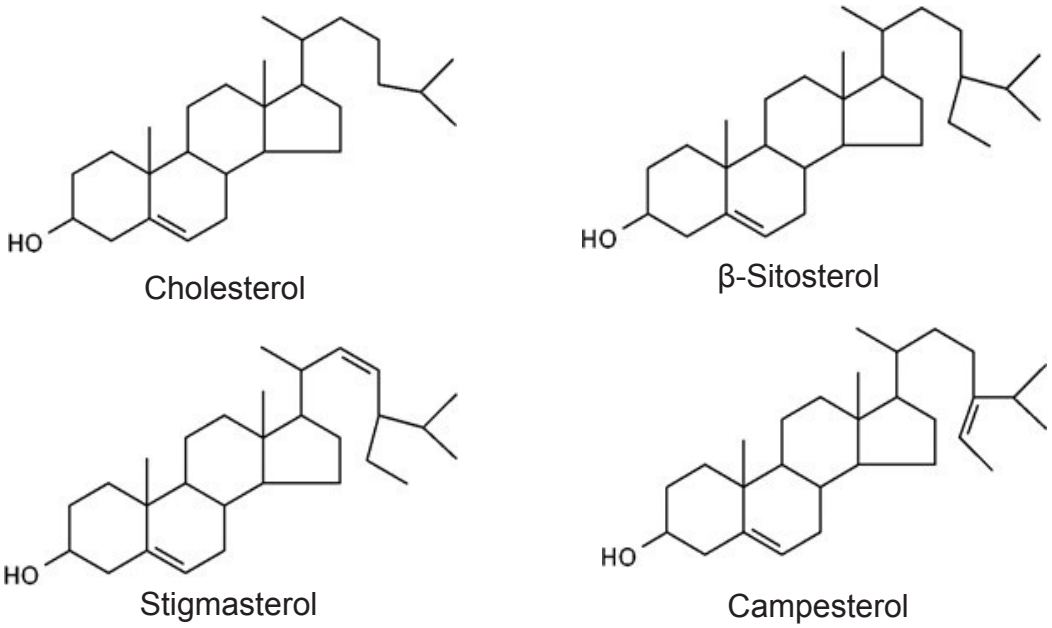

Figure 2. Chemical structures of the main phytosterols

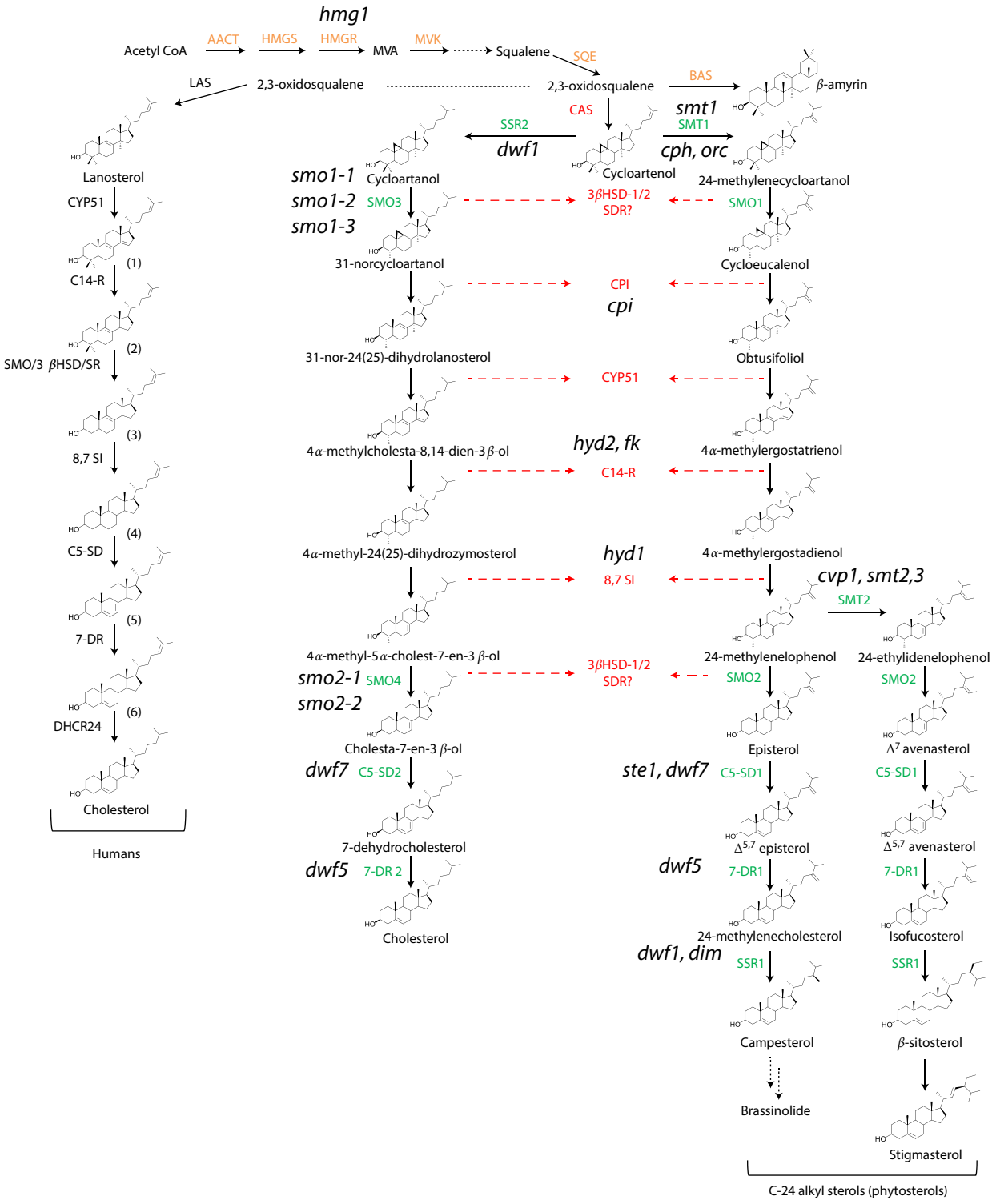
hmg1
Acetyl CoA
AACT
HMGS
HMGR
MVA
MVK
Squalene
SQE
2,3-oxidosqualene
LAS
BAS
β-amyrin
CAS
smt1
SSR2
SMT1
dwf1
cph, orc
Lanosterol
Cycloartenol
24-methylenecycloartanol
smo1-1
smo1-2
smo1-3
SMO3
3βHSD-1/2
SDR?
SMO1
CYP51
(1)
C14-R
31-norcycloartanol
Cycloeucalenol
CPI
cpi
(2)
SMO/3 βHSD/SR
31-nor-24(25)-dihydrolanosterol
Obtusifoliol
CYP51
(3)
8,7 SI
4α-methylcholesta-8,14-dien-3β-ol
4α-methylergostatrienol
hyd2, fk
C14-R
(4)
C5-SD
4α-methyl-24(25)-dihydrozymosterol
4α-methylergostadienol
hyd1
8,7 SI
cvp1, smt2,3
(5)
7-DR
SMT2
4α-methyl-5α-cholest-7-en-3 β-ol
24-methylenelophenol
24-ethylidenelophenol
smo2-1
smo2-2
SMO4
3βHSD-1/2
SDR?
SMO2
SMO2
(6)
DHCR24
Cholesta-7-en-3 β-ol
Episterol
Δ7 avenasterol
dwf7
C5-SD2
ste1, dwf7
C5-SD1
C5-SD1
Cholesterol
7-dehydrocholesterol
Δ5,7 episterol
Δ5,7 avenasterol
dwf5
7-DR 2
dwf5
7-DR1
7-DR1
24-methylenecholesterol
Isofucosterol
dwf1, dim
SSR1
SSR1
Campesterol
β-sitosterol
Brassinolide
Stigmasterol
Humans
C-24 alkyl sterols (phytosterols)

Figure 3. The biosynthetic pathway of the major sterols in plants and its comparison to the cholesterol biosynthetic pathway in huma Enzymes taking part in both the plant cholesterol and the C-24 alkylphytosterols biosynthetic pathways are depicted in red whereas enzym specific for one of these pathways are marked in green. MVA, the triterpenoid precursor and other triterpenoid pathway enzymes are presented orange.Enzymes catalysing human cholesterognesis are shown in black and bold. Arabidopsis mutants are shown in lower case black.

MV; mevalonicacid; AACT, acetylCoAacetyltransferase; HMGS, 3-hydroxy-3-methylglutaryl-CoAsynthase; HMGR, 3-hydroxy-3-methylglutarCoAreductase; MVK, mevalonatekinase; SQE, squaleneepoxidase; BAS, -amyrinsynthase; CAS, cycloartenolsynthase; LAS, lanosterolsyntha SMT, sterolC-24methyltransferase; SMO, C-4sterolmethyloxidase; SDR, sidechainoxidoreductase; CPI, cyclopropylsterolisomeraCYP51, sterolC-14demethylase; C14-R, sterolC-14reductase; 8,7SI, sterol 8,7 isomerase; C5-SD2, sterolC-5(6)desaturase2; 7-DR2 dehydrocholesterolreductase2; C5-SD1, sterolC-5(6)desaturase1(DWARF7); 7-DR1,7-dehydrocholesterolreductase1(DWARFSSR2, sterolsidechainreductase2; DHCR24, C24-sterolreductase. In human cholesterogenesis, the intermediates are:(1)4,4-dimethylcholes 8,14(15), 24-trien-3-ol; (2)4,4-dimethylcholesta-8, 24-dien- 3-ol; (3)cholesta-8,24-dien-3_-ol; (4)cholesta-7,24-dien-3_-ol,(5 dehydrodesmosterol; (6) desmosterol. (**From Sonwane et al., 2016**).

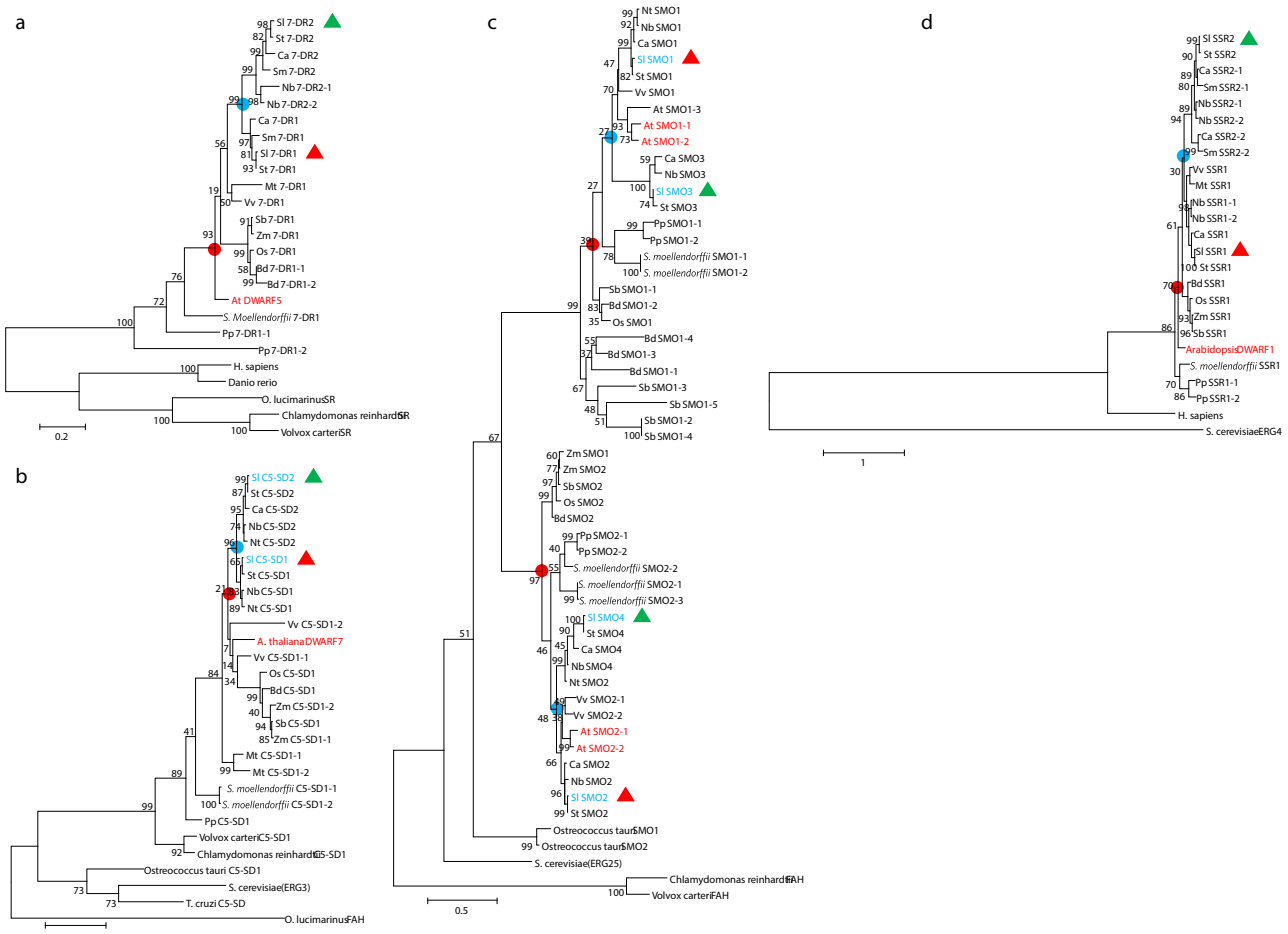

Figure 4. Phylogenetic analysis of enzymes involved in sterol metabolism in plants. a) 7-dehydrocholesterol reductases b) Tomoto genes involved in cholesterol and other phytosterol biosynthesis are marked with green and red triangles, respectively. Blue circles show a phylogenetic duplication fork, while red circles show clade separtation of cholesterol from other phytosterol enzymes. The red font indicates Arabidopsis genes.

Note that the Arabidopsis genes show clade separation into both cholesterol and other phytosterol biosynthetic genes of tomato, indicating that one gene may be involved in both pathways, particularly with the DWARF genes. (**From Sonawane et al. 2016**)

Hypothesis 1: Exogenous sterols are taken up by a novel pathway by the elongating cells of roots (Chapter 2).

The nature of sterol transport in the plants is unknown. Different organs have different amounts of sterols and different sterol compositions. Table 1 shows that the shoot apices of both monocots and dicots have an order of magnitude higher cholesterol level than stem or leaf tissue. This corresponds to the finding that squalene synthase, one of the early key enzymes in sterol biosynthesis, Figure 3, is primarily expressed and active in the shoot apical meristem (Devarenne et al., 2002). This tissue-specific expression of a key enzyme in sterol biosynthesis gives rise to the hypothesis that some sterols, in particular cholesterol, are made in the shoot apex and are translocated via the phloem to the roots. For this to occur, we hypothesize that transport proteins that bind the sterols can be found in the source tissues and move them via the phloem vasculature through the organism. This movement would be similar to sterol transport in animals, where, for example, there are sterol-binding proteins in the vasculature (low density and high density lipoproteins) that move cholesterol through the organism.

Table 1. Cholesterol levels (ng/mg dry weight) in tissues of *Hordium vulgare* (monocot), *Lolium temulentum* (monocot), and *Xanthium strumarium* (dicot) during flowering from (Garg et al., 1987).

Day	Meristem	H. vulgare			L. temulentum			X. strumerium	
		Leaf	Stem	SAM	Leaf	Stem	SAM	Stem	SAM
1	Vegetative	48	452	9860	54	185	5680	29	310
2	Transitional	38	267	8330	33	123	5150	22	224
3	Floral	32	257	5580	30	93	3240	17	232
4	Floral	22	131	3260	21	44	2950	18	225
5	Floral	25	123	3750	17	31	1820	12	213
6	Floral	29	83	1180	16	22	1500	10	178
LSD P=0.05		4	17	33	2	7	33	2	16

Although this mode of transport of sterols has not yet been demonstrated, there is a sterol-binding protein, At1g23130, in the phloem of Arabidopsis, an ortholog of Bet_v_1, the major birch pollen allergen with an archetypal sterol-binding domain (Barbaglia et al., 2016). The movement of cholesterol through the phloem has been established (Behmer et al., 2013). Ecdysis of phytophagous, phloem-feeding insects such as aphids depends on this source of cholesterol because these insects generally do not have the enzyme pathway to make sterols, but require cholesterol as a precursor for the molting hormone, ecdysone (Behmer et al., 2013). We also hypothesize that in both the source and sink tissues, movement of cholesterol into or out of the phloem would be via another set of proteins with sterol-binding domains, the StART-like proteins (steroidogenic acute regulatory protein-related lipid transfer-like proteins), which will now be introduced.

StART-like proteins were recently described in yeast (Gatta et al., 2015). They are similar to the StART protein family in mammals. Members of that family, StARD2, StARD7, and StARD10, can bind a PC molecule in a hydrophobic pocket, mediating the inter-membrane transfer of PC in vitro (Olayioye et al., 2005). Budding yeast do not have StART proteins; however, Gatta et al. (2015) found that Ysp2p, one of six StART-like proteins in yeast, has two StART-like domains both of which bind sterol. Along with three other yeast StART-like proteins Ysp1p Sip3p and Lam4p, these proteins are located membrane contact sites (MCSs) between the endoplasmic reticulum (ER) and the plasma membrane (PM), which are known to be sites of phospholipid and sterol exchange in yeast (Prinz, 2010). MCSs are not a necessary condition for sterol import . Their function requires anchoring of the protein to the ER (via two trans-membrane domains) and to the PM (via lipid-binding plekstrin homology, PH, domains) in such a way that their StART-like domains on the ER- resident protein can reach out across the MCSs. They then either directly bind the sterol or transfer it to other cytosolic sterol-binding proteins (Dittman and Menon, 2017). The loss of Ysp2p, Ysp1p or Sip3p reduces the rate of transfer of sterol out of the PM in yeast in cells without oxysterol binding proteins (Gatta et al., 2015). StART-like proteins may also function outside of an MCS because when most, if not all, of the MCS tethers are deleted from yeast, sterol transport still occurs (Quon et al., 2018).

Our current, but untested, hypothesis is that the StART-like domain-containing orthologs in plants also transfer sterols from the PM to the inside of the cell. There are three, At3g59660 (BAGP-1), At5g50170 (late embryogenesis StART-like protein, LES), and At1G03370 (pollen, leaf, and root epidermal Start-like protein, PLRS). The localization of their expression (Waese et al., 2017) is consistent with their hypothesized function. LES is expressed during late embryo development and in the proto- and metaphloem of the root. It is both PM-localized (via PH domains) and ER-localized (double transmembrane domain, probably similar to those found in other MCS proteins such as reticulons). PLRS is PM- and ER-localized (PM via a PH domain and ER via a double transmembrane domain, with a cryptic nuclear localization domain within the PH domain) and found in potential sterol source tissues, e.g., leaves, and potential sink tissues, e.g., pollen and the root epidermis. BAGP-1 is in the secretory pathway, with a single transmembrane domain, ending up on the PM or in the cell wall (after proteolytic cleavage). It is probably not localized to MCSs. It is most highly expressed in mature root epidermis and in senescent leaves and is a regulator of cell death and defense responses (Li et al., 2016; Lorrain et al., 2004). Future work will determine if these proteins act in this way to deliver sterols from source to sink tissues in plants. However, if StART-like proteins, or other sterol transporters on or off membrane contact sites are involved in internalization of sterols transported through the tissue, then the internalization of sterol, or retrograde transport from the PM to inside the cell, should occur by non-endocytic means. This is the hypothesis we test here.

There are three basic mechanisms put forward for lipid and sterol transport in plant cells, Figure 5 (Li-Beisson et al., 2013), vesicular traffic, traffic at MCSs, and cytoplasmic exchange of lipids. Sterols and phospholipids are transported by default through endomembrane pathway via vesicles. Anterograde vesicular transport from inside the cell where phospholipids and sterols are made in the ER to the plasma membrane is responsible, at least in part, for transport of newly-synthesized lipid (Blom et al., 2011; Lung et al., 2017; Moreau et al., 1998; Silvestro et al., 2013). However, anterograde transport of sterols to the vacuole from the ER may by-pass the Golgi (Viotti et al., 2013). Retrograde vesicular transport from the PM to inside the cell has been suggested to be mediated by endocytosis (Boutte and Grebe, 2009; Stanislas et al., 2014). To be sure, endosomes contain sterols and the process of endocytosis can be altered by altering sterols (Men et al., 2008; Pan et al., 2009). However, in animals and fungi, this pathway is not the mechanism for retrograde transport of sterols from the PM (Lahiri et al., 2015; Maxfield and Menon, 2006; Wustner et al., 2011) and sterol content of endosomes may be modulated by contact between the endosomes and ER (van der Kant and Neefjes, 2014). Nonetheless the retrograde vesicular transport route of sterol transport is the one currently held by most plant cell biologists. This idea of sterol transport is based on the notion that retrograde transport is simply the consequence of membrane recycling and that retrograde transport only serves that role in the plant. As seen above, there is evidence that sterols are transported around the plant and both anterograde and retrograde transport specialized to that transport, over and above that of simple membrane recycling, are quite likely.

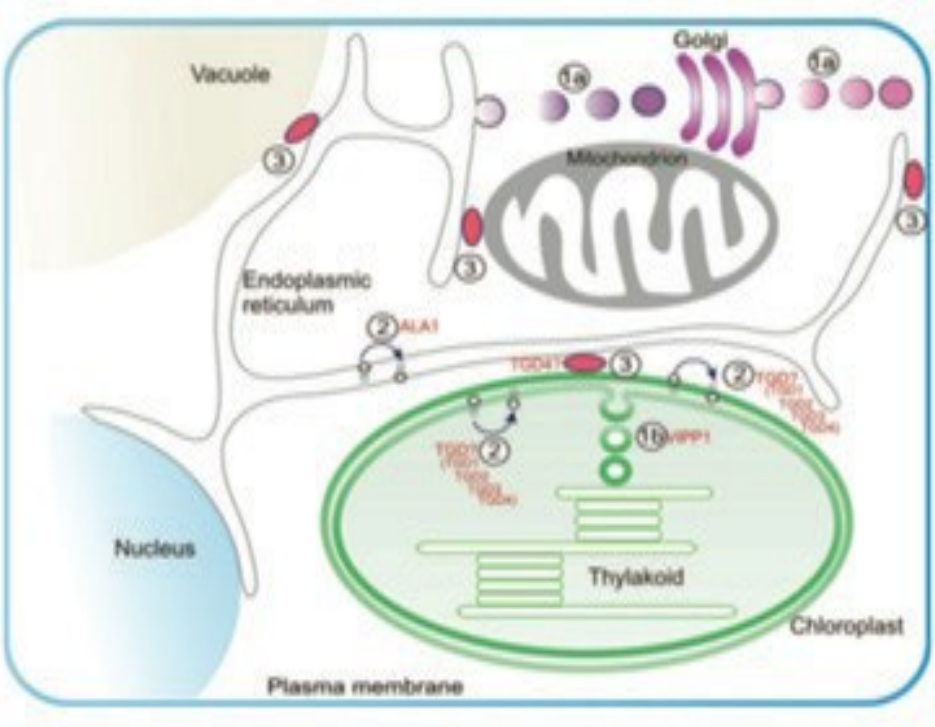

Figure 5. Summary of Intracellular Lipid Transport Processes in Arabidopsis. Process 1a. A vesicular mecha- nism is proposed for trafficking of membrane proteins and certain lipids between cellular organelles in the secretory pathways. Process 1b. The vesicular transfer of lipids from the inner chloroplast envelope to the thylakoids. Process 2. Polar lipid flipping across the ER and outer and inner chloroplast envelope mem- branes. Process 3. Lipid transfer through membrane contact sites between ER and plastids, mitochondria, plasma membrane, or vacuoles. Abbreviations: ALA1, aminophospholipid ATPase; TGD1, permease-like protein of inner chloroplast envelope; TGD2, phosphatidic acid-binding protein; TGD3, ATPase; TGD4, protein involved in lipid transport; VIPP1, vesicle-inducing protein in plastids (**From Li-Beisson et al., 2013**)

MCSs have been implicated in retrograde sterol transport in yeast (see above). It has been known for many years that the plant ER forms MCS with several organelles including the PM (Staehelin, 1997). Image analysis of persistency maps revealed the presence of non-plasmodesmatal ER-PM MCSs in plants expressing fluorescent ER- localized fusion proteins (Sparkes et al., 2009). Since then, several ER-specific proteins that interact with the PM have been identified as components of different subsets of MCSs. Orthologs of VAPs (VAMP-Associated Proteins) known to be at the ER-PM contact sites in animals (Quon et al., 2018; Rocha et al., 2009) are found at ER-PM contact sites in plants (Wang et al., 2014). VAP27, Vamp-Associated Protein 27, is an interacting partner of the ER-localized RTNLB3 (reticulon-like protein B3) and RTNLB6 (Kriechbaumer et al., 2015). VAP27 can bind microtubules, RTNLBs, and NETWORKED3C (NET3C) thereby linking the actin cytoskeleton to the ER contacts. Also, orthologs of the E-SYTs (extended synaptotagmins) found at ER-PM contact sites in yeast and animal cells (Schauder et al., 2014), have been found at the ER-PM MCSs in plasmodesmata (Salmon and Bayer, 2012), a specialized subset of ER-PM MCSs. The related protein, SYT1 (also SYTA, synaptotagmin 1), is found at plasmodesmata in plant cells as well as in non-plasmodesmatal MCSs (Kriechbaumer et al., 2015; Lewis and Lazarowitz, 2010; Perez-Sancho et al., 2015; Schapire et al., 2008; Uchiyama et al., 2014; Yamazaki et al., 2010). Sterols themselves may be a regulator of the MCSs, since in yeast, the depletion of sterol from the cells causes a large increase in the level of E-Syt and in the number of MCSs (Quon et al., 2018). These workers also found that the level of PI4P (phosphatidyl inositol – 4 phosphate), the cytoplasmic-side phospholipid in the plasma membrane to which the PH domains of MCS proteins bind, was key to maintaining sterol transport,

and, indeed, viability in cells lacking several MCS-localized proteins. The StART-like protein candidates for sterol transport have such PH domains.

The third mechanism of lipid traffic in cells, the cytoplasmic transfer of lipid by carrier or transfer proteins, is not a mutually exclusive pathway with either the vesicular traffic pathway or the MCS pathway. Indeed, phosphoinositides use such carrier proteins in plants (Vincent et al., 2005). Also there is evidence for movement of sterols in animal cells using this pathway and cytoplasmically-localized STAR- domain containing proteins (Dittman and Menon, 2017). The extent to which these participate in movement of sterols in plants is unknown, but it should be kept in mind as we examine the retrograde pathway for internalization of sterols, that some of the sterols may be sequestered by cytosolic proteins.

We hypothesize that sterols follow pathways other than endocytic for intracellular transport. In our study, we have defined the uptake of sterol in Arabidopsis root and hypocotyl cells and further characterized its transport pathway by using the fluorescent sterol probes, Bch (Bodipy Cholesterol) and DHE (Dehydroergesterol) as tracers, delivered in the presence of the sterol-carrying agent, methyl-β-cyclodextrin (MβCD), using modifications of protocols used in sterol transport studies in animals (Wustner et al., 2011). These tracers are superior to filipin, which is the only established tool for sterol visualization in plants. Filipin binds to free sterols, i.e., sitosterol, stigmatosterol, campesterol and cholesterol (Boutté et al., 2011). Filipin can be used in live cell imaging or on fixed cell samples, but there is a major issue in the internalization of filipin-sterol complexes in live cell samples: it inhibits endocytosis of styryl dye such as FM4-64, which is endocytic tracer (Boutté et al., 2011).

As described in Chapter 2, we found that the immediate target of sterol internalization was the PM and the nuclear envelope (NE), a subdomain of the ER. Interestingly, there is no accumulation of sterols in other subdomains of the ER, a result which may be expected because even though it is the primary site of sterol biosynthesis within the cell there is proportionally less sterol in the ER than in other endomembrane compartments. After long-term treatment, the BCh is found in lipid bodies, probably as acyl esters.

In the course of these experiments we found that the sterol tracers could only be delivered effectively to the plants by the use of MβCD. Since this approach had not been used previously in plants for delivery of sterols to plants (although MβCD had been used to perturb plants with sterol extraction), we were curious about whether or not we could chemically complement some of the mutations in the sterol biosynthetic pathway with added end-products of those pathways delivered to the plants by MβCD,. As described below, this approach was successful and produced the initially surprising result that addition of exogenous sterols cause a decrease in the total sterols in plants.

Hypothesis 2: Addition of exogenous sterols negatively feeds back on sterol biosynthesis with consequent effects on development (Chapter 3).

Mutations in the genes of Arabidopsis of sterol biosynthesis, shown in Figure 3, produces a change in endogenous sterol profiles, Table 2. In most cases, the relative abundance of β-sitosterol goes down, while the relative abundance of other sterols, such as stigmasterol and cholesterol goes up.

The change in sterol profiles is accompanied by severe dwarfing and reduced germination. This has led to the speculation that some of the elevated sterols, such as stigmasterol, could have growth-controlling effects on their own (Aboobucker and Suza, 2019).

Table 2. Endogenous sterols analysis, % of total free sterols of wild type plants and sterol biosynthetic mutant plants (Carland et al., 2010; Clouse, 2002; Nakamoto et al., 2015; Souter et al., 2002; Suzuki et al., 2004; Willemsen et al., 2003). The enzymatic steps that are changed in each mutant is shown in Figure 3. NR = not reported.

	wt	*smt1*	*fk*/*hyd2*	*cvp1*/*smt2*	*dwf1*/*dim1*	*hyd1*	*hmg1*	*cpi*
β-Sitosterol	75	25	2	45	30	4	33	1
Campesterol	18	22	Trace	NR	1.8	12	12	NR
Stigmasterol	2.2	Trace	322	2	1.2	182	1.5	NR
Cholesterol	2.5	15	NR	12.5	1.5	NR	2	NR
Brassinosteroids	1.8	NR	2.5	4.8	NR	NR	NR	1.8

Many of the embryo-lethal mutants of *Arabdopsis thaliana*, such as *hyd1*, *fackel* or *hydra2, smt1* and *smt2*, cannot be chemically complemented by brassinosteroids alone (Clouse, 2002; Diener et al., 2000), nor could they be complemented by the addition of other end products of the pathway. The failure of the sterol biochemical complementation could be due to the lack of their bioavailability after exogenous

application, which can be related to the lipophilic nature of end product sterols (brassinolides are water soluble). In our experiments on sterol uptake, we found that we could make the sterols bioavailable with MβCD (see Chapter 2). When MβCD is used as a carrying agent and mutant seeds are incubated in several different sterols, the tested mutants were chemically complemented and showed normal growth (see Chapter 3). A surprising result from this work was that as the external sterol concentration increased, we saw a return of the mutant phenotype in mutant lines. We also found that at these higher concentrations the wild type plants also showed these developmental defects. We assayed several of the mutant phenotype changes including vascular development changes, changes in root hair length and abundance, changes in PIN2 localization, changes in root growth, and changes in chloroplast development. All of the developmental changes are phenocopied at high (100 μM) sterol. Our hypothesis was that the addition of super-abundant sterols causes feedback inhibition in the sterol biosynthetic pathway. To test this hypothesis, we assayed sterol levels in planta at these high concentrations of external sterol. The result is consistent with the hypothesis of negative feedback regulation, Figure 5; in planta sterol levels diminish and the proportions of different sterols change with external sterol addition. In addition to genetic approaches, pharmacological approaches have also been used to inhibit the biosynthesis of sterols by inhibition of specific enzymes (Benveniste, 1986; Mercer, 1993). The compounds such as N-alkylmorpholines and azole derivatives interfere with the sterol biosynthesis by inhibiting cycloeucalenol- obtusifoliol isomerase (CPI) and the obtusifoliol 14-demethylase (CYP51), Figure 3, respectively. These compounds cause similar developmental defects to those seen in plants with mutations in these enzymes

(Benveniste, 2004). We used sterol supplementation to determine if an external sterol concentration (100μM) that causes the same developmental effects as mutants in the endomembrane pathway operated on the same or different pathways as the chemical inhibitors. The expected outcome of these experiments was that if external sterol operated on the same pathway, there would be no change in the level of developmental change caused by the drugs, whereas if the treatment inhibited a different pathway, the developmental change would be aggravated. In general, the results were consistent with an interpretation that external sterols caused feedback inhibition along the same pathway as the drugs. One developmental effect that we found upon applying exogenous sterols was the inhibition of germination, an effect phenocopying the embryo-lethal mutants in the sterol biosynthetic pathway. This effect showed promise as a pre-emergent herbicide, so we pursued the third central hypothesis, as described below.

Hypothesis 3: Exogenous sterols are an effective pre-emergent herbicide with a novel mode of action (Chapter 4).

Our initial experiments on the effects of addition of exogenous sterols on seed germination revealed that it might be used as a pre-emergent herbicide. However, if such an effect were to be useful and thereby patentable, we had to design a system where we could translate the experiments done on Arabidopsis to crop plants. To control the precise amount of sterol in the media, we needed a system, which did not change the sterol concentrations, but could occur outside of the petri dish. Soils, in particular aluminum silicates, can absorb sterols (Atanassova and Doerr, 2010), so we pursued approaches in hydroponics that could be adapted to crop plants.

To this end, we wrote a review article on translating hydroponic systems used for Arabidopsis to crop plants (Kumar and Griffing, 2019). This chapter is basically the outcome of setting up a translational system for drug, or herbicide, discovery. It could also be used in the future for gene discovery through rapid cycling (shorter life cycle) approaches covered in the review.

Many of the growth conditions under which the model organism, *Arabidopsis thaliana,* thrives may not translate to crop plants. Crop plants may differ from Arabidopsis in their specific uptake and translocation properties, metabolism, specific life cycle, and environmental requirements for growth (Kraehmer et al., 2014). During the optimization of our hydroponic system several goals have been set: a) to achieve homogeneous growth across the plant spectrum, b) to easily control and monitor nutritional components, c) to acquire data in situ or harvest plants easily for measurement and analysis, and d) to minimize the cost of system set-up. A simple, easy and cheap hydroponic system is first demonstrated for Arabidopsis growth from a young seedling to mature plant. Our hydroponic adaptation was based on low cost, efficient systems, which employ reusable and sterilizable plastic materials (Arteca et al., 2000, Tocquin et al., 2003., Alatorre-Cobos et al., 2014). These systems were shown to be effective for the growth of other model plants such as the dicots, Nicotiana tabacum, and Solanum lycopersicum, and the monocot, Setaria viridens. The methods do not require transplanting seedlings grown on agar or different media as is done with traditional hydroponic systems (Nguyen et al., 2016). Furthermore, they do not require constant pumping of nutrients through the system as is commonly used in commercial hydroponic systems, such as the continuous flow systems, aeroponics, or the nutrient film technique

(Kumar and Griffing, 2019).

Non-pumped or still systems such as these are also superior for maintaining sterility or controlled microflora. Single-tube hydroponics, which is a slightly modified version of the above, enables efficient usage of space and facilities in labs for small-scale crop seed production. Small amounts of seeds from a variety of crops can be harvested from plants growing in single-tube hydroponics, but in the initial studies, the crops studies were limited to those under 60 cm, due to growth chamber limitations (Kuroda et. al., 2015). This type of hydroponics for short plants such as leafy vegetables has been used for commercial production. The annual yield per unit area of hydroponic systems of lettuce can be 10 times higher than conventional soil-based or flow hydroponic systems (Barbosa et al., 2015). We replace this single-tube system with a single-cup system that provides more nutrient media per plant, individual adjustment of planting conditions for each plant, ease of transport to the greenhouse for tall or vining species, and ease of positioning with a superstructure that can support tall plants. One of the concerns that is under investigation is the contamination of the media and plant root system as covers of the aseptically-grown plants are removed as the plants grow. Investigations are under way to examine if the addition of the competing bacterium, Bacillus amyloliquefaciens Strain D747, can minimize the effects of other contaminants because it produces a competitive biofilm on root surfaces and produces a variety of antibiotics (Xu et al., 2013).

Using our single-cup system, we have successfully grown Arabidopsis to maturity in the presence or absence of external sterol. A remarkable outcome is that if the plants are germinated and grown for the first week without sterol, the addition of subsequent

concentrations of sterol that would inhibit growth and germination do not influence growth. This appears ideal for use as a pre-emergent herbicide. Crop plants and weeds do well in the single-cup system. Initial experiments show that crop plants and weeds are inhibited in germination and growth when the sterol is included during imbibition. Crop plants grown in the absence of sterols show excellent growth in the single-cup system. Experiments to show the effect of sterols on crop plants and weeds post-imbibition are underway.

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CHAPTER II

EXOGENOUS STEROLS ARE TAKEN UP BY A NOVEL PATHWAY BY THE ELONGATING CELLS OF ROOTS

Introduction

The movement of sterols, and in particular, cholesterol, in the plant is of interest for a variety of reasons. First, although the claim of "cholesterol-free" is made in the United States for many plant-based food products (based on Food and Drug Administration guidelines), plants do indeed have cholesterol (1) and the genetics of the pathway for the synthesis of cholesterol has recently been outlined (2). Although cholesterol is about 6% of the sterol content in Arabidopsis, it can be higher in other plants, such as in Solanaceous species, where cholesterol is commonly esterified and forms toxic steroidal alkaloids through the GAME9 (GLYCOALKALOID METABOLISM) pathway (3). Second, some of the cholesterol that is made in the shoot is exported to the phloem where it is translocated to the root (4, 5). Why the plant has this kind of selective transport of cholesterol is unknown, but phloem-feeding insects rely on this pathway for the production of the molting hormone, ecdysone, not being able to make cholesterol themselves (5). Altering the movement of cholesterol in plants could have a profound impact on the life cycle of phloem-feeding insects and is therefore of great agronomic interest. Third, many of the embryo-lethal mutants in Arabidopsis, such as Fackel and Hydra2, are mutations in the $\Delta^{8,14}$ sterol C-14 reductase which is in the precursor pathway leading to the production of cholesterol, stigmasterol, and brassicasterol, and cannot be chemically compensated by brassinolides alone (6).

Fourth, mutations in later stages of the sterol biosynthetic pathway show mislocalization of PIN2, the auxin transporter, and have defects in cell plate formation (7).

The change in the localization of PIN2 and the change in endocytosis of other proteins and endocytic labels that accompany these mutations in sterol biosynthesis have been interpreted as evidence for the vesicular movement of sterols from the plasma membrane (PM) to the endosomal compartment via endocytosis (8, 9). Early work (10) supporting this interpretation, using in vivo labeling with the fluorescent polyene antibiotic, filipin, which binds sterols in the plasma membrane and also labels endocytic structures, has been reevaluated. Filipin apparently inhibits endocytosis (11) and when added to root hairs, causes aberrant endocytic structures to form (12). This is probably a consequence of the formation of sterol-filipin aggregates in the plasma membrane that perturb membrane function (12).

Hence, the importance of sterols for the process of endocytosis does not necessarily mean that vesicular endocytosis is the main mechanism for internalization of plasma membrane sterols. There are other potential mechanisms for the delivery of sterols to endosomes including transport by cytoplasmic sterol-binding proteins (13) and transport to recycling endosomes via the trans-Golgi network, and transport to endosomes via endoplasmic reticulum (ER)-to- endosome membrane contact sites (MCS) (14). If vesicular endocytosis is not the mechanism for internalization of sterol, the other main candidate for sterol (and other lipid) transport from the membrane into the cell in plants are putative ER-PM MCS (15). Although ER-PM MCS or anchor sites have been visualized using persistency mapping (16) and molecular components of the MCS have been identified (17-19), biosynthesis or transport come from the observations that plasmodesmata are enriched in

the function of the ER-PM MCS in plants has not been elucidated. Tantalizing evidence that the specialized ER-PM MCS occurring in the plasmodesmata might be involved in sterol sterols and that plasmodesmatal reticulons, ER proteins involved tubulation of the ER (20), can bind to sterol methyl transferase 1 (SMT1) as a partner (21). The strongest evidencethat ER-PM MCS are engaged in sterol transport, however, comes from other systems, such as yeast, where proteins involved in sterol transport, other than oxysterol binding proteins, have been identified (22).

Therefore, we undertook an investigation into the mechanism of sterol entry into the plant cell to determine whether vesicular or non-vesicular uptake is the primary route of entry of sterols. For this, we use the fluorescent sterols, BODIPY-cholesterol (BCh) and dehydroergosterol (DHE). Our data are more consistent with a non-vesicular entry of sterols than by endocytic vesicular uptake of sterols. The immediate target of internalization is a subdomain of the ER, the nuclear envelope (NE), which labels within 5 minutes of exposure to fluorescent sterols. Interestingly, there is no accumulation of sterols in other subdomains of the ER, a result which may be expected because even though it is the primary site of sterol biosynthesis within the cell (23), there is proportionally less sterol in the ER than in other endomembrane compartments (24).

Results

BCh and DHE track endogenous sterols

BCh and DHE (10 μM) label a region of native sterol accumulation. Sterols accumulate at the tip of emerging root hairs (12). As shown in Figure 1, both BCh and DHE label the tip of emerging root hairs, supporting the hypothesis that they are effective tracers of

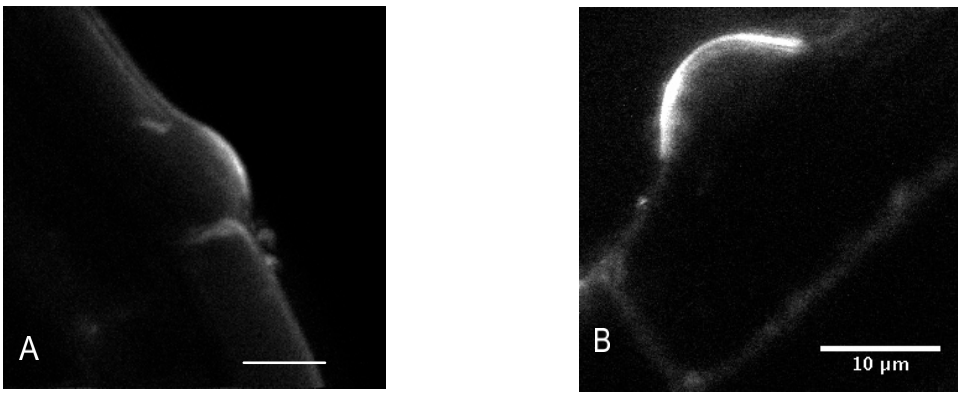

Figure 1. Labeling of root hair tip with DHE. A) Emergent root hair at the base of an epidermal cell labeled for 20 min with DHE. B) Emergent root hair labeled with BCh for 20 min. Scale bar = 10 micrometers.

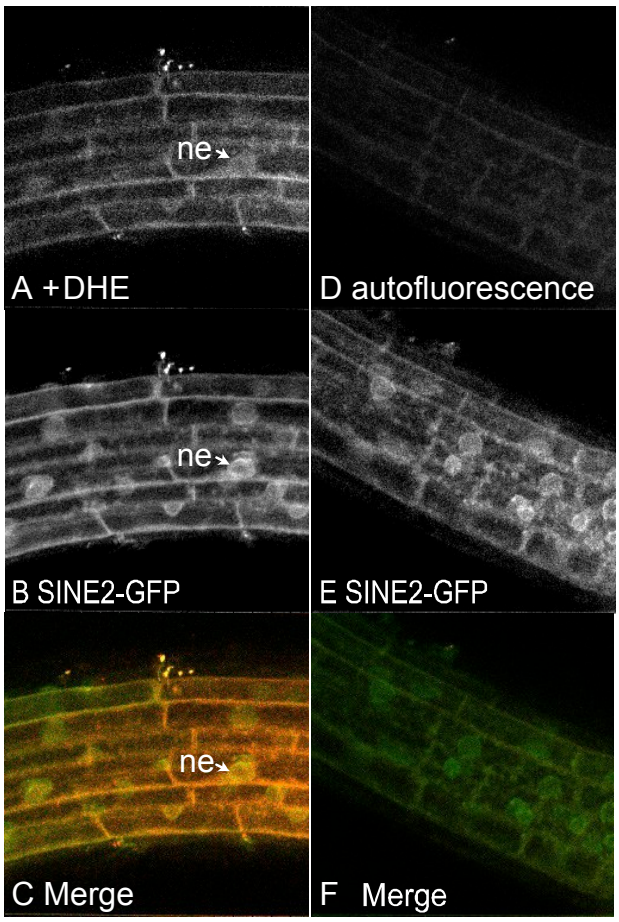

Figure 2. Labeling of the nuclear envelope with DHE. A-C) Seedlings expressing SUN2-GFP were incubated for 30 min in DHE and fluorescence after multiphoton excitation measured. A) Emission at 405/20nm for DHE, B) Emission at 525/50nm for SINE2-GFP, C) Overlay of the DHE signal (red) and the SINE2-GFP signal (green). D-F) SUN2-GFP expressing seedlings were incubated in buffer for 30 min prior to multiphoton excitation. D) Autoflu- orescent emission at 405/20nm. E) Emission at 525/50nm for SINE2-GFP, F) Overlay of autofluores- cent signal (red) and SINE2-GFP signal (green). Representative of three experiments with ap= 0.007 for the difference between the nuclear enve- lope DHE fluorescence and the autofluorescence. Scale bar = 60 micrometers.

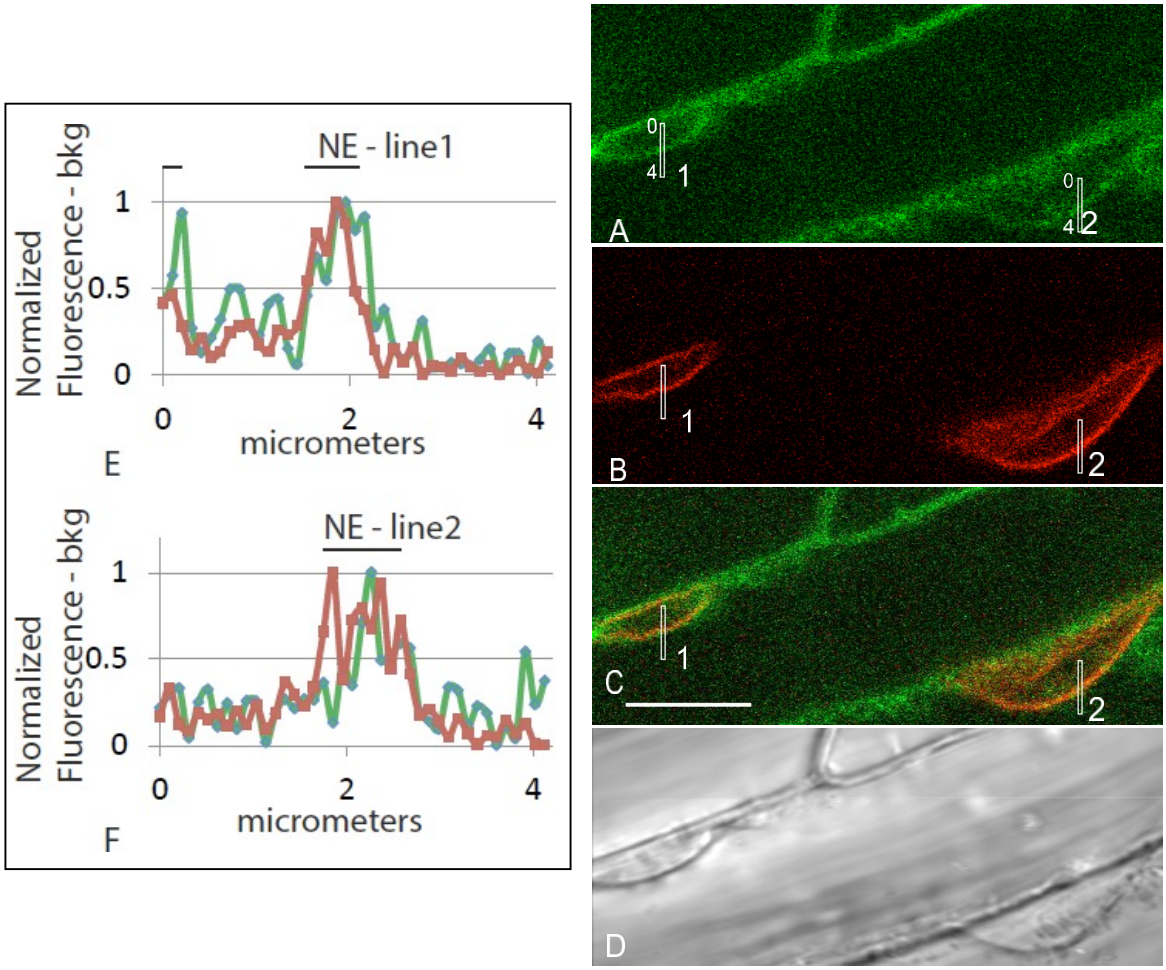

Figure 3. BCh label after 30 min and colocalization of the label with the nuclear envelope marker, SUN-RFP. A) BCh labels the plasma membrane and the nuclear envelope of cells in the elongation zone of roots. B) SUN-RFP expression in the nuclear envelope of the cells shown in (A). C) Merged fluorescence micrographs (A) and B). Scale bar = 10 micrometers. D) DIC image of the fluorescent region in (A-C). E) Fluorescence in the 4 micrometer line outlined in region 1 of (A-C) that crosses the nuclear envelope. F) Fluorescence in the 4 micrometer line in region 2 of (A-C). In (E) and (F) the fluorescence intensity in the BCh channel is green, whilst that in the SUN-RFP channel is red.

endogenous-sterol-distribution.

BCh and DHE label the nuclear envelope (NE) and plasma membrane (PM) of elongating cells of the root

Uptake of the DHE into the NE and PM after 30 min can be seen in Figure 2, where DHE uptake was detected with multiphoton microscopy. To confirm the NE localization, colocalization was done with SINE2-GFP, an NE protein (25), Figure 2A-C. There is little NE autofluorescence at the wavelength of detection of DHE (405nm, 20nm bandpass), Figure 2D-F.

The accumulation of fluorescent sterol in the NE is also seen with BCh. Elongating root cells of plants expressing SUN-RFP (26) were treated for 30 min with BCh and colocalization with the NE was seen, Figure 3 A-D. Line scans across two separate nuclei, Figure 3 E-F, reveals that the peaks of SUN-RFP and BCh closely correspond at the NE.

The time course of internalization of the fluorescent sterols and their appearance in the NE is shown in Figure 4. Labeling of the NE with DHE and BCh can be seen within 5 minutes. Labeling with both DHE and BCh reaches saturation fairly quickly, within 30 min. Although some of the label may be in the thin cytoplasmic layer between the vacuole membrane and the NE, the uptake into the NE shown in Figure 4 was determined by subtracting the intensity of sub-PM cytoplasmic label found in nearby regions.

BCh label in or near the cortical ER

When plants expressing mCherry-HDEL in the endoplasmic reticulum are treated with BCh for 16 h, there may be some co-localization between the ER and the BCh. It is most obvious in the NE, Figure 5. Some of the BCh label remains on the plasma membrane and,

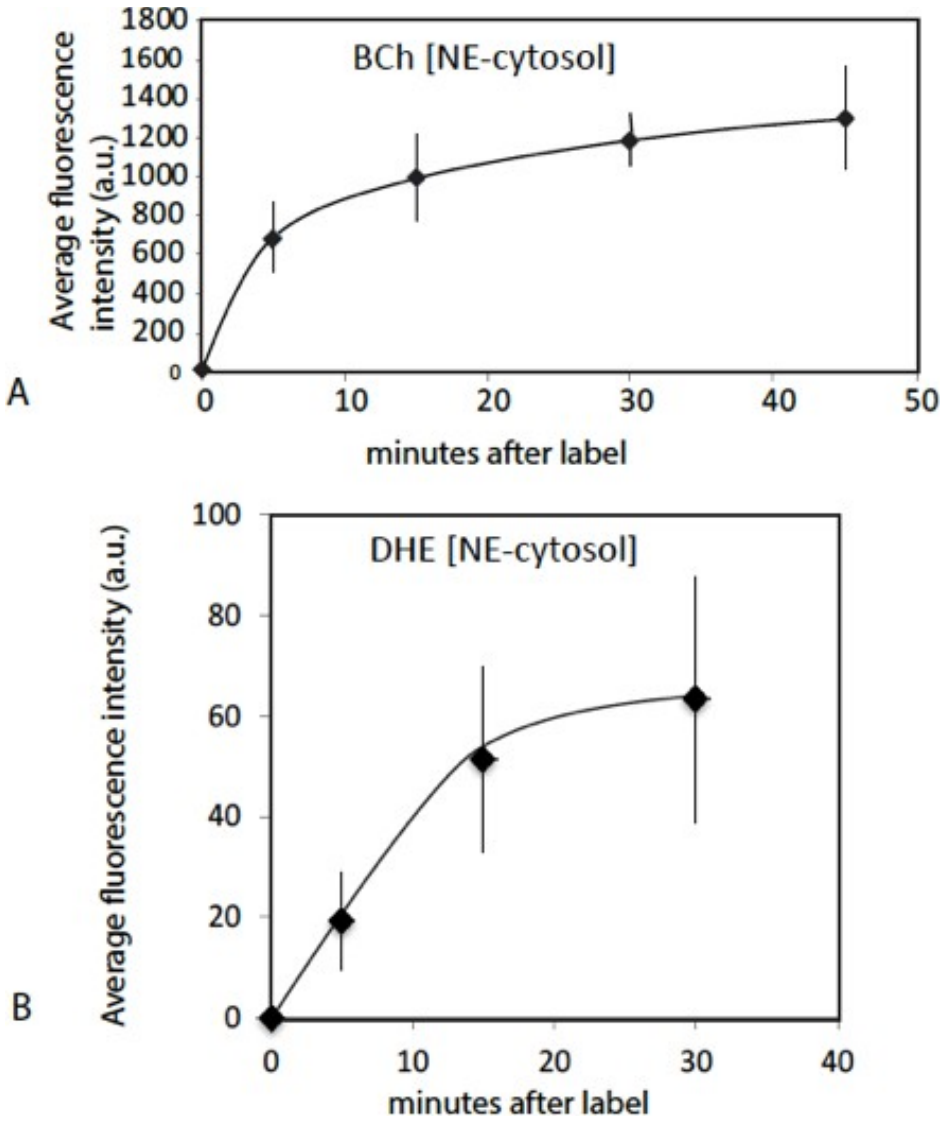

Figure 4. Uptake of BCh and DHE into nuclear envelope over time. A) Average fluorescence intensity of BCh taken over a region containing the nuclear envelope with the adjacent cytoplasmic fluorescence subtracted. Error bars are standard deviation of five separate samples. B) Average fluorescence intensity of DHE uptake into nuclear envelope. The fluorescence background in the cytoplasmic region has been subtracted. Error bars are standard deviation of five separate cells.

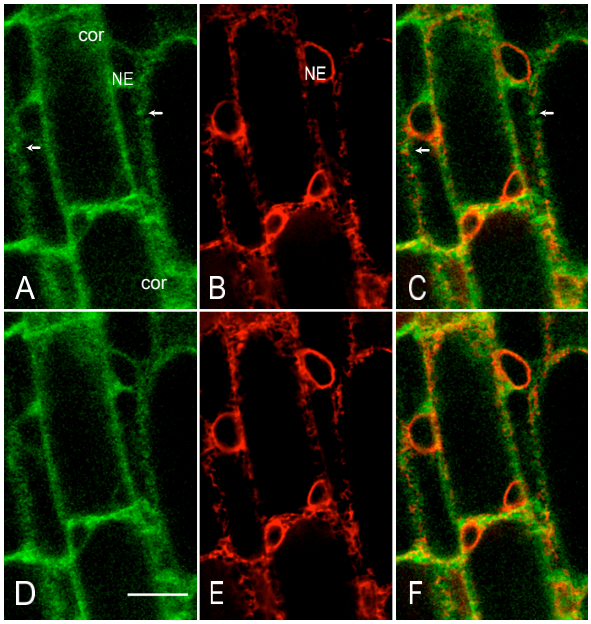

Figure 5. Comparison of BCh label (16 hr) with mCherry-HDEL labeled ER. A-C series taken at 0 s. D-E same area taken at 10 s. A, D , appearance of BCh label. B,E, localization of ER. C,F merge. Arrows in A and C point to putative oil bodies. NE= nuclear envelope. cor= cell cortex. Scale bar = 10 micrometers.

right underneath it in the cortical reticulum, some of the BCh label is juxtaposed with the signal from the ER, Figure 5.There are mobile and immobile elements and vesicles containing BCh that are not colocalized with ER. Some of the mobile elements are probably oil bodies (see below).

BCh does not label early or late endosomes

Very little labeling of endosomes by BCh was seen when endosomes were visualized with fluorescent fusion protein markers, Rha1 (RabF2a)-mCherry (27) for the late endosomes and VTI12-mCherry for the early endosomes/trans-Golgi (27), Figure 6. The elongating roots of intact seedlings expressing these fusion protein markers were labeled with BCh for 5-30 min. In all cases, the BCh does not appreciably label endosomes. However, BCh does label the NE and PM, Figures 6 B, D.

The absence of BCh label in endosomes was confirmed with co-incubation of 5-7-day-old seedlings with 4 μM FM 4-64 and 10 μM BCh for 30 min. Epidermal cells of the elongating region of the root were examined in near-tangential optical sections of the outer periclinal cytoplasm, Figure 7. Internal label with BCh showed little colocalization with FM 4-64. Several of the regions labeled with BCh were relatively immobile (im, Figure 6C), although there were some moving punctae (mo, Figure 7C). Some of the immobile punctae are refractile in bright field, Figure 7D.

Internalization in induced auxilin-like2 overexpression

To address the possibility that the absence of Bch in endosomes may be a consequence of a low but rapidly transported level of Bch in endosomes that is not detected with confocal

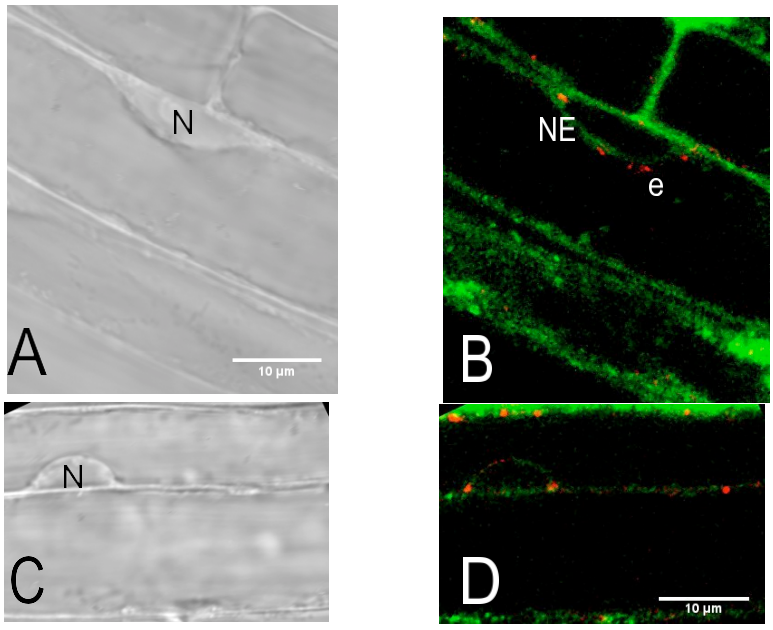

Figure 6. Lack of endosome label with BCh A,B) Sructures labeled after 30 min of incubation in BCh (green) in plants expressing the early endo- some/trans-Golgi marker, VTI12-mCherry (red) A) DIC micrograph showing nucleus (N). B) Fluores- cence of Bch (green) labels the nuclear envelope (NE) and the plasma membrane/cell wall of the elongating root cells. Endosomes (e) are outside the nuclear envelope in the cytoplasm. C, D) Structures labeled with BCh (green) after 30 min in plants expressing RabF2a-mCherry. C) DIC image showing nucleus (N). D) Fluorescence image showing label in nuclear envelope (NE) by Bch and late endosomes (e) in elongating root cells. Scale bar = 10 micrometers.

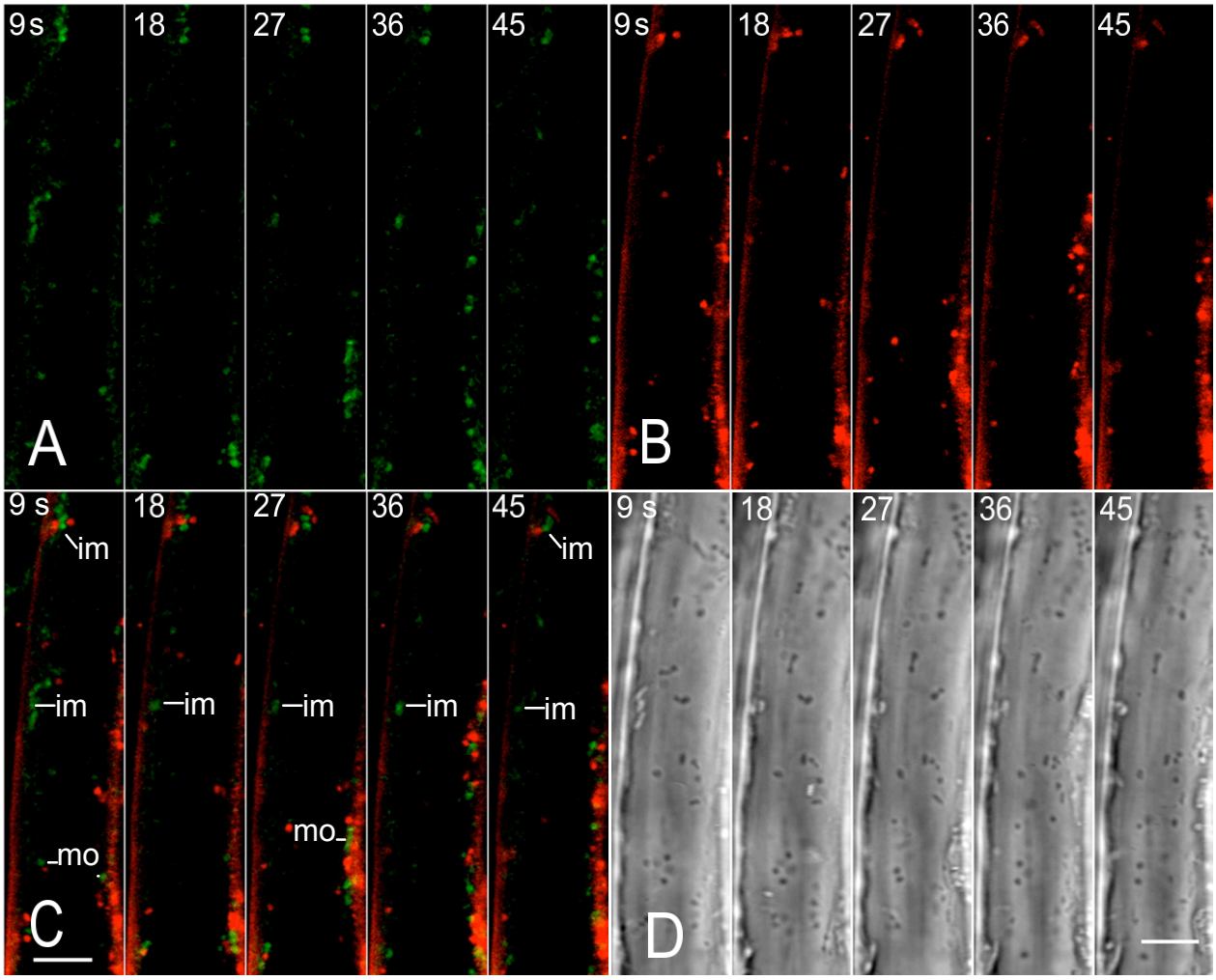

Figure 7. Labeling of elongating root cells with BCh (green, A) and FM4-64 (red, B) after 30 min of co-incubation. A) Green fluorescent structures associated with the PM and in the cytoplasm. BCh labels immobile (im) and motile (mo) structures B) Red fluorescent, mostly motile, endosomes labeled with FM 4-64 after 30 min. C) Merged image of (A) and (B) - punctate structures do not colocalize. D) Bright field image. Images taken at 9 second intervals. Scale bars = 5 micrometers.

microscopy, we examined the uptake of Bch after inhibiting endocytosis with the β-estradiol inducible expression of auxilin-like 2 (28), Figure 8. After 30 min incubation in BCh, the level and pattern of cytoplasmic label in elongating root cells doesn't change from that seen in wild type plants (Figure 8A), in wild type plants pre-incubated in β-estradiol (Figure 8B), in Aux2-like plants (Figure 8C), or in Aux2-like plants pre-treated β-estradiol (Figure 8D). In contrast, uninduced Aux2-like plants show normal levels of internalization of FM4-64 (Figure 8E), but greatly reduced uptake in β-estradiol-induced Aux2-like plants (Figure 8F). The quantification of internalization in β-estradiol-induced and uninduced Aux2-like plants with shows significant reduction of internalization of FM4-64 in induced Aux2-line, but no significant reduction of internalization of BCh in the induced Aux2-line (Figure 8G).

The nature of the punctate label with BCh in root cells

The label with BCh near the plasma membrane and nuclear envelope is somewhat discontinuous, Figure 6, and, when interior, punctate, Figure 7. BCh can be found in the highly- refractile lipid bodies after overnight (12-20 hr) incubation, Figure 9. Co-localization with nile red confirms the oil body label. Following uptake, the BCh is probably esterified and BCh-esters would accumulate in the lipid body. Long-term incubation in BCh has no effect on growth, Supplemental Figure 1.

BCh uptake in hypocotyl cells

The labeling pattern with BCh after overnight incubation is different in hypocotyl epidermal cells and elongating root epidermal cells. BCh is not taken up as rapidly from the plasma membrane in hypocotyl cells. Internalized BCh is only visible as punctae after

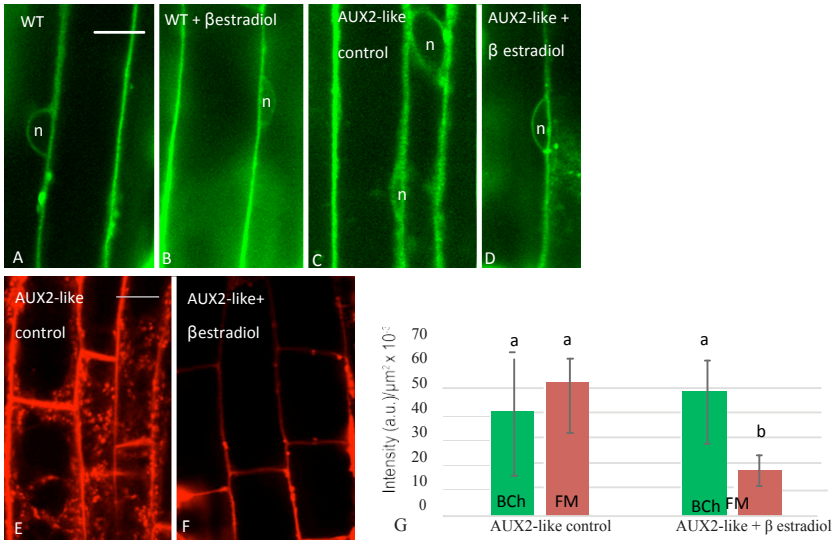

Figure 8. Confocal micrographs of elongating root cells following: (A-D) 30-min labeling with BCh in A) the wild type (WT) Arabidopsis line, B) WT line pretreated with 9 μM β-estradiol, C) the auxilin-like 2 (AUX-like 2) line, and D) the AUX-like 2 line pretreated with 9 μM β-estra- diol, (E-F) 30-min labeling with FM4-64 in E) the AUX-like 2 line and F) the AUX-like 2 line pretreated with 9 μM β-estradiol. G) Quantitation of label intensity per square micrometer of cytoplasm in 4-5 different cells in 3 different plants. (b) Column values <0.05p compared to (a) column values, n = nucleus. Scale bars= 10 micrometers.

overnight (16 h) treatment with BCh. However, unlike root cells, the punctae are not refractile oil bodies, instead, they appear in regions juxtaposed to Golgi, Figure 10A-B. They often track with the Golgi, Figure 10B, sometimes becoming superimposed with the Golgi, Figure 10B, arrowheads. The movement of such BCh punctae is similar to that of ER-Exit Sites, or ERES, which can either move with, but are separate from, the Golgi (29), or directly colocalize with the Golgi (30).

Long-term incubation may also label the vacuole membrane in some, but not all, epidermal cells of the hypocotyl, Figure 10B-D. Punctate labeling at the crosswalls can correspond to sites of ER traversal, i.e., plasmodesmata, Figure 10B. There is no colocalization of BCh with mitochondria, Figure 10C.

Discussion

Although filipin is not an accurate tracer of the physiological internalization of sterols (12), it does accurately label sterol-enriched membranes in fixed tissue and surface sterols in living plants (11). The root hair tip localization of the two tracers, dehydroergosterol (DHE) and Bodipy-cholesterol (BCh), Figure 1, is similar to that achieved with brief treatment with filipin. This indicates that a modified sterol (BCh) and a foreign sterol (DHE) can be used to accurately trace local sterol distribution. DHE is an analog of ergosterol, a fungal sterol that acts as a signal for the plant cell to initiate a defense response (31), so long term (> 1 hr) treatments are not shown. In the presence of 10 μM BCh, on the hand, plant growth is normal, Supplementary Figure 1.

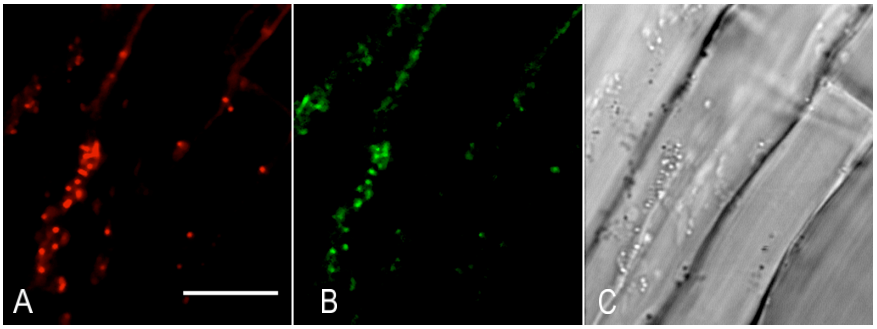

Figure 9. Co-localization of BCh with oil bodies of elongating root cells after 1.5 days of incubation in BCh. A) Nile-red-stained oil bodies. B) BCh staining of oil bodies. C) Brightfield image showing that the oil bodies are highly refractive. Scale bar = 10 µm.

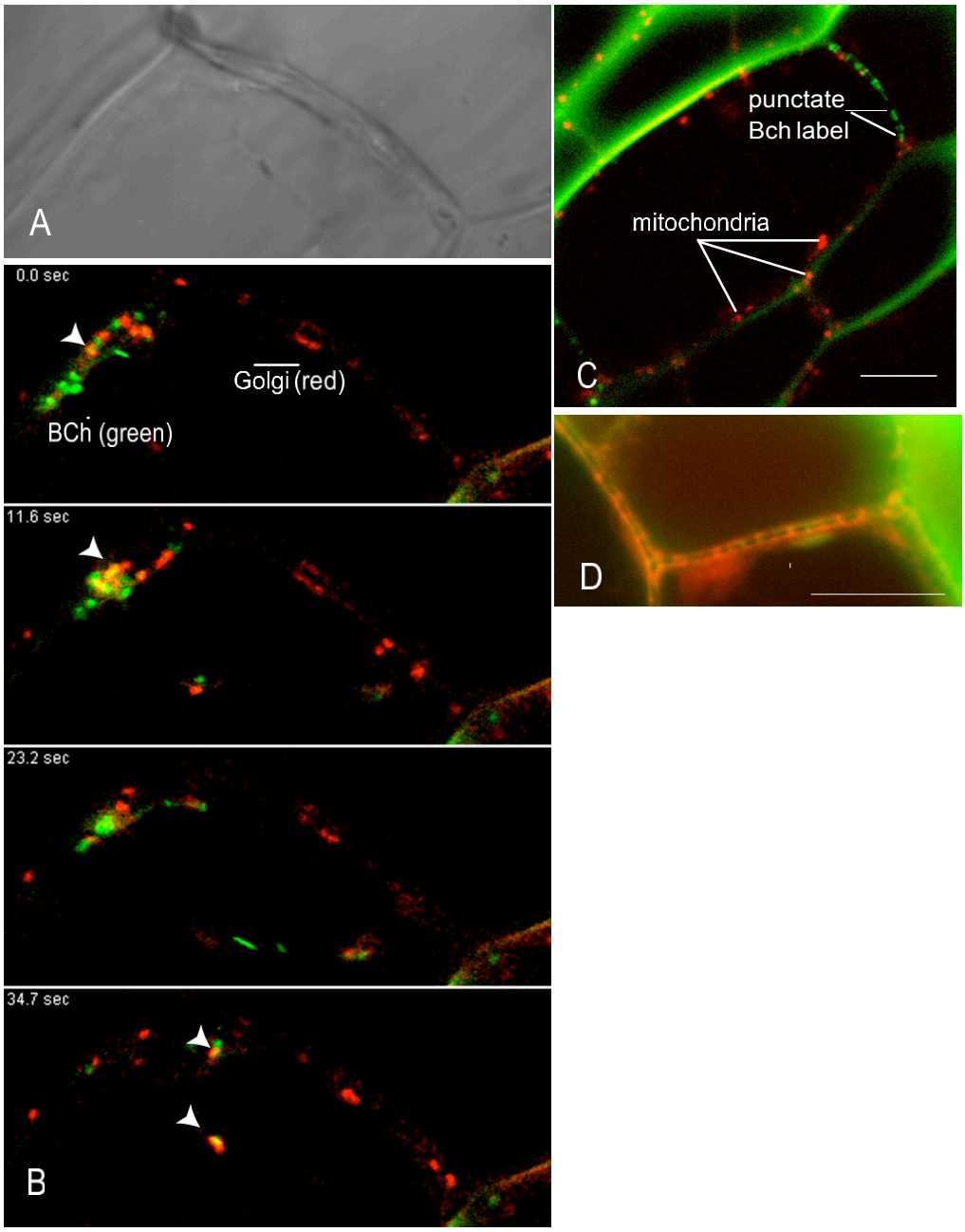

Figure 10. Hypocotyl labeling of near-Golgi structures and plasmodesmata by BCh. A) Bright field image of cytoplasm, transvacuolar strand, and cross-wall of Memb12-mCherry expressing hypocotyl treated with 10 µM BCh for 16 hours. B) Time series of movement of BCh-labeled structures (green) and MEMB12-mCherry (red) labeled Golgi (red). The green label moves with the red label, inidicating that they are move with the Golgi. C) Hypocotyl cell expressing COX-mCherry (red) labeled overnight (16 h) with BCh (Green).

Mitochondria (red) do not colocalize with the BCh, but BCh does label punctate structures on the cross- walls. D) Hypocotyl cells expressing mCherry-KDEL (red) labeled overnight (12 h) with BCh (green). Some of the punctate BCh label at the crosswalls colocalize (arrow) with ER, indicating that some of the BCh label is in plasmodesmata. Scale bars = 10 micrometers.

As shown in Figure 2, Figure 3 and Figure 4, BCh and DHE both quickly label the nuclear envelope. Initial (5-10 min) label does not occur in motile, streaming punctae, but is found at the plasma membrane and the nuclear envelope. The uptake into the NE is fast. The data do not exclude diffuse uptake into the cytoplasm, but when the level of cytoplasmic fluorescence is subtracted from NE fluorescence, Figure 4, the NE label appears higher than cytoplasmic label and saturates over time. Furthermore, when a line plot of labeling intensity by BCh is compared with a marker for the NE, there is little label in the cytoplasmic region outside the region marked by the SUN-RFP, NE signal.

In order to label cells with sterols, they had to be in the presence of the delivery agent, MβCD. Although MβCD has not been used previously in sterol uptake experiments in plants to our knowledge, it has been used to deplete sterols from the plant membranes (32, 33). MβCD apparently dimerizes when it binds sterols (34) and can either extract or supply sterols to a membrane.

The appearance of sterols in the NE is not so surprising when one considers that the concentration of cholesterol (35) and other sterols (36) in the nuclear envelope is higher than that of the ER in general. The rest of the ER does not accumulate sterols during their synthesis (37). The absence of the sterols in the ER during their biosynthesis indicates that there is rapid transport out of the ER. This is consistent with the observations in Figure 5, showing that the ER does not accumulate BCh. There are hypotheses for the function of the accumulation of sterols and other lipids in the NE in, for instance, yeast whereby their presence is postulated to be a storage mechanism for lipid during the elaboration and growth of membrane that occurs during cytokinesis (38, 39).

The label seen after 30 min incubation in BCh does not colocalize with early or late endosomes, as assessed by markers for these organelles, Figure 6. Although BCh does label some motile and immotile punctae at this time, Figure 7, this label does not colocalize with FM 4-64, a marker of membrane-bound endocytosis (40). The presence of BCh would be expected were there endocytosis of sterols through a common, clathrin-mediated endocytic pathway.

However, if the endocytic delivery pathway were extremely transient, as is postulated for the delivery of sterols out of the ER upon their synthesis in the ER, then only by inhibiting endocytosis could it be ruled out. This was addressed by following the initial uptake of BCh in plants where endocytosis was inhibited with the induced overexpression of Auxilin2-like protein (28). With the induction of overexpression of Auxilin2-like protein with β-estradiol, endocytosis of FM 4-64 is inhibited, Figure 8E-G. However, the uptake of BCh is not, Figure 8A-D,G. The remaining level of FM4-64 label quantified in Figure 8G may be the consequence of the accumulation of FM4-64 in plasma membrane domains that appear with inhibition of endocytosis

The non-vesicular mode of uptake of sterols can be found in other systems, where it is proposed that the sterols are internalized by specific transporters that are associated with ER- PM MCS (22, 41). Of particular interest is the work of Gatta et al., (22), where it was shown that internalization of sterols in yeast occurred in a genetic background where all seven oxysterol binding proteins, also thought to be involved in sterol transport, had been deleted.

The sterol transport capability of yeast cells seemed to be conferred, at least in part, by StART- like proteins, since mutations in these proteins inhibited the uptake of sterols. In that work the internalization of DHE and cholesterol was monitored by the internal formation of sterol esters and delivery to oil bodies. The first appearance of sterol internalization was at 10-20 minutes. Here, we have shown that internalization can be detected within 5 min of exposure to BCh, Figure 2, Figure 3 and Figure 4. Intriguingly, some of the StART-like proteins in plants have a transmembrane spanning domain in the middle of the protein (22), which may form a single hairpin structure in the cytoplasmic leaflet of the ER. Plant ER proteins known to reside in the cytoplasmic leaflet of the ER, i.e., the membrane-bending reticulons (42) and the large GTP- binding protein involved in ER fusion and/or bundling, Root Hair Defective 3, RHD3 (43), have a double hairpin structure. If these StART-like proteins associate with these other proteins on the cytoplasmic side of the ER and are localized to ER-PM MCS, they are excellent candidates for a sterol transporter in plants. In fact, it has recently been shown that one of the reticulons localized to the outer member of the ER in plants, RTN20, changes sterol dynamics (44).

The compartment of neutral lipids, the oil body, is a well-known place in plants for the storage of lipids and modified sterols and presumably originate from ER. Overproduction of sterols produces oil bodies containing higher levels of sterol esters (45). Sterol homeostasis is mediated by phospholipid sterol acyltransferase 1 and plants deficient in this enzyme do not accumulate as much lipid in oil bodies (46). BCh colocalization with oil bodies, Figure 9, indicates that the extra sterol is taken up and stored in the oil bodies. The observation is consistent with the model that oil body sterol esters, as in yeast, arise

from the ER and are stored inside the oil bodies (22). Hypocotyl cells label with BCh differently from the elongating cells of the root. BCh also labels the nuclear envelope of hypocotyl cells, but it takes longer, 1-2 hours. However, after overnight treatment, instead of labeling oil bodies, it labels other punctate organelles. After these long term treatments with BCh, punctae associated with the Golgi label in the hypocotyl, Figure 10A-B. These could be ERES that track behind the cis-region of the Golgi, Figure 7, but their identity awaits further work. That ERES could accumulate sterols would be explained by the observation that oxysterol binding proteins are found at this site (47) and may be involved in sorting sterols into the endomembrane pathway. Punctae also appear at the apical cell wall of hypocotyls also label when exposed to the BCh for longer than 5 hours, Figure 10C-D. Although these can, at times, be found in association with plasmodesmata where ER traverses the wall, it is not associated with all such sites, Figure 10D. That BCh may accumulate in plasmodesmata could be explained by the observation that plasmodesmata are known to be enriched in sterols (48).

The differing uptake and sequestration sites of BCh in root cells and hypocotyl cells may relate to the transport of sterol through the plant. Sterols are transported by the phloem (5) and presumably would follow the same pathway marked for fluorescent labels of phloem unloading (49) via the phloem-pole pericycle, thereby delivering it to the elongating cells of the root. In fact, when initially screening cells for BCh uptake, the elongating cells of the root were found to be the most active. The nature of the transported sterol to these cells via the phloem is unknown, but very likely involves a sterol binding protein or other binding partner(s) which would be necessary to solubilize this very hydrophobic molecule. Proteins unload in the phloem-pole pericycle through specialized funnel-shaped

plasmodesmata (49). The uptake of MβCD-solubilized sterol reported here would monitor a process by which the epidermal cells, several steps removed from the transport of sterols out of the phloem, acquire sterols. Interestingly, we saw little evidence of plasmodesmatal associations of BCh in elongating root cells, while in hypocotyl cells, it is common. We hypothesize that delivery of sterols to the hypocotyl and the root differs and may involve different activities of plasmodesmatal connections in the two tissues.

Materials and methods

Plant materials and Growth conditions

The wild-type and transgenic seeds of *Arabidopsis thaliana* were surface-sterilized with 70% ethanol and planted on ½ strength Murashige and Skoog (Caisson labs, USA) 1% agar (Sigma- Aldrich, USA) medium containing vitamins and phosphates, and the pH was adjusted to between

5.6 and 5.8. Following cold, dark treatment for 48 h in Petri dishes the seedlings were grown for 4-5 d at 22^{o} under the continuous white light in vertical position and treated for analysis. The following homozygous transgenic Arabidopsis lines tagged with fluorescent fusions were used in this study: RFP and GFP targeted to nuclear envelope (SUN-RFP and SINE2-GFP) (25, 26) , mCherry-tagged vacuolar protein (VAMP711-mCherry) (27), mCherry-tagged late endosomal marker (RabF2a-mCherry) (27), mCherry-tagged trans-Golgi network/early endosomal marker (VTI12-mCherry) (27), mCherry-tagged Golgi network (MEMB12-mCherry) (27), mCherry-COX1- tagged mitochondria (Mito-mCherry) (courtesy of David Logan, Universite' d'Angers, France), and mCherry-HDEL (50). The β-estradiol-inducible Auxilin2-like Arabidopsis line was from the Friml lab (28).

Bch (Bodipy-cholesterol), Dehydroergosterol (DHE), FM4-64, Filipin and Nile Red labeling

Bch (Top-Fluor, Avanti Polar Lipids, USA) and DHE (Sigma-Aldrich. USA) were prepared as a stock solution with a slight modification from that described (51). BCh was dissolved in 100% ethanol to 1.7 mM in the stock solution and DHE at 10 mM in the stock solution and combined with methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich, USA) at a

molar ratio of 1:3 to a final concentration of 10 μM BCh or DHE and 30 μM MβCD. The probe was either sonicated for 30 minutes and vortexed for 15 minutes before use, or just vortexed for 30 min before use. The styryl dye FM 4- 64 (Invitrogen, USA) at a final concentration of 4 μM was used for dual labeling with Bch. Filipin (Sigma-Aldrich, USA) was made up in a DMSO stock solution (15 mM) and diluted 1:500 in water for treatment. For oil body staining, seedlings were incubated in 2μg/ml Nile red (Sigma-Aldrich, USA) solution (made from 2mg/ml stock in acetone).

Confocal microscopy and image analysis

Confocal laser scanning microscopy imaging was performed on intact Arabidopsis wild-type (Col-0) and transgenic seedlings grown on agar plates. Whole seedlings were stained for the time indicated. Imaging was done on an Olympus Fluoview 1000 Confocal imaging system equipped with X60, 1.2 NA water immersion objective. Images and time-lapse videos for analysis were taken using a 20 μs/pixel dwell time. BCh (λex = 497 nm, λem = 507 nm, (51) was examined with the 488nm line of an Argon ion laser and fluorescence was recorded between 500-530nm. mCherry and RFP fusion proteins were examined with a 543 He-Ne laser and fluorescence recorded between 585-685 nm. FM4-64 labeling also used the 543 nm He-Ne laser and fluorescence recorded at 640-700 nm (52). Analysis and post-processing of images was performed with ImageJ (53) FIJI (54), Adobe Photoshop® and Adobe Illustrator ®, (CS 6, Adobe Systems, San Jose, CA, USA). The relative intensity of NE has been calculated by subtracting the cytoplasmic integrated density per unit area from the NE integrated density per unit area.

Two-Photon Laser Microscopy

Fluorescent observation of DHE was carried out with two-photon laser scanning microscopy. For two-photon imaging, 10 fs pulses centered at 800 nm with a bandwidth of 133 nm were pre- compensated and coupled to a 20X, 1.0 NA objective with x-y scanning mirrors. 25 mW average power was used on live samples. Fluorescence signals were separated by a dichroic long pass mirror at 430 nm. Wavelengths below 430 nm passed through a BG-39 filter and wavelengths above 430 nm passed through a bandpass filter centered at 450 nm with a bandwidth of 60 nm to separate the DHE fluorescence and cellular autofluorescence, respectively, that were subsequently detected with photomultiplier tubes.

Supplemental Figure 1. Absence of growth effects on seedlings grown in the presence of BCh

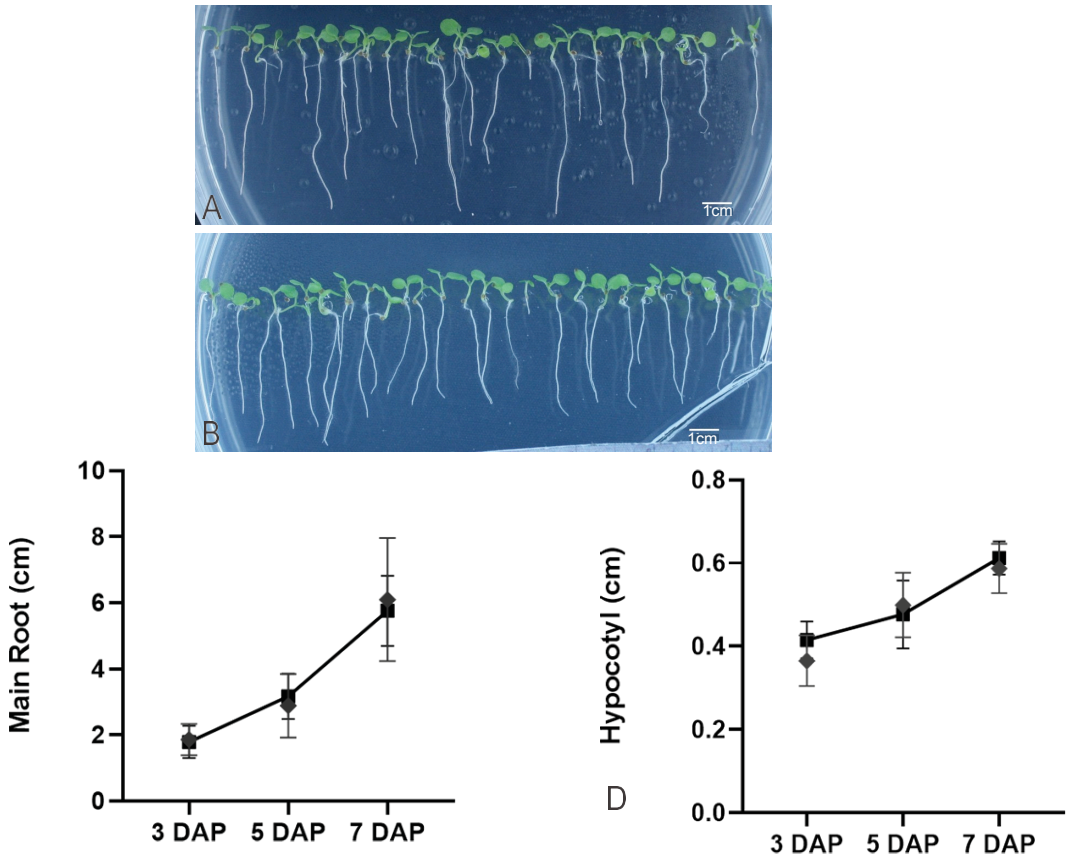

Figure 1. Absence of growth effects on seedlings grown in the presence of BCh. A) Plate of untreated Arabidopsis seedlings. B) Plate of Arabidopsis seedlings grown in the presence of 10 μM BCh. C) Root length measured over 3,5 and 7 days after planting (DAP). D) Hypocotyl length measured over 3, 5, and 7 days after planting (DAP).

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CHAPTER III

ADDITION OF EXOGENOUS STEROLS NEGATIVELY FEEDS BACK ON STEROL BIOSYNTHESIS WITH CONSEQUENT EFFECTS ON DEVELOPMENT

Introduction

A major mechanism by which sterols influence plant growth and development is through the activity of an end-product of the sterol pathway, the brassinosteroids (BRs). BRs act as hormone signals that, when absent (or their receptor is defective), produce extreme dwarfism and interfere with etiolation, producing phenotypes in the dark that show constitutive photomorphogenesis. BR abundance is regulated through negative feedback inhibition on the transcription of enzymes in its biosynthetic pathway (Schaller, 2003).

Mutants (*dwf1*, *dwf5*, *dwf7)* in the pathway giving rise to campesterol, the precursor to BRs, produce dwarfing that can be chemically complemented by the addition of BRs to the medium (Choe et al., 1999; Choe et al., 1999; Choe et al., 2000).

However, there is also evidence that sterols produced at earlier stages in the BR pathway or in pathways not leading to BRs are important in plant growth and development. There are embryo-lethal (non-germinating) mutations, such as *fackel/hydra1* or *hydra2,* and non- lethal mutations such as*, smt1, smt2/3,* in the sterol biosynthetic pathway of *Arabidopsis* that produce different relative concentrations of the main sterols, cholesterol, β-sitosterol, and stigmasterol. These mutations produce dwarfing, but cannot be chemically complemented by BRs (Grebenok et al., 1998; Diener et al., 2000; Schrick et al., 2000; Schrick et al., 2002; Souter et al., 2002; He et al., 2003). One effect of these mutations is to change the sterol profile, which may, in turn, change plant growth and development (Clouse, 2002). These mutants could not be rescued with individual sterols, such as stigmasterol and β-sitosterol

(Carland et al., 2010). However, these results need to be revisited, as indicated by our successful chemical complementation of *hydra 1* with sitosterol, reported below, using a method by which sterols become internalized in plant cells.

A change in the sterol profile can also arise from treatment of plants with inhibitors of sterol biosynthetic enzymes. Lovastatin, an inhibitor of HMG-CoA reductase, one of the first enzymes in sterol biosynthesis, not only changes the sterol profile of plants, but also shuts down the isoprenoid pathway and cytokinin production (Crowell and Salaz, 1992). Chemical inhibitors such as 15-aza-steroid (Benveniste, 2004) of the enzyme 18,14-Sterol-Δ14-Reductase coded by the FACKEL gene, phenocopy the *fackel* mutation. The phenotype is also copied by the drug, fenpropimorph (He et al. 2003), which inhibits cyclopropyl sterol isomerase, an enzyme two steps earlier in the pathway, see Chapter I, Figure 3. In the work described below, addition of the end-products of these pathways, except for BRs, through our delivery method do not change the phenotype of inhibitor-treated seedlings, indicating that pharmacological treatments may have multiple sites of action, and that non-BR end-products work at least in a subset of them. BRs produce additional phenotypic effects, indicating that they work through a separate pathway.

The hypothesis that individual sterols may act as plant growth regulators, separate from the effects of BR, is supported by several observations. β-Sitosterol is implicated in cell plate formation and polarized growth (Nakamoto et al. 2015). Stigmasterol is involved in the regulation of HMG-CoA reductase (the enzyme inhibited by lovastatin), and when at elevated levels, can induce the expression of proteins involved in cell morphogenesis (Aboobucker and Suza 2019). Overexpression of the enzymes for cholesterol increases the endogenous free cholesterol in Arabidopsis and produces dwarfed plants (Sonowane et al.

2016). One molecular mechanism of the action of sterols, their influence on cellulose synthase activity (Schrick et al. 2012), may be involved, but does not explain different effects on different organs.

It is important to note that the sterol profile of plants changes during development in different tissues (Schrick et al. 2011). In peas, embryos contain primarily β-sitosterol, with small amounts of cholesterol and stigmasterol, while in mature plants, that ratio is diminished, with stigmasterol and cholesterol increasing (Schrick et al. 2011). The differential effect of added cholesterol on the growth of plants pre-germination vs. post- germination is discussed in terms of a model whereby free sterols vary with growth and development through changes in intermediate biosynthetic enzyme gene expression and activity.

Materials and Methods

Plants and plant growth conditions

Cholesterol (C3045, Sigma-Aldrich, USA), stigmasterol (S2424, Sigma-Aldrich, USA), and β- sitosterol (567152, Millipore-Sigma, USA) were prepared as stock solutions with a slight modification from that described (Holtta-Vuori et al., 2008). Sterols were dissolved in 100% ethanol at 15 mM concentration and combined with methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich, USA) at a molar ratio of 1:3 to a final concentration of 1-300 μM sterol and 1-900 μM MβCD. The probe was either sonicated for 30 minutes and vortexed for 15 minutes before use, or just vortexed for 30 min before use.

The wild-type and transgenic seeds of *Arabidopsis thaliana* were surface-sterilized with 70% ethanol, rinsed in sterile distilled water (+/- sterol) and incubated at 4 C in the dark for 48 h.

The imbibed seeds were then planted either in petri dishes or in hydroponic containers. For petri dish growth, 1% (w/v) agar (Sigma-Aldrich, USA) was dissolved in ½ strength Murashige and Skoog medium containing vitamins (Caisson labs, USA) (+/- sterol), and the pH was adjusted to between 5.6 and 5.8. The seedlings were grown in vertically-placed Petri dishes containing this medium for 5-10 d at 22o under the continuous white light (50 µmol m-2sec-1 photosynthetically active radiation). Hydroponic culture was a modification of that described by Alatorre-Cobos et al. (2014). The seeds were placed on a mesh inserted into the bottom of a sterile polypropylene 2 oz cup with a lid (TY-M2-100, Bingwu, sold by Amazon. Com) inset into either a 4 oz sterile polypropylene cup (TY-M4-N50 Bingwu, sold by Amazon.com) or a black 5.5oz sterile polypropylene Dart Conex cup (B07BK1DPPS, Table Top King, sold by Amazon.com). Enough medium (½ strength Murashige and Skoog medium containing vitamins (Caisson labs, USA) (+/- sterol), and the pH was adjusted to between 5.6 and 5.8) was added to bring it up to the level of the mesh. They were grown for 5 days to maturity (~75 days) in a 18:6 h photoperiod at 22C with 100 µmole cm-2sec-1 photosynthetically active light. Following growth, were then harvested for sterol analysis, leaf venation analysis, or fresh and dry weight analysis, or photographed for image analysis of root, hypocotyl, and stem growth and color.

Sterol analysis

Plants were weighed (fresh wt) and lyophilized. Sterols were initially extracted from lyophilized plants with the addition 5 ml of 100% MeOH (pre-equilibrated to hexane), plus 5 ml 100% hexane (pre-equilibrated to 50% MeoH/water). Additionally, 10 µg of cholestane was added to each sample (this served as an internal standard). Next, each sample was

shaken vigorously for several seconds, followed by incubation at room temperature for 24 h in the dark. The hexane fraction (containing free and acylated sterols) was then separated from the MeOH/water fraction (containing the glycosylated sterols), and both fractions were evaporated to dryness using nitrogen. For each sample, the hexane fraction was processed further for quantification of either the free sterols or acylated sterols, while each MeOH/water fraction was processed further for quantification of glycosylated sterols.

For free sterol analysis, 50% of the hexane fraction was taken, conjugated, and analyzed by GC–MS. For acylated sterol analysis, the remaining 50% of the hexane fraction was resuspended in 100 µl of clean hexane and 8 ml of 70% MeOH-water containing 5% KOH was added, and then incubated in a shaking water bath (225 rpm) at 55°C for 2.5 h. This replaces the lipid moiety at C3 with a free hydroxyl group. The MeOH-water fractions were resuspended in 8 ml 100% methanol containing 10% HCl, and then incubated in a shaking water bath (225 rpm) at 55°C for 2.5 h, to remove the carbohydrate moiety present at C3; it was replaced with a free hydroxyl group. Subsequently, all fractions contained free sterols, which were extracted from the chemically treated samples with water-equilibrated hexane; the hexane layer was then washed to neutrality with hexane- equilibrated water. The recovery rate of our internal standard (cholestane) was 92 ± 5%. The level of detection for GC–MS was tens of nanograms; detection at this low level was made possible using selected ion chromatogram software, and selected ion-monitoring software [GC–MSD ChemStation (Agilent Technologies)].

The sterols contained in the three fractions were converted to their respective trimethylsilyl ether (TMS) deriviatives, to ensure the inertness of the free C3 hydroxyl, by overnight incubation with a 2:1 excess volume v/v of BSTFA + TMCS, 99:1 (Sylon BFT; Supelco Inc.

Bellefonte, PA, USA). All conjugated sterols were processed by gas chromatography – mass spectroscopy (GC–MS), using an Agilent 6850N GC coupled with a 5973 mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC–MS was equipped with a fused capillary EC-5 column (30 m; Alltech, Nicholasville, KY, USA) with a 0.25 mm internal diameter and 0.25 μm film thickness. The running conditions were: inlet 280°C, transfer line 290°C, column 80°C (1 min), ramp at 10°C min-1 to 240°C, 240 to 300°C, ramp of 5°C min-1, with helium (1.2 ml min-1) as carrier gas. The Agilent 5973 mass selective detector maintained an ion source at 250°C and quadrupole at 180°C. Sterols were identified and quantified by GC–MS using selected ion monitoring (SIM) protocols for each steroid identified (Rahier and Benveniste, 1989). Authentic sterol standards were purchased commercially [from Sigma Chemical (St. Louis, MO, USA), and Steraloids Inc. (Newport, RI, USA)].

Photography and image analysis

Plants in petri dishes or on a moist towel were photographed on a copy stand, using a Canon EOS Ti1 using an 18-55mm lens. The image included an internal scale and color card. Analysis and post-processing of images was performed with ImageJ (Schneider et al., 2012) FIJI (Schindelin et al., 2012), Adobe Photoshop® and Adobe Illustrator ®, (CS 6, Adobe Systems, San Jose, CA, USA).

Results

Altered sterols in seedlings treated with free sterols

When seedlings are germinated and grown in 100 μM cholesterol or stigmasterol, there is a

differential effect on endogenous free and esterified sterols. Both show a reduction in free and esterified cholesterol, while only growth in exogenous 100 µM cholesterol reduces the level of β-sitosterol, Figure 1. Of the treatments with exogenous sterols below 100 µM did not alter it cholesterol levels, Figure 1. Treatment with exogenous stigmasterol produced an increase in endogenous stigmasterol, while the amount of seedling stigmasterol varied after treatment with exogenous cholesterol, Figure 2.

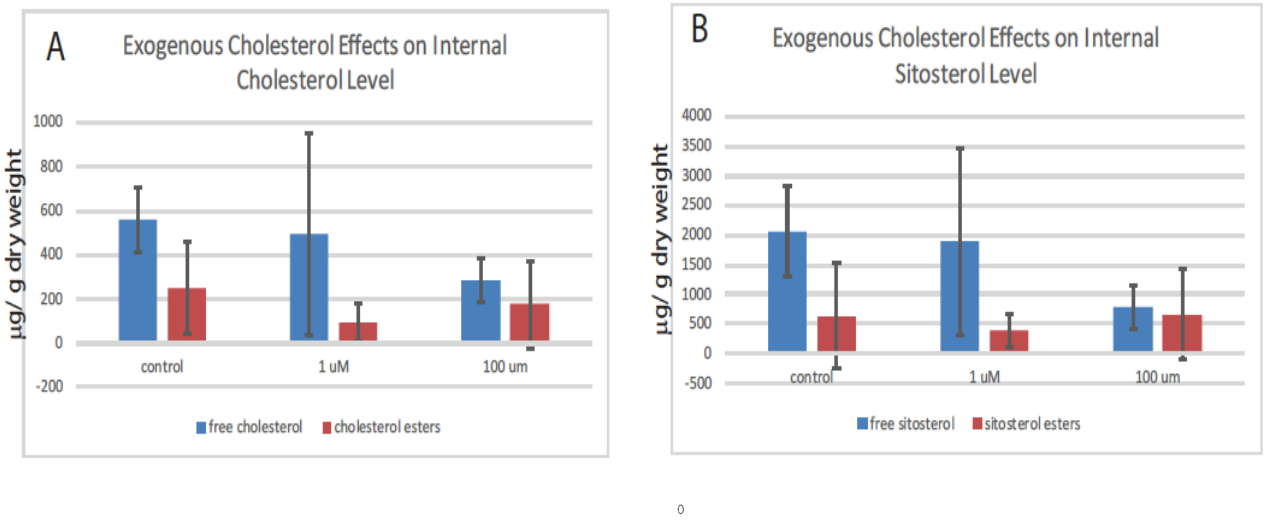


Figure 1. Micrograms per gram dry weight of cholesterol and sitosterol after treating seedlings for 7 days with exogenous cholesterol at 1 and 100 micromolar concentra- tions. A) Effects of treating with exogenous cholesterol on internal cholesterol levels. B) Effects of treating with exogenous cholesterol on sitosterol levels.

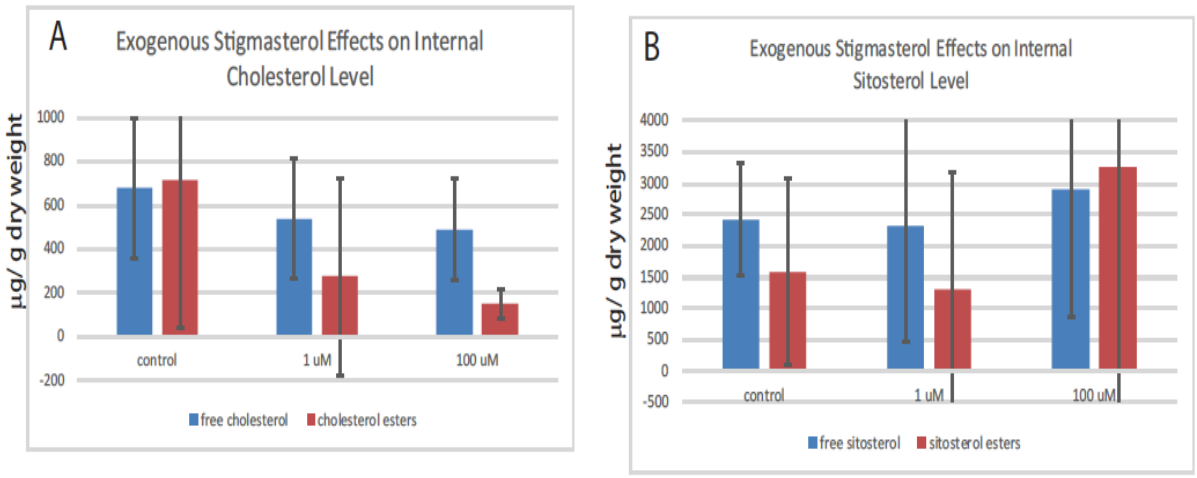


Figure 2. Micrograms per gram dry weight of cholesterol and sitosterol after treating seedlings for 7 days with exogenous stigmasterol at 1 and 100 micromolar concentra- tions. A) Effects of treating with exogenous stigmasterol on internal cholesterol levels. B) Effects of treating with exogenous stigmasterol on internal sitosterol levels.

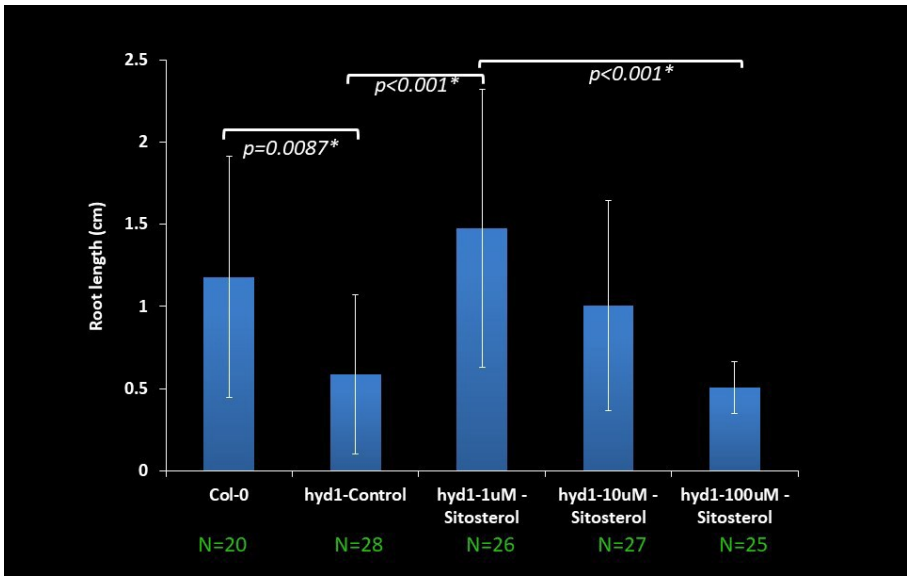


Figure 3. Chemical complementation of *hyd1* mutation at low, but not high, exogenous sterol concentrations. Root length was mea- sured six days after planting.

Hyd1 plants can be chemically complemented with exogenous sterols

To test whether the biologically active sterol pool in the tissue is affected by the application of exogenous sterols, we attempted to chemically complement the growth defects arising from mutations in the sterol biochemical pathway. Treatment of *hydra1* plants was successful, Figure 3. However, it was only successful at 1 μM applied sterol. Higher concentrations caused dwarfing, Figure 3, a reappearance of the initial dwarfed phenotype of the mutant. As shown above, Figures 1 and 2, higher concentrations of applied sterols generally decrease tissue sterols. Working on the hypothesis that low concentrations of applied sterols increase the biologically active sterol pool, while higher concentrations cause feedback inhibition of sterol biosynthesis, we examined the effects of exogenous sterols on the growth of wild type plants, with the expected outcome that high concentrations of applied sterols would phenocopy mutants in the biosynthetic pathway of sterols. They should also show similar growth to plants treated with chemical inhibitors of sterol biosynthesis.

Applied sterols alter germination, root growth, and root development

Cholesterol and stigmasterol at 100 μM inhibit seed germination, delaying it two days or completely inhibiting it, whilst treatment at lower concentrations does not affect germination, Figure 4. Germination is also delayed or inhibited in *hyd 1* or *hyd2/fk* mutants (Schrick et al. 2000). Five-day-old plants grown in 100 μM cholesterol or stigmasterol have qualitatively stunted growth with short roots, stems and small leaves, Figure 5, phenocopying *hydra1* mutants.

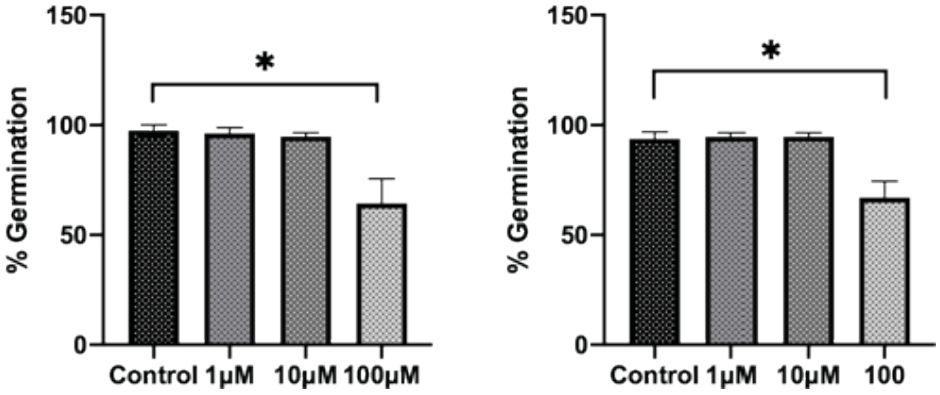


Figure 4. Seed germination percentage of Arabidopsis planted on 100 uM of sterol. A) Percentage of seed germination on 100 µM of cholesterol supple- mented agar media. B) Percentage of seed germination on 100 µM of stigmasterol supplemented agar media. Data has been recorded 5 days after planting (DAP)of seeds. (n=100-120).

phenocopying *hydra1* mutants. Plants grown at lower concentrations of sterol show quantitative changes in growth and development, but not the severe dwarfing phenotype. As shown by Souter et al. (2002) *hydra 1* and *hyd2/fk* mutant plants have severe root growth defects, *hydra 1* showing minimal root growth by day 7 (Short et al. 2018). Analysis of the root growth during day 2 and day 3 post-germination reveals a large difference between 100 uM treatment with either stigmasterol or cholesterol and the treatment with lower concentrations of sterol and the control plants, Figure 6a. Total Total shoot area also declines, Figure 6b-c after treatment with either cholesterol or stigmasterol.

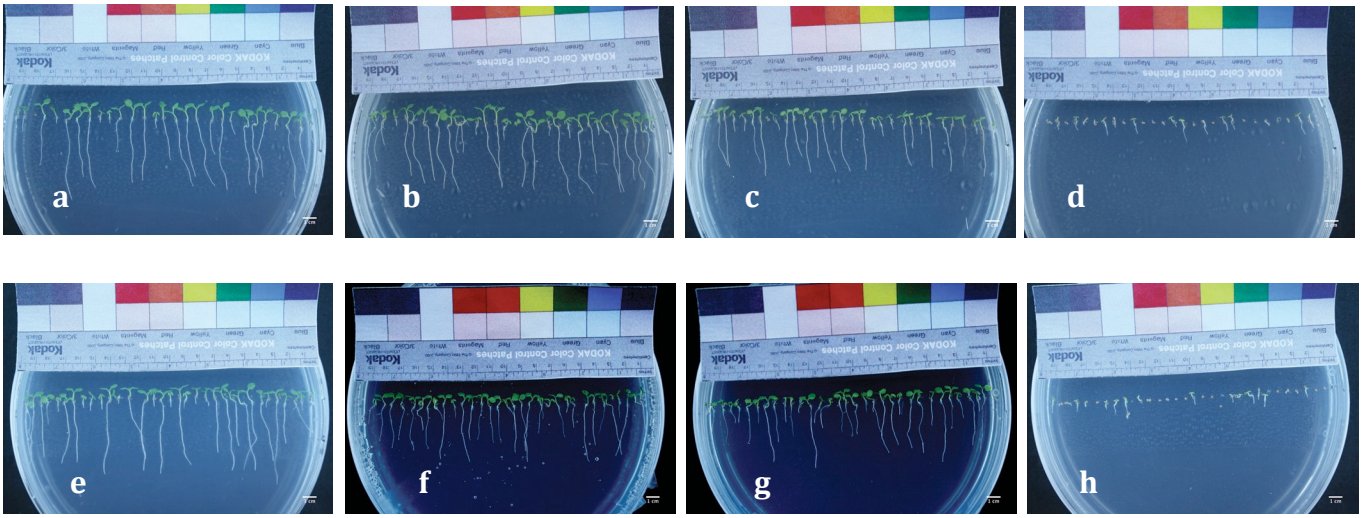
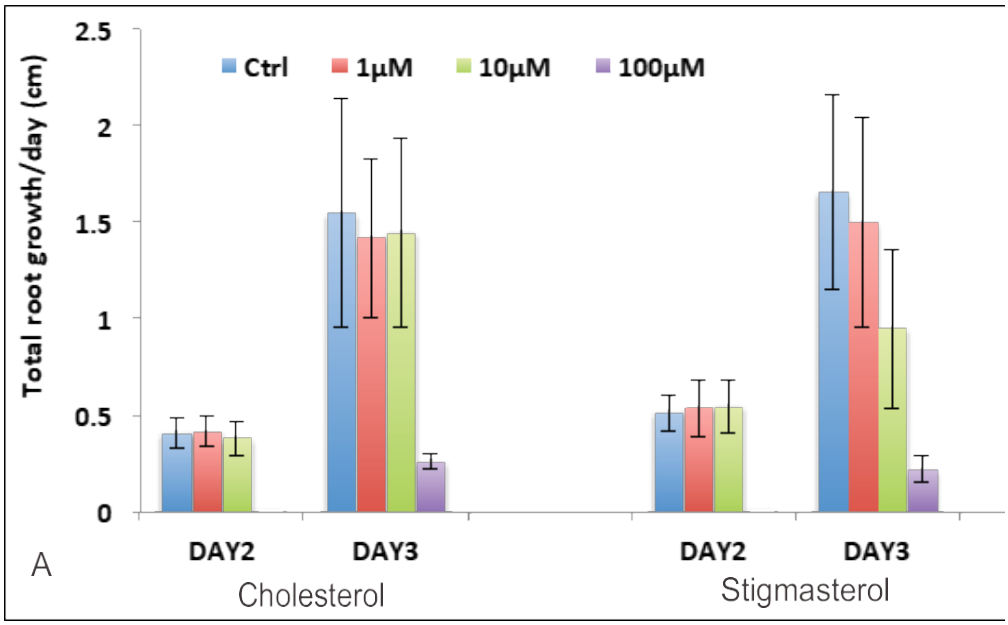


Figure 5. Effect of exogenous supplementation of cholesterol and stigmasterol on growth of the plants. a-d) 5-day-old seedlings of Arabidopsis after supplementation with cholesterol at various concen-trations. e-h) 5-day-old seedlings of Arabidopsis after supplementation with stigmasterol at various concentrations. Scale bars= 1 centimeter.



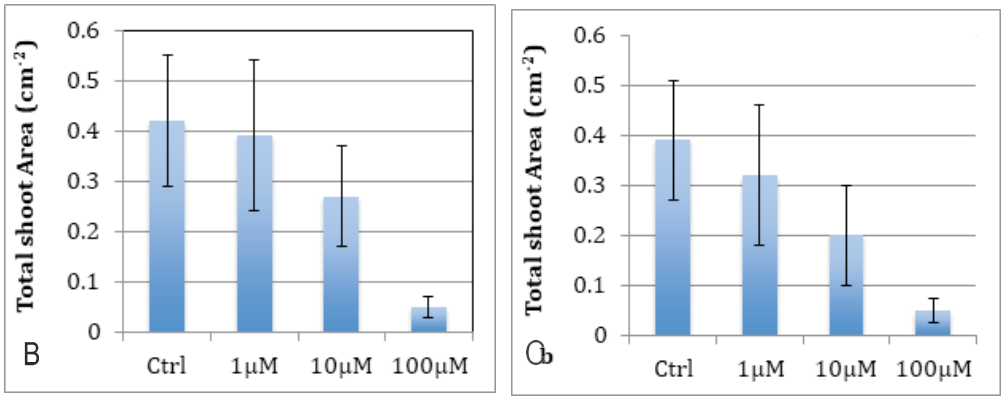


Figure 6. Total shoot and root developmental defect with exogenous sterols appli- cation. A) Root length analysis of Arabidopsis plants after cholesterol and stigmas- terol supplementation. B) Total shoot area quantification of 5-days old seedlings of Arabidopsis after exogenous cholesterol application. C) Total shoot area quantifica- tion of 5-days old seedlings of Arabidopsis after exogenous stigmasterol applica- tion.

The altered growth of the root in *hyd 1* can be attributed to the mislocalization of PIN2 proteins (Short et al. 2018) and redistribution of auxin. To determine the role of sterol supplementation in PIN protein localization, PIN1:GFP transgenic line was grown in 100 uM cholesterol-supplemented media. In untreated control root apical meristem cells (root tip) PIN1 localize mainly at the plasma membrane (PM) (Figure 7a) in a polarized fashion, however, cholesterol-supplemented seedlings show disturbed PIN1 localization to the cytoplasm and more uniform PM labeling (Figure 7b).

Changes in auxin distribution change cell morphogenesis, cell patterning and root growth. The 100 µM sterol-exposed seedlings show changes in cell patterning, developing additional

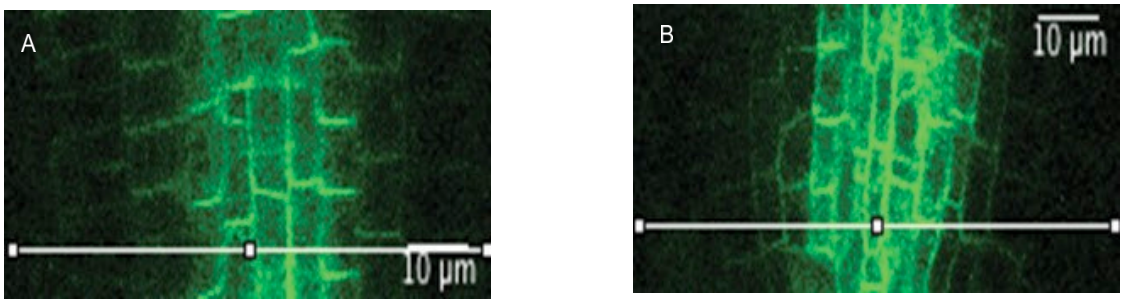


Figure 7. PIN1:GFP localization of plants grown with cholesterol supplemented media. 5-days old seed- ling of PIN:GFP transgenic line A) control and B) with 100 µM of exogenous cholesterol. Scale bars= 10 micrometers.

abnormal layers of cells in the root meristemetic zone when compared with untreated roots (Figure 8). Sterols are also required for the polarized growth of root hairs (Ovecka et al. 2010) and abnormal hairs form in *hyd1* mutants (Souter et al. 2002). When wild type seedlings grow in 100 µM cholesterol or stigmasterol, there is a lower density of root hairs and they do not grow as long (Figure 9).

Applied sterols change vascular development

Sterol biosynthetic mutants have been isolated using altered vascular development in the cotyledon as a selection assay. Cotyledons of wild-type plants possess a closed network of three to four loops of vascular veins, but the *cvp 1* (COTYLEDON VASCULAR PATTERN 1) mutants lack C24 sterol methyl transferase 2 and have open networks (Carland and Nelson, 2004). This is phenocopied in seedlings treated with 10 and 100 µM cholesterol, Figure 10.

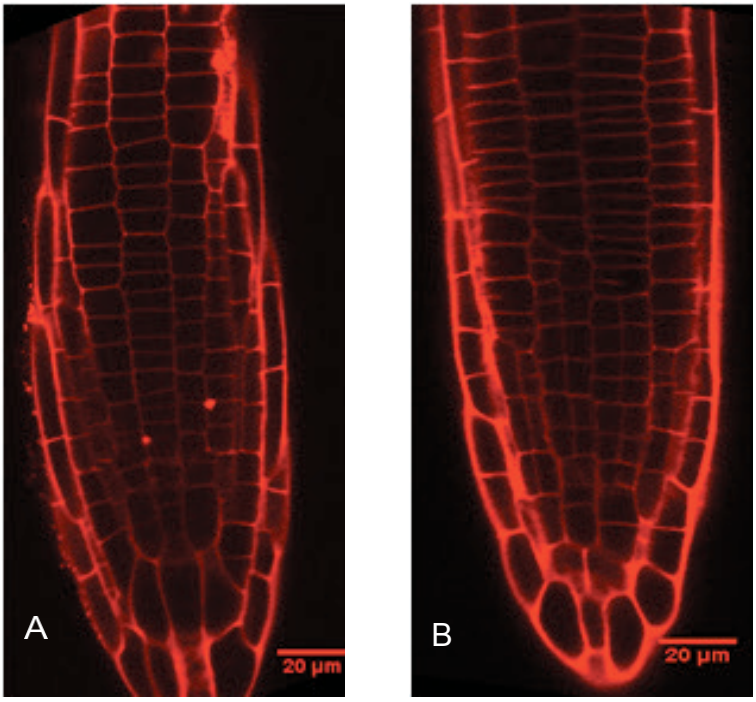


Figure 8. Root meristem cells anatomy of 5-days old seedlings grown on exogenous applied stigmasterol at 100 µM. a) Confocal image of propidium iodide labeled seedling of wild-type plant without sterol. B) Wild-type seedling with 100 µM of stigmasterol showing multilayered flat cells and labeled with propidium iodide. Scale bars= 20 micrometers.

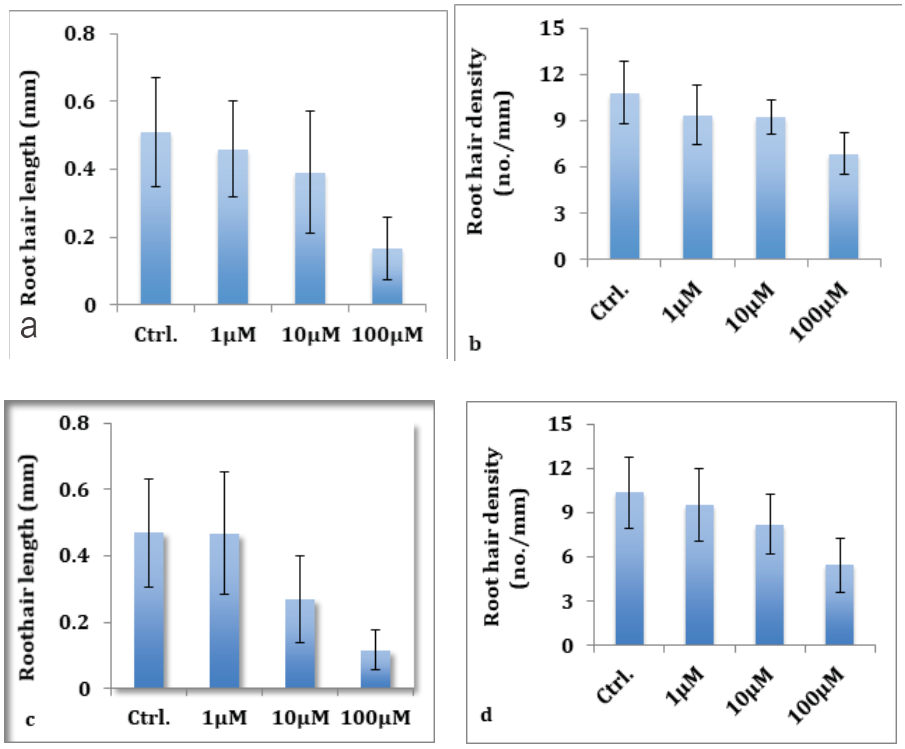


Figure 9. Root-hair growth analysis of 7-days old seedlings after growing on cholesterol and stigmasterol. a-b) root hair length and its density after plants are grown on cholesterol media. c-d) root hair length and its density after plants are grown on stigmasterol media.

Applied sterols change greening and chlorophyll content

Pre-germination addition of 1-100 uM stigmasterol or cholesterol produces more chlorotic seedlings, Figure 11. The chlorotic phenotype is more apparent at low concentrations of applied sterol than other effects, such as vein net opening or effects on root growth (see above). To further explore the cause of yellowing, we measured the *in situ*/ *in vitro* chlorophyll contents in the sterol grown seedlings and control samples via *in situ* optical measurements (Parry et al., 2014). Total chlorophyll was extracted from 9-days-old seedlings of sterol grown (pre-germination) plants and from control. Compared with the control, 1µM

and 10 μM cholesterol and stigmasterol showed reduced the levels of chlorophyll a+b, while treatment with 100 μM of either sterol showed near-complete inhibition of chlorophyll biogenesis, Figure 11.

Comparison with chemical inhibitors of the sterol pathway

Inhibitors of the enzymes involved in sterol biosynthesis include lovastatin, 15-azasterol, and fenpropimorph. Lovastatin inhibits HMGR, so also inhibits many other pathways besides sterol formation, such as the isoprenoid pathway involved in cytokinin formation. Fenpropimorph treatment phenocopies *fk –J79/hyd2*, but inhibits several enzymes in the sterol pathway including cycloeulenol-obtusfoliol isomerase, C-14 reductase, C-8,7 isomerase, and Δ7-reductase (He et al. 2003). On the other hand, 15-azasterol inhibits only the sterol C- 14 reductase (Schrick et al. 2002). All of these inhibitors diminish root growth, but not hypocotyl length, at 10 days after planting, Figure 12. As shown above, Figures 5-9, both root growth and total shoot area (need hypocotyl length from previous data!!) is inhibited by addition of 100 uM sterols. While addition of 100 uM cholesterol to plants along with lovastatin and fenpropimorph does not further inhibit root growth, there is an additional inhibition of root growth when added along 15-azasterol, Figure 12. This indicates that there are additional steps, besides the sterol C-14 reductase, in the sterol biosynthetic pathway that are inhibited with 100 uM cholesterol. The absence of an additional inhibition on root growth with 100 uM cholesterol after treatment with lovastatin and fenpropimorph is consistent with the idea that its targets include earlier enzymes in the pathway.

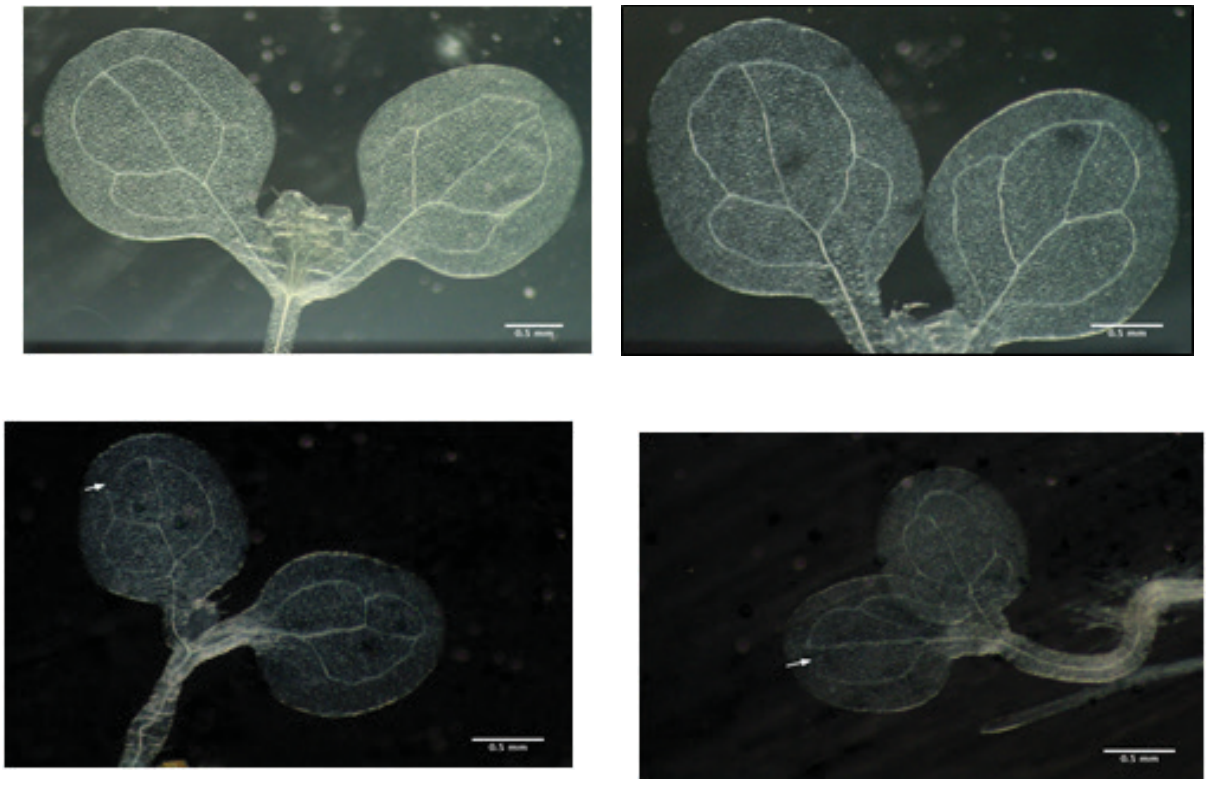


Figure 10. Effect of sterol on the symmetry of cotyledon vein patterning at different concentrations. a) Wild type plants 7-days after growth without supplemented cholester- ol. b) Plant with 1 µM cholesterol in media. c) Plant with 10 µM of exogenous cholesterol shows discontinuous vein pattern (white arrow). d) Plant grown on 100 µM cholesterol supplemented media and shows defect in vein patterning (white arrow). Scale bars = 0.5 millimeters.

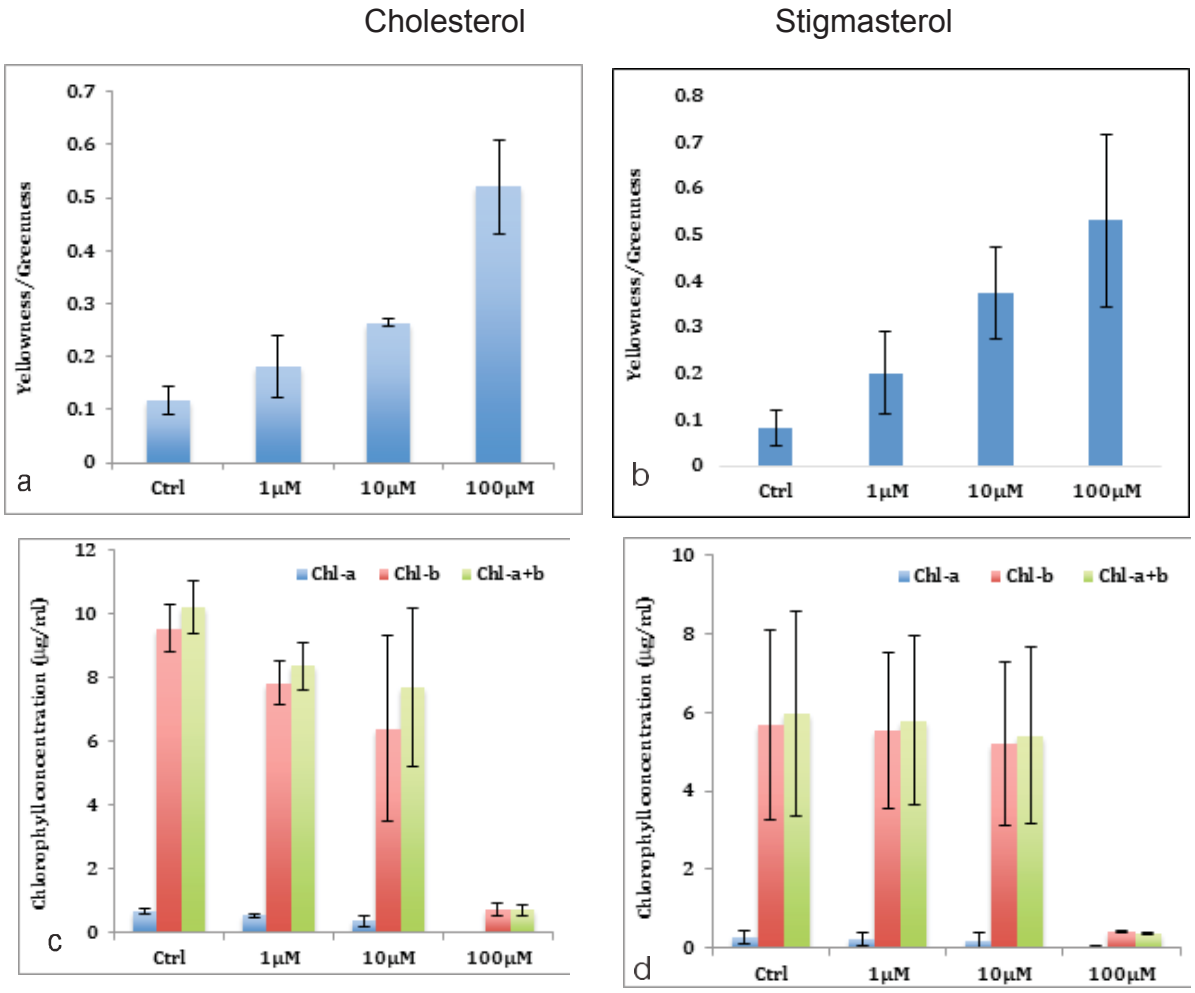


Figure 11 Analysis of leaf color and chlorophyll concentrations of exogenous sterols grown plants. a-b) Analysis of leaf phenotype of 5-days old seedlings, ratio of yellow- ness and greenness at different concentrations of cholesterol and stigmasterol. c-d) Analysis of chlorophylls (chl-a and chl-b) of 7-days old seedlings grown on cholesterol and stigmasterol.

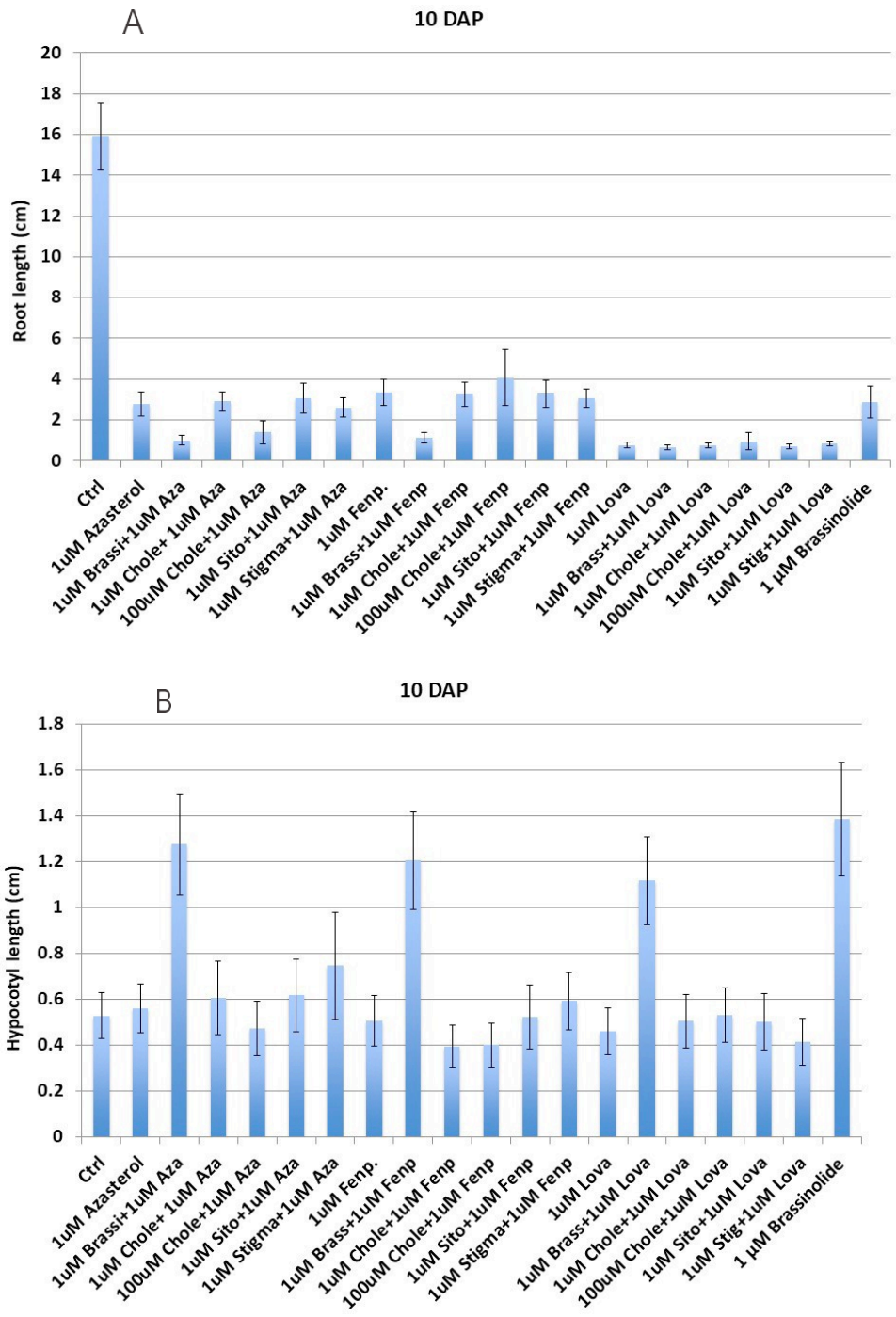


Figure 12. Effects of added sterols and brassinolide on growth inhbition by sterol biosynthesis inhibitors. A) Effects on root length. B) Effects on hypocotyl length.

This amplification of response by applied cholesterol is not as apparent in hypocotyl length, but there is a small additional effect of both 1 uM and 100 uM on shoot length in 15-azasterol treated seedlings. As described by others, (He et al. 2002, Schrick et al. 2004), addition of brassinolide appears to work in a different pathway because it has an additive effect on the root growth when given with all of the sterol biosynthesis inhibitors, Figure 12.

Discussion

Sterols are essential membrane constituents and hence, any changes in major sterols or altered sterol compositions affect membrane properties and their normal function. These changes in the membrane physiology influence diverse developmental aspects. So far in, previous studies, genetic mutation or treatment with chemical inhibitors have been used to study the physiological and developmental aspects of plants after sterol profile modification. Using the exogenous application of cholesterol and stigmasterol, we present evidence that it mimics effects on growth and development in Arabidopsis achieved with genetic mutations in the sterol pathway and treatment with chemical inhibitors. With the evidence that exogeneous application of sterols causes alteration in the sterol biosynthesis and uptake pathways, Figures 1 and 2, we come to the conclusion that exogenous sterols produce negative feedback on the sterol biosynthetic pathway, Figure 13. The changes in the total amount and ratio of sterols after treatment of plants are greatest at 100 uM cholesterol and stigmasterol, Figures 1 and 2. To test whether these treatments were simply generally inhibitory, we examined the effects of exogenous sterols on the growth of *hyd1* mutants, Figure 3. At low concentrations, i.e., 1 uM, the mutation could be chemically complemented

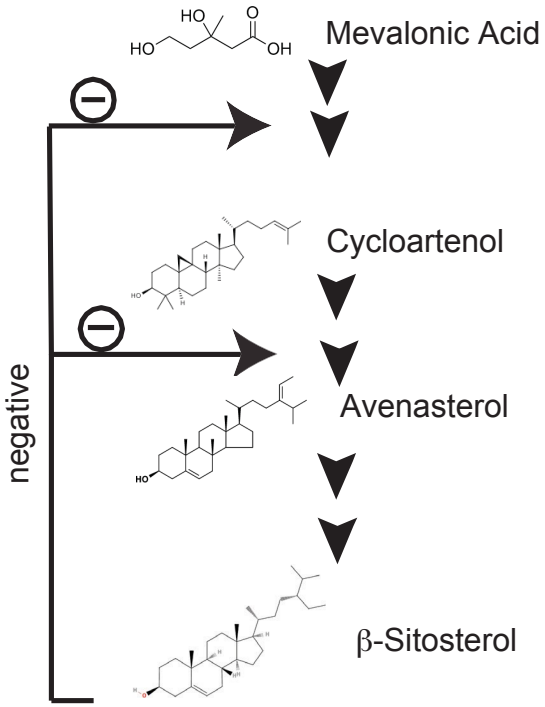


Figure 13. General outline of the negative feedback effects added sterol has on sterol biosynthesis, probably having an effect at multiple steps in the pathway either through direct inhibition of enzyme activity or on transcriptional regulation of enzymes.

with exogenous sterols, indicating that they enter the biologically active pool of sterols. Higher concentrations show the dwarfing phenotype, when germination occurs. The developmental phenotypes accompanying treatment with higher concentrations of sterols are not the result of general inhibition of growth, but specific to the developmental pathways found in mutants defective in the sterol biosynthesis pathway which also show dwarfing and embryonic developmental effects that reduce and delay germination including *smt1*(Diener et al., 2000), *fk*/*hydra2*(Jang et al., 2000; Schrick et al., 2000; Souter et al., 2002), *hydra1*(Souter et al., 2002), and *cotyledon vascular pattern1* (*cvp1*)/*smt2*(Schaeffer et al., 2001; Carland et al., 2002). The reduction in germination with increasing sterols is seen in Figure 4. The delayed and reduced germination of Arabidopsis. seeds and dwarf phenotype at a higher concentration (100 μM) of cholesterol and stigmasterol is consistent with work in tobacco where the genes of cholesterol biosynthetic pathway were overexpressed (Sonawane et al., 2016). The transgenic pCHOLESTEROL plants have also a similar phenotype to that found in sterol mutants which that accumulate cholesterol in relation to other phytosterols (Arnqvist et al., 2003).

Scanning electron microscopy has revealed that sterols mutants (*smt1*, *cvp1* and *cvp1smt3*) root epidermal cells have altered alignment of longitudinal cells and abnormal cell expansion and morphology (Carland et al., 2010). Our results with cholesterol and stigmasterol at 100 μM have demonstrated severe root growth defects, Figures 5 and 6.

Staining with propidium iodide to show the root developmental anatomy revealed defects in the cell division and elongation in stigmasterol (100 μM) supplemented plants, Figure 8. The stigmasterol plant roots show altered transverse cell alignment where cells are smaller and more abundant in division and elongation zones. Previously, sterol deficiencies have

been associated with the activation of genes for cell expansion and proliferation (He et al., 2003). This could come about from changes in PIN protein disposition. The PINs, which are auxin efflux carrier proteins have been proposed as candidates for sterols enriched nanodomain-localized proteins (Carland et al., 2002; Betts and Moore, 2003). PINs regulates the directional transport of auxin and mutation in PINs or mislocalization in PINs causes polarity defects in roots, embryo and veins (Robert and Friml, 2009). Interestingly, PINs protein family members (PIN1, PIN3) have been shown mislocalized in *smtorc*, *hyd1*, *hyd2/fk* and *cpi* mutant (Willemsen et al., 2003; Men et al., 2008; Pan et al., 2009; Pullen et al., 2010). Sterol analysis carried out in these mutants found β-sitosterol level significantly reduced but cholesterol level was higher than normal.

PIN2 protein also failed to redistribute asymmetrically after cytokinesis in the endocytosis-defective *cpi* mutant, indicating sterol-dependent PIN protein asymmetry establishment (Carland et al., 2010). Figure 8 shows similar PIN1:GFP mislocalization to that in sterol mutants in root cells of Col-0 grown on exogenously supplemented cholesterol at a higher concentration (100 μM). Although earlier work (Chapter II) reveals that sterols at 10 uM concentration do not influence endocytosis, it would be interesting to examine the sterol dependent endocytosis in plants treated with higher concentrations of sterols.

The effects of high external sterol concentration are also consistent with the known dependence of root hair tip growth on sterols (Ovečka et al., 2010), and see Chapter II, Figure 1. Both root hair initiation (as revealed by root hair density) and tip growth are inhibited, Figure 9.

The vascular pattern of cotyledons is controlled by *CVP1* (*cotyledon vascular patterning1*)/*SMT2* gene (Carland et al., 2002). Similar to the *cvp1*/*smt2* mutant where

cholesterol accumulates at the cost of β-sitosterol, treatment with 100 μM cholesterol produces defective veins patterning of cotyledons, Figure 10. The high concentration of exogenous sterols supplementation also leads to pale yellow or albino phenotype compared with relatively lower concentration and control plants, figure 12. Sterol imbalance or depletion causes the defect in plastids morphology. Isoprenoid compounds, which derived mainly from MVA pathway and MEP pathway are important for the chloroplast function (Andrade et al., 2017) and chlorophyll biosynthesis. Mutants in the isoprenoid pathway/ sterol synthesis pathway like *fkp1-1*(Ishiguro et al., 2010), *hmg1- 1*(Suzuki et al., 2004; Heintz et al., 2012), *ippi2*(Okada et al., 2008), *sqe1-5*(Pose et al., 2009) and *cas1-1*(Babiychuk et al., 2008) show defective chloroplast development, chlorosis, altered chloroplast proteins, pale green/albino phenotype and reduced chlorophyll and carotenoid pigments. The effect of external sterols is similar to that achieve with silencing the farnysyl diphosphate synthases in Arabidopsis (Manzano et al. 2016), where yellowing occurred in addition to reduced germination and growth, Figure 11. Seedlings with miRNA down-regulated farnesyl diphosphate synthases (*fps1*/*fps2*), which give rise to farnesyl phosphate, a precursor of all plant sterols and plastidial isoprenoids, have reduced chloroplasts and a chlorotic phenotype when induced post-germination, but when induced pre-germination produce severe dwarfing and seedling lethality (Manzano et al. 2016). The chlorotic symptoms in 100 μM cholesterol and stigmasterol treated seedlings is a consequence, at least in part, of depletion in chlorophyll concentration, Figure 11. The one possible function could be the role of sterols in the architecture of plastidial membrane, which has been reported in the previous work where sterols and lipids were found in the plastidial outer membrane. The second possibility is that sterols are part of the nanodomain that makes up ER-plastid

contact sites. The ER is involved in making sterols, but it does not store sterols (see Chapter II). Interestingly, specific interactions via contact sites between ER-plastids have been characterized (Andersson et al., 2007; Griffing, 2011; Schattat et al., 2011), which facilitates inter-organellar exchange of metabolites such as lipids, sterols and other non-polar compounds (Benning, 2008; Mehrshahi et al., 2014; Block and Jouhet, 2015). The experiments with inhibitors of sterol biosynthesis support the interpretation that exogenous sterols interfere with sterol biosynthesis at several levels. The chemical inhibitors of sterol biosynthesis, 15- azasterol (Rahier et al., 1980; Schmitt et al., 1980) and fenpropimorph (Mercer, 1993) shows similarity in root morphology (Schrick et al., 2004). In our experiments, there was an additional inhibition of root growth when 100 uM cholesterol was added with 15-azasterol, indicating that added sterol operates on other enzymes of the pathway not inhibited by 15-azasterol, Figure 12. However, when 100 uM cholesterol was added to plants treated with drugs that influence sterol biosynthesis earlier in the pathway, fenpropimorph and lovastatin, no additional effect on root growth was seen, Figure 12. In conclusion, we propose that the addition of sterols with the appropriate solubilization agent, MβCD, produces a negative feedback on the biosynthesis of sterols, Figure 13, as is the case with end product inhibition of cholesterol biosynthesis in animal cells (Nes, 2011). As with cholesterol biosynthesis in animal cells, the sensitive biosynthetic enzymes, and perhaps transcription factors, at which the end products and intermediates act is probably complex. However, given that the addition of sterol does not add to the developmental alteration induced by specific inhibitors of the sterol biosynthetic pathway, it probably acts in the same general way.

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CHAPTER IV

EXOGENOUS STEROLS ARE AN EFFECTIVE PRE-EMERGENT HERBICIDE WITH A NOVEL MODE OF ACTION

Introduction

Some of the first genetic studies on the Arabidopsis sterol biosynthetic pathway revealed that mutants early in the pathway were embryo lethal. The mutants, *smt1*/*cephalopod (cph*), *fackel*(*fk*)/*hydra2*, *hydra1* and *cvp1*/*smt2* produced severe embryonic developmental defects and/or embryo lethality (see Chapter I, Figure 3 for their location in the pathway). They changed the sterol profile of these plants, often causing an accumulation of cholesterol and campesterol in the imbibed seed. Less severe mutant alleles produced morphological developmental defects such as short roots and hypocotyls and abnormal cotyledons (Diener et al., 2000; Grebenok et al., 1998; He et al., 2003; Schrick et al., 2000; Schrick et al., 2002; Souter et al., 2002). The inability of BR application to rescue this class of mutants distinguishes them from a second class of mutants, occurring later in the pathway. However, the rescue with BRs of these late-pathway sterol mutants, such as *dwf7*, *dwf5*, and *dwf1* (see Chapter 1, Figure 3), was often only partial and they had defects the production of other sterols besides BR, Chapter I, Table 2. These late-pathway sterol mutants had less of an effect on embryo development, but produced abnormal cell division and cell expansion resulting in dwarfing (Choe et al., 1999a; Choe et al., 1999b; Choe et al., 2000).

Besides the absence of an effect of BRs, the early pathway mutants could not be rescued with other end product sterols, such as cholesterol, stigmasterol and β-sitosterol (Carland et al., 2010; Clouse, 2002). However, as shown in Chapter III, low concentrations (less than 1 μM) of added cholesterol or stigmasterol rescues the *hyd1* mutant when the sterol is

delivered for uptake along with MβCD. Furthermore, when sterols are applied at higher concentrations with MβCD, changes in development occur, sometimes in a dose-dependent manner, with a more severe phenotypes occurring at higher concentrations. One of the changes in development occurring at high concentrations is the inability of seeds to germinate, or if germinated, produce plants with severe dwarfing. These phenocopied the mutants in the early stage of the sterol pathway. As discussed in Chapter III, this is probably the result of feedback inhibition of the sterol biosynthetic pathway by its end products. The hypothesis was then developed that these results with Arabidopsis could be translated to other plant species, and, extending that line of reasoning, proposing that the encapsulated sterols (sterol plus MβCD) could be used a pre-emergent herbicide. Using these results, we have made a provisional patent claim (Griffing and Kumar, 2019) and are testing the sensitivity of a wide variety of plants, both crop and weed species, to the effects of encapsulated sterols. To translate this work from Arabidopsis to crop and weed species, we have developed a low-cost hydroponic method to deliver a specific quantity of sterols to the plants as they grow and develop. The set-up is the product of a survey of hydroponic systems that can be adapted to crops from use with Arabidopsis, see Appendix (Kumar and Griffing, 2019). This chapter provides some of the preliminary results of those studies.

Materials and methods

Chemicals

Cholesterol (C3045, Sigma-Aldrich, USA), stigmasterol (S2424, Sigma-Aldrich, USA), and b- sitosterol (567152, Millipore-Sigma, USA) were prepared as stock solutions with a slight modification from that described (Holtta-Vuori et al., 2008). Sterols were dissolved in 100% ethanol at 1.5 mM concentration and combined with methyl-β-cyclodextrin (MbCD) (Sigma- Aldrich, USA) at a molar ratio of 1:3 to a final concentration of 1-300 bM sterol and 1-900 μM MβCD. The probe was either sonicated for 30 minutes and vortexed for 15 minutes before use, or just vortexed for 30 min before use. Defguard, a suspension of Bacillus amyloliquefaciens D474, was purchased from Amazon.

Plant materials

We routinely use Arabidopsis Columbia-0 wild type plants (Lehle Seeds, Austin TX) for these experiments. *Hyd2* homozygous, and *fk-5D8*, *hyd1-E508*, and *atm1* heterozygous Arabidopsis seeds were a generous gift from Kathrin Schrick (Kansas State University, Manhattan, KS). Corn (*Zea mays*) c.v. Delectable, pea (*Pisum sativum*) c.v. Little Marvel and bean (*Phaseolus vulgaris*) c.v. Blue Lake Pole were obtained from Everwild Farms Inc., Sand Creek WI. Cotton seed (*Gossypium hirsutum*) seed was a generous gift from Kerthi Rathore (Texas A&M University, College Station, TX). Weed seed (Waterhemp, *Amarathus tuberculatus*; Horseweed, *Erigeron canadensis*; Annual bluegrass, *Poa annua*) was a generous gift from Muthu Bagavathiannan (Texas A&M University, College Station, TX).

Petridish procedure

For petri dish growth, 1% (w/v) agar (Sigma-Aldrich, USA) was dissolved in ½ strength

Murashige and Skoog medium containing vitamins (Caisson labs, USA) (+/- sterol), and the pH was adjusted to between 5.6 and 5.8. The seedlings were grown in vertically-placed Petri dishes containing this medium for 5-10 d at 22o under the continuous white light (50 µmol m-2sec-1 photosynthetically active radiation).

Hydroponic procedure

For Arabidopsis, plant seeds were surface sterilized prior to imbibition. For other seeds, imbibition was over 48 hr, 24 h in the dark at 4C, and 24 h in the dark at 21 C followed by surface sterilization with 10% (v/v) sodium hypochlorite bleach (Clorox). The imbibed seeds were planted in hydroponic containers. Hydroponic culture was a modification of that described by Alatorre-Cobos et al. (2014). The seeds were placed on a mesh inserted into the bottom of a sterile polypropylene 2 oz cup with a lid (TY-M2-100, Bingwu, sold by Amazon. Com) inset into either a 4 oz sterile polypropylene cup (TY-M4-N50 Bingwu, sold by Amazon.com) or a black 5.5oz sterile polypropylene Dart Conex cup (B07BK1DPPS, Table Top King, sold by Amazon.com). Enough medium (½ strength Murashige and Skoog medium containing vitamins (Caisson labs, USA) (+/- sterol), and the pH was adjusted to between 5.6 and 5.8) was added to bring it up to the level of the mesh. Corn plants were transferred to 8/16 oz cup system at 3 weeks (Dart Solo MicroGourmet MN16-0100 16 oz. Contact Clear Polypropylene Deli Container and Dart Solo MicroGourmet MN8-0100 8 oz. Contact Clear Polypropylene Deli Container from Amazon. com). Arabidopsis was grown for to maturity (~75 days) in a 18:6 h photoperiod at 22C with 100 µmole cm-2sec-1 photosynthetically active light.

Photography and image analysis

Plants in hydroponics were photographed under controlled lighting on a dark background,

using a Canon EOS Ti1 using an 18-55mm lens. The image included an internal scale and color card. Analysis and post-processing of images was performed with ImageJ (Schneider et al., 2012) FIJI (Schindelin et al., 2012), Adobe Photoshop® and Adobe Illustrator ®, (CS 6, Adobe Systems, San Jose, CA, USA).

Results

Evaluation of hydroponics for translational studies

To test the ability of our hydroponic system to handle short- and longer-term growth of large-seeded crop plants that grow to a height of 60 cm or more, seeds were imbibed in water and surface sterilized. The seeds were placed aseptically on a mesh in a small (2 oz) sterile cup, which was then inserted into a larger (4 oz) sterile cup, filled with 70 ml sterile, half-strength MS media. The results for Arabidopsis, corn, and beans shows that plants show normal growth pattern and phenotype in this system, Figure 1. The rate of growth is faster for Arabidopsis in hydroponics than in agar, producing four sets of rosette leaves at week 2, compared with two sets in agar. Growth also appears to be slightly faster than most soil-grown plants.

Once plants germinate and grow to a height of more than 5 cm, the sterile lid is removed and the plant media (70 ml) is inoculated with 100 microliters of *Bacillus amyloliquefaciens* D474, which not only competes with other bacteria, but produces a variety of anti-fungal agents (Xu et al., 2013). This is particularly important in plants open to the environment and treated with cholesterol, which encourages the growth of fungi. At this stage, the plants can be transferred to larger cups, an 8 ounce insert in a 16 ounce cup, Figure 2.

The uptake and/or breakdown of the sterols by the bacteria and fungi has yet to be determined. However, *Bacillus subtilis* can convert cholesterol to 4-cholesten-3-one, the first breakdown product in the microbial cholesterol metabolism pathway (Giorgi et al., 2019). Other soil microfloral clades take up and metabolize cholesterol and other sterols (Garcia et al., 2012; Mohn et al., 2008). The most effective end product in inhibiting pre-

emergent germination may be β-sitosterol, which is known to have anti-fungal activity on its own (Moosavi et al., 2019). Inoculation with *B. amyloliquefaciens* may not be necessary for those future studies.

Effectiveness of sterols as a pre-emergent inhibitor of germination

As shown in Chapter III, addition of 100 μM sterols to the growth medium of Arabidopsis when added during germination produces severe dwarfing and other development defects typical of interference with the sterol biosynthetic pathway. This is in those plants that actually succeed in germinating. About 40% of the seeds do not germinate, Figure 3. Increasing the sterol concentration to 300 μM, completely inhibits the germination of the seeds, Figure 4. To test the germination of other plants in the presence of 300 μM sterol, an initial test with corn was conducted. Seeds imbibed and germinated in the presence of 300 μM sterol have swollen radicles, but no germination, as in petri dish-grown Arabidopsis, Figure 5. Future tests will assess the effect of pre-emergent treatment with sterols on a variety of other crop plants and weed species.

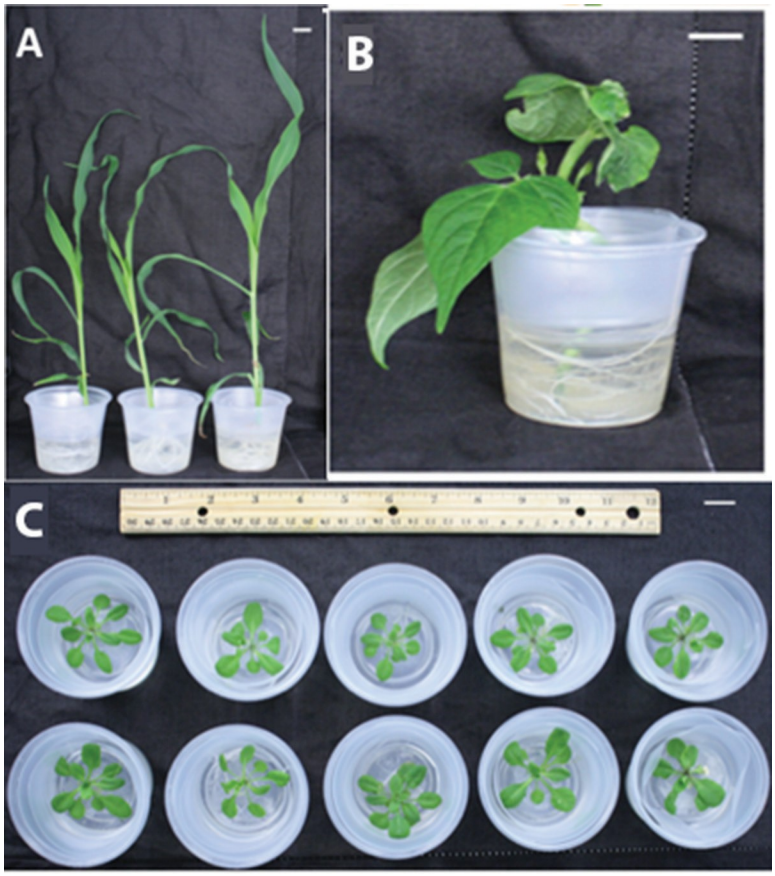

Figure 1. Hydroponically-grown plants using 2oz/4oz cup system. A) Corn plants 3 weeks old. B) Bean plant 2.5 weeks old. C) Arabidopsis plants 2 -weeks old.

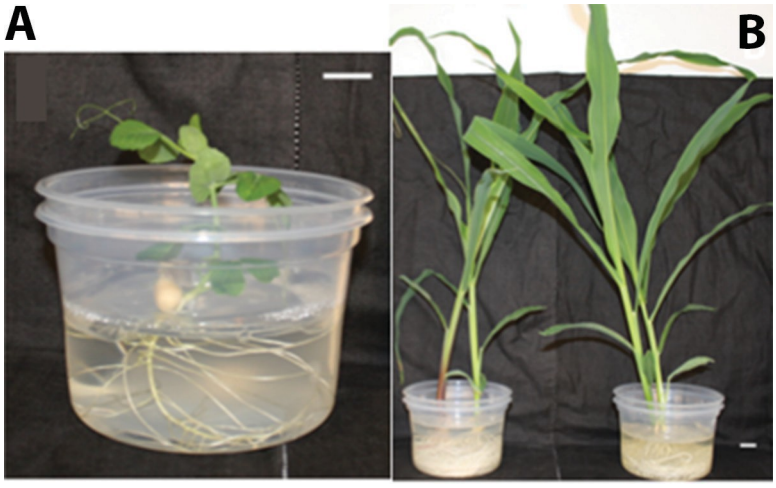

Figure 2. Hydroponically-grown plants in 8oz/16oz cup system. A) Pea plant two weeks old B) Corn plants 3.5 weeks old..

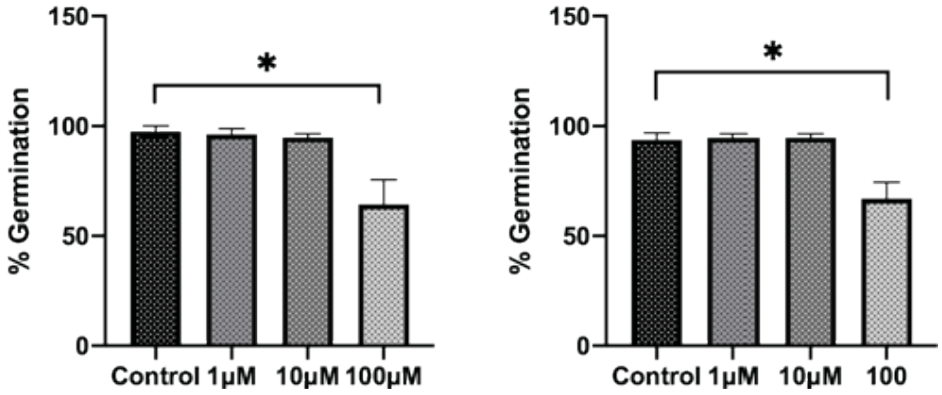

Figure 3. Seed germination percentage of Arabidopsis planted on 100 uM of sterol. A) Percentage of seed germination on 100 µM of cholesterol supple- mented agar media. B) Percentage of seed germination on 100 µM of stigmasterol supplemented agar media. Data has been recorded 5 days after planting (DAP) of seeds. (n=100-120).

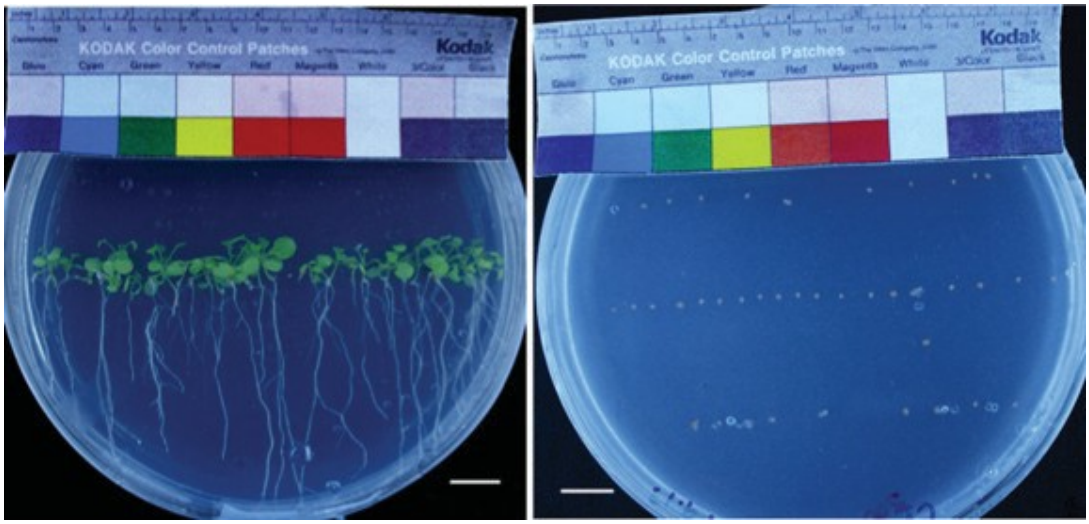

Figure 4. Seed germination test of Arabidopsis planted on 300 µM of sterol. A) Wild-type seedlings grown on normal agar media. B) Wild-type seedlings grown on 300µM of choles-

-terol supplemented agar media. Data has been recorded 2-weeks after planting of seeds.

Analysis of post germination treatment of Arabidopsis seedlings with sterols

As shown in Chapter III, 100 μM cholesterol, when included in the germination medium, severely dwarfs the plant, causing changes in vasculature, inhibiting root growth, and altering chlorophyll production by the seedling. We tested the hypothesis that it would not have the same effects on the growth and development of plants germinated in the absence of applied sterols. Arabidopsis seedlings germinated in hydroponic systems without sterols grew normally for two weeks. At that time, 100 uM cholesterol was added to half of the samples and allowed to continue to grow for two more weeks, reaching seed set, Figure

The development of the treated plants does not change in the presence of added sterol. To quantify of the effect of sterols added after germination, root growth was measured. Treating seedlings in 100 μM sterol during germination, severely reduces root growth Figure 2, Chapter III. However, if the same concentration of sterol is added to plants four days after germination on sterol-free medium, there is no change in root growth, Figure 7. Future work will extend these studies to include 300 μM sterol treatments.

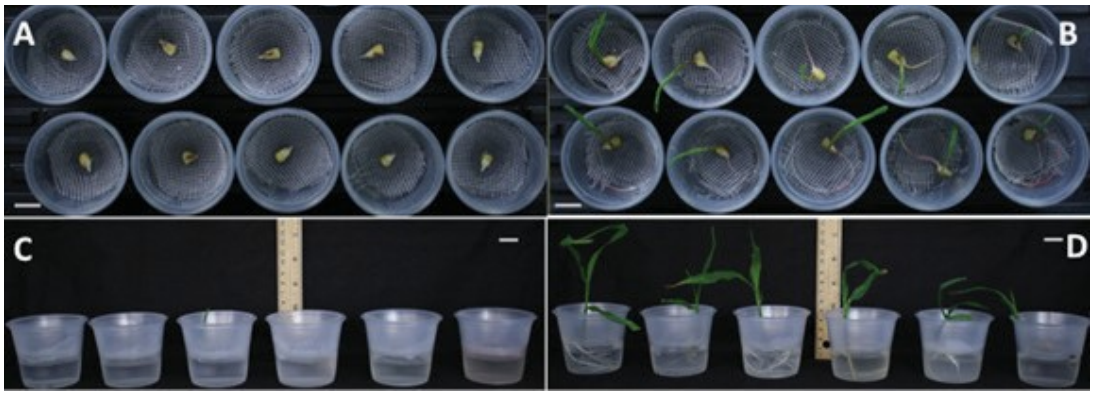

Figure 5. Hydroponically grown corn plants. A-B) one-week old corn plants. A) Corn seeds after one week of planting on MS-media supplemented with 300µM cholesterol. B) Corn plants after one week of planting on normal MS-media. C-D) Two-weeks old corn plants. C) Corn seeds after two-weeks of planting on MS-me- dia supplemented with 300 µM cholesterol. D) Corn plants after two-weeks of planting on normal MS-media. Scale bars= 2 cm.

Discussion

One of the challenges of bringing discoveries in Arabidopsis to real world applications is that Arabidopsis is quite a different creature from our crops and most of our weeds. The most important crops in Texas are cotton, corn, hay, grain sorghum, and wheat. There are important non-crops that require herbicides such as the turf grass in golf courses and lawns. The most pernicious weeds in Texas are *Amaranthus palmeri* (careless weed or pigweed), *Ambrosia trifida* (giant ragweed), *Erigeron canadensis* (horseweed or marestail), and *Poa annua* (annual bluegrass). All of these are quite different from the brassicaceous, *Arabidopsis thaliana*. To translate our findings done on Arabidopsis in agar to crop and weed species requires a system where we can repeat the work without agar and in larger containers, amenable to growing large plants, while regulating root microflora and nutrients.

To this end, we recently reviewed hydroponic systems that work with Arabidopsis that can also adequately grow crop and weed species in a way where they can be phenomically characterized, see Appendix (Kumar and Griffing, 2019). With that background, we developed a modification of existing systems that works to grow Arabidopsis and crop plants, Figures 1 and 4. Not only does the system work for growing the plants, but also the results achieved with Arabidopsis appear to translate to a quite distantly related monocot, corn. Initial work with weed seeds is slow because the percent germination of our weed seeds is low in soil, agar, and hydroponics.

The advantage of this hydroponic system is that it can be adapted to larger-scale phenomic studies, while maintaining each individual plant separately. For initial studies, imaging of the plants under standard lighting at a distance of 70 cm above the plant produces a high-resolution image of the Arabidopsis rosette, Figure 1. In the future, we plan to take images of the rosettes of 100 -200 plants once per day using a series of automated, fixed-position cameras that wirelessly communicate with an image server. For monocots such as corn or bluegrass, camera mounting will also be in the horizontal dimension against a dark, movable screen. Four key properties of sterol treatment make it a desirable pre-emergent herbicide. First, it completely inhibits the germination of seeds grown in its presence. The concentration for complete inhibition is between 100 and 300 μM sterol, Figures 3 and 4. Although this concentration is higher than some other herbicides, it is cheap and non-toxic. The estimated cost for this would be $9-25 per acre at an application rate of 6 liters per 1000 square feet using commercial sources, e.g., Sigma-Aldrich, well within the range of existing agrichemical costs of up to $100 per acre for herbicide.

Second, it is broad-spectrum (affects both monocots and dicots), Figure 5, thereby superior to dicot weed killers such as atrazine and Dicamba. Third, it is a true pre-emergent herbicide, not affecting the growth of already germinated plants, Figures 6 and 7. Fourth, it has a new mode of action. As described in Chapter III, the application of sterols during germination reduces the sterol biosynthetic capability of the plant, altering the amount and ratio of endogenous sterols necessary for seed germination. Recent reviews of the state of herbicide research provide a summary of the modes of action of herbicides (Kraehmer et al., 2014a; Kraehmer et al., 2014b). Of the seventeen known modes of action of herbicide, thirteen have greater than 1% market share. They include the following targets (in order of market share with examples):

1) 5-Enolpyrvylshikimate 3-phosphate synthase (Glyphosate 21% market share),
2) Acetolactate synthase, ALS (Chlosulfuron 17% market share)
3) Very long chain fatty acid synthesis, VLCFA (Alachlor 11% market share),
4) Photosystem II inhibitors, (Atrazine 10% market share),
5) Acetyl coenzyme A carboxylase, ACCase (Diclofop 8% market share),
6) Auxin (Dicamba 8% market share),
7) 4-hydroxyphenylpyruvate dioxygenase, HPPD (Pyrazolynate 5% market share)
8) Photosystem I (Paraquat 4% market share)
9) Protoporphyrinogen IX oxidase, PPO (Nitrophen 3% market share)
10) Tubulin (Trifluralin 3% market share)
11) Glucan synthase, GS (Glufosinate 2% market share)

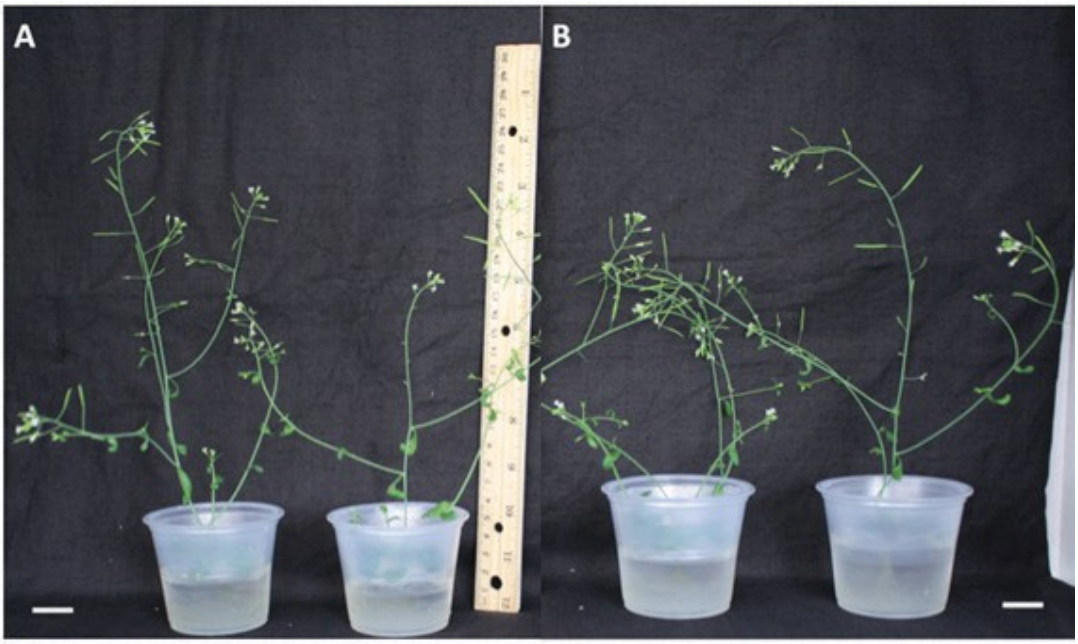

Figure 6. Hydroponically grown Arabidopsis. A) 4-weeks old Arabidopsis plants on normal MS-media. B) 4-weeks old Arabidopsis growing on MS-media supple-mented with 100µM cholesterol after growing on normal MS-media for 2-weeks. Scale bars= 2 cm.

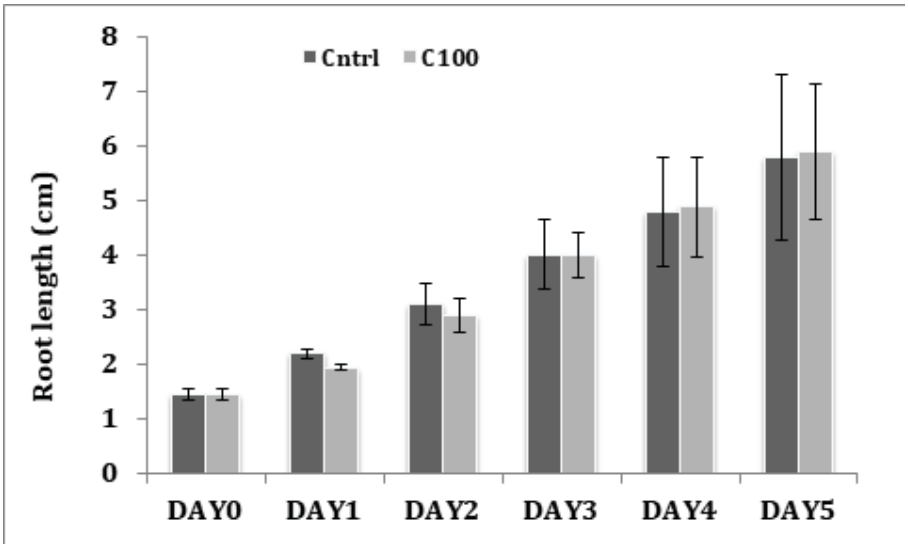

Figure 7. Root growth analysis of Arabidopsis seedling grown 4 days on normal agar media and there after transplanted on 100 uM of cholesterol. DAY 0= day of transplanting. (n=150-160)

12) Phytoene desaturase, PDS (Norflurazon 1.5% market share)

13) 1-Deoxy-D-xylulose 5-phosphate synthase, DXS (Clomazone 1.2% market share)

All of these were discovered before 1985. Other herbicides, with another 14 modes of action have been discovered since then (at a cost of $250 million per herbicide by 1995), but none have come to market for reasons such as high application rates, incomplete weed spectrum, and high costs of production. Also the limited amount of chemical variation of these new mode of action inhibitors for their targets further hampers optimization for efficacy and low field application rates (Kraehmer et al., 2014a). Other classes of chemicals, called safeners, have been developed that prevent known herbicides from injuring crop plants. They are often marketed as seed treatments or herbicide tank mixes (Kraehmer et al., 2014a).

The sterol biosynthetic pathway has not been ignored as a potential mode of action for herbicides. Some of the inhibitors of the pathway, such as 15-azasterol, fenpropimorph, and lovastatin (see Chapter III), were initially developed as herbicides (Benveniste, 2004). However, the toxicity of these chemicals preclude their use, since they target the same enzymes used for sterol biosynthesis in animals. Lovastatin, is a founding member of the class of cholesterol-lowering drugs, the statins, commonly used for treatment of high cholesterol in people.

The novelty of using the end products of the plant sterol pathway to specifically inhibit the sterol biosynthetic and/or uptake pathways during germination in plants provides an avenue to exploit this mode of action as a non-toxic herbicide. Although toxicity has not been a general concern for the end-user of herbicides, given the guidelines for use and application by the Environmental Protection Agency in the US, the growing concern of the toxicity of the most commonly used herbicide, glyphosate (Acquavella et al., 2016; Zhang et al., 2019),

puts this feature of herbicides in the spotlight. The low toxicity of, for example, β- sitosterol, found in the clinical studies that have studied its use as a nutritional supplement in humans (Cicero et al., 2002) bode well for using it as a potential herbicide, even at relatively high application rates. The other component of the herbicide, MβCD, is used to reduce toxicity of a variety of drugs (Mantik et al., 2019) and has demonstrated no significant toxicity in studies on rats and dogs (Bellringer et al., 1995).

The importance of this work to understanding plant biology lies in the observation that seed germination is exquisitely sensitive to changes in external sterols, Figures 3-5, when delivered with MβCD, but non-germination plant growth is relatively insensitive, Figures 6 and 7 (at least when it is applied for uptake by roots). As discussed in Chapter I, the mechanisms of movement of sterols throughout the tissues of the plant are unclear, but there we presented a model for movement throughout the plant, starting in the source tissues, such as the shoot apical meristem, and ending with sink tissues, such as the elongating root cells. The uptake is generally non-vesicular, as described in Chapter II. In Chapter III, we have shown that there is end product inhibition of sterol production in growing seedlings. How these end products are sensed by cells which take them up and how they are perhaps transported to the sites of synthesis in the plant are unknown.

Likewise, we do not know how sterols move throughout the embryo and influence the stages of germination. It is quite likely that sterol transport and availability are intimately linked to the cellular transport of PIN proteins (see Chapter III) and ABC transporters that, in turn, control auxin movement and set up polarity gradients in the emerging seedling. These are the subjects of exciting future studies.

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CHAPTER V

OVERALL SUMMARY

In our study, we have tried to answer some of the very important aspects of sterol biology in plants, i.e., how it moves inside the plant cell and how exognenous sterols affect the growth and development of the plant, where we have examined concentration-specific effects of sterols on the growth and development. In these studies, we have used typical lab procedures to grow Arabidopsis plants, which, for seedlings is an MS-based agar media and, for mature plants, is a soil-based potting system. As we began to explore the herbicidal properties of exogenous sterols, we translated the results to crop and weed species and, in the process, tested our own version of a hydroponic system in which the concentration of herbicide was accurately controlled. This low-cost hydroponic system successfully produced mature plants from a variety of plants (Arabidopsis, corn, pea, bean). Our hydroponic system was based on the use of a single cup, which provides us more leverage in the cultivation of tall crop plants compared with a single tube-based system (Kumar and Griffing, 2019). In our single cup hydroponic system, we have tested varieties of seeds and they have grown well and we were able to perform our herbicide tests successfully. Initial experiments show that crop plants and weeds are inhibited in germination and growth when the sterol is included during imbibition. Crop plants grown in the absence of sterols show excellent growth in the single-cup system. This has formed the basis for a provisional patent.

Unlike in mammals, where the cellular uptake mechanism and organismal transport of sterols is known, in plants, the transport mechanisms of sterols to its site of action are not well established. Previous researchers suggested endocytosis as a primary pathway for

internalization and transport of sterols in plant cells. Our study has not only revealed that this pathway was not used by sterols, and that the plant cell transports sterols using a novel mechanism of transportation. To explore this uptake pathway, we used fluorescent sterols (dehydroergosterol, DHE, and Bodipy- cholesterol, BCh), which are structural analogs of endogenous cholesterol. The movement of these sterols was tracked in real-time using confocal (BCh) and two- photon imaging (DHE).

The initial site of labeling with these sterol dyes is the PM and, followed the nuclear envelope (NE) and non-endocytic vesicles, primarily oil bodies. Except for the PM, these organelles are not associated with the endocytic pathway but are derived from or are part of the endoplasmic reticulum (ER) (Figure 1). This result was confirmed by the experiments with the over-expression of auxillin 1 (AUX1 line), which blocks most of the endocytosis. The Bch internalization and its nuclear envelope labeling in AUX1 over-expresser line support our hypothesis that the transport of sterol via the non-endocytic route. The discovery of this novel trafficking pathway, which differs from vesicular endocytosis, suggests the involvement of different pathways of sterol transport. These include potentially important participating membrane contact sites (MCSs) between the ER and the PM and other organelles, and cytoplasmic carrier proteins or transporters (Figure 1). The potential role of MCSs and cytoplasmic carrier proteins appears more realistic when we look at work in mammalian cells or yeast. Sterol transport in mammalian and yeast cells involve MCS (ER-PM) and carrier proteins or StART-like proteins. The presence of orthologs of the animal proteins found at contact sites, e.g. VAPs, and of ER proteins involved in transport of sterols from the PM in yeast (StART

domain containing) proteins in plants further strengthen the hypothesis that MCSs and carrier proteins play a role in non- endocytic sterol transport in plants (Figure 1).

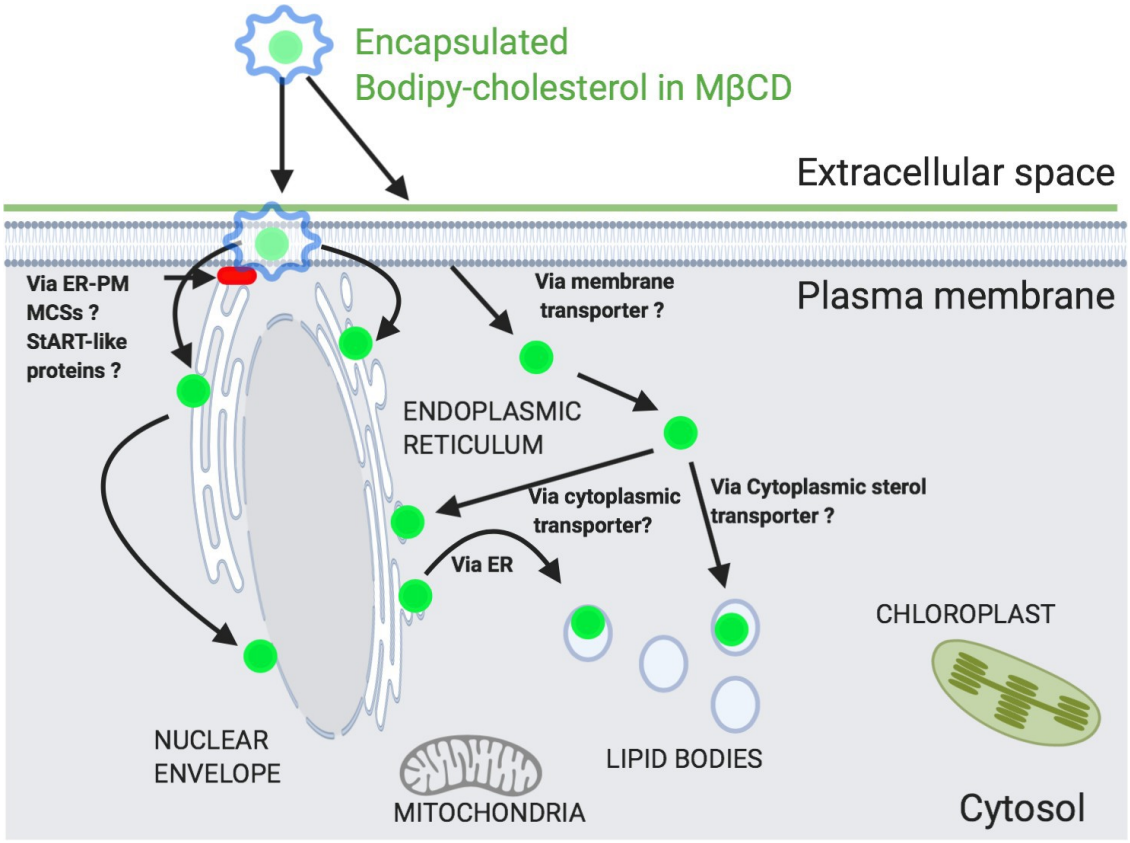

Figure 1. Uptake pathway of Bodipy-cholesterol (Bch) in the elongation zone of root cells. MβCD; Methyl-β-cyclodextrin.

Our finding has also opened the path of further investigation of mutants of sterol binding proteins like Bet-v-1 and StART-like (BAGP-1) proteins (Barbagila et al; 2016, Li et al; 2016, Waese et al; 2017), or in the proteins that tether the ER to the PM, such as VAP27 and NET3C (Wang et. al; 2014). Mutants in the sterol-binding proteins should show diminished transport through the plant or into the cell. NE after labeling with Bch dye. Similarly, mutants of ER-PM MCSs members like VAP27 and NET3C may show reduced Bch labeling, thereby validating the role of ER-PM MCSs in the transport of sterol across the membrane.

As shown in work from Behmer's lab (Behmer et al; 2013), the transport of cholesterol from its site of synthesis in the shoot apex to the root occurs via phloem, but the molecular mechanisms of transport have not been established (Figure 2). A likely candidate for phloem transport is Bet-v1, since it has been found in high abundance in the phloem (Barbagila et al; 2016) and has an "archetypal" sterol- binding domain. Loading of sterols at the site of synthesis and downloading at the site of use may involve movement through the ER, MCSs or cytoplasmic transporters. To get cholesterol from its site of synthesis in the stem to its site of use in the will require connecting these dots.

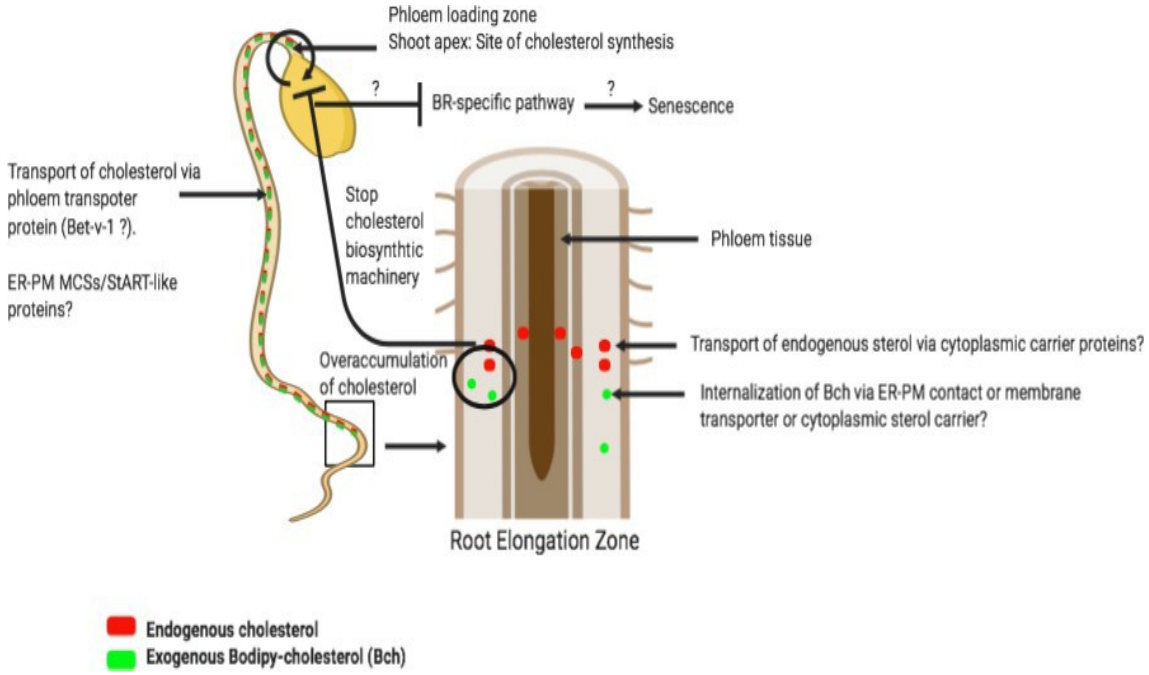

Figure 2. Transport mechanism of endogenous sterol synthesized at the shoot apex and exogenously supplied bodipy-cholesterol and their potential carriers involved in the pathway.

In this work, our initial challenge was delivering of sterols to the cells. We always found poorly labeled cells, but we discovered that, like in animal cells (Vuori et al; 2008), solubilizing the sterol in MβCD, achieved good cell labelling. We optimized the labeling after some modification in the existing animal cell protocols. This success gave rise to the question as to whether or not this approach would help in chemically complementing the mutants of the sterol biosynthetic pathway, which had, to date, not been successful. In those experiments, mutant complementation of *hyd2* was successful, but that further increasing exogenous sterols levels had a negative impact on the growth and development of control Arabidopsis plants. These higher exogenous sterol levels also produced a decrease in the total sterols, indicating that, like in animal cells, sterol biosynthesis in plant is inhibited by the end products of the pathway.

In plants, a complex sterol biosynthetic pathway produces the different sterols (Figure 3). Most of the genes/enzymes of the biosynthetic pathway are known and the mutants of those genes are characterized (Clouse et al; 2002). A mutation of the genes of the sterol biosynthesis pathway produces a change in the ratio of endogenous sterol profiles. In most of the mutant studies, the ratio of β-sitosterol goes down, however, other important sterols such as; stigmasterol and cholesterol ratio go up. Imbalance in the ratio of endogenous sterol produces dwarfing, poor germination and sometimes it causes embryo lethality. In earlier studies the phenotypes of these mutants, e.g., *hydra1*, *hydra2*/ *fackel*, *smt1* and *smt2* have been characterized (Clouse, 2002; Diener et al., 2000). These defects were phenocopied at a higher concentration (100 μM) of exogenous sterols, further support our hypothesis that at a higher concentrations. Sterol triggers the negative feedback on in the sterol biosynthetic pathway. Another possible explanation could be

that the over-accumulation of endogenous sterol results in less brassinosteroid production shown by the add-back experiments with brassinosteroid (Chapter 3, Figure. 12), additional brassinosteroids did not reverse the negative effect of the exogenous sterols on plant growth.

To further understand the potential site of this negative inhibition, we checked to see if adding exogenous sterol produced exaggerated effects by known inhibitors of sterol biosynthesis. Since the target sites of drugs are known (Figure 3), we developed a hypothesis that if the sterol with drug targets the same place in the pathway then there would be no change in the sterol outcome and therefore no change in the phenotype compared with drug and sterol alone. If they act on different targets of the pathway then we would observe a more pronounced phenotype compared with the individual treatments. Only azasterol with cholesterol (100 mM) produced an additive effect on the phenotype. Because azasterol selectively inhibits a single step in middle of the pathway (C14-reducatse, Figure 3), we suggest that cholesterol may be acting at a different, earlier step in pathway (Figure 3).

During the study of developmental effect with a higher concentration of sterol, we also have observed that excess of sterol significantly inhibits the germination like phenocopying the embryo lethal mutants. This result has motivated us to further investigate the effect of sterol on the germination of seeds and its effect on already grown plants. The results were very surprising and interesting as well because on the one hand sterol reduces seed germination whereas on another hand it did not affect the growth and development of already grown plants. This led us to a path or journey of working on and developing a potential pre-emergent herbicide that could be used after crop plants were

already growing. In conclusion, we will use these tools and discoveries to not only discover the pathways of sterol transport in the entire plant, but also to increase our understanding of how sterols affect plant growth and interaction with the biotic environment. Although the discovery of a potent, new organic herbicide is very exciting, much of the basic science behind its mechanism of action remains undiscovered. Future work will test the models shown in Figures 1-3 and thereby identify further new strategies for controlling crop growth, weed growth, and the important role sterols play in the relationship between plants and their animal and fungal pests.

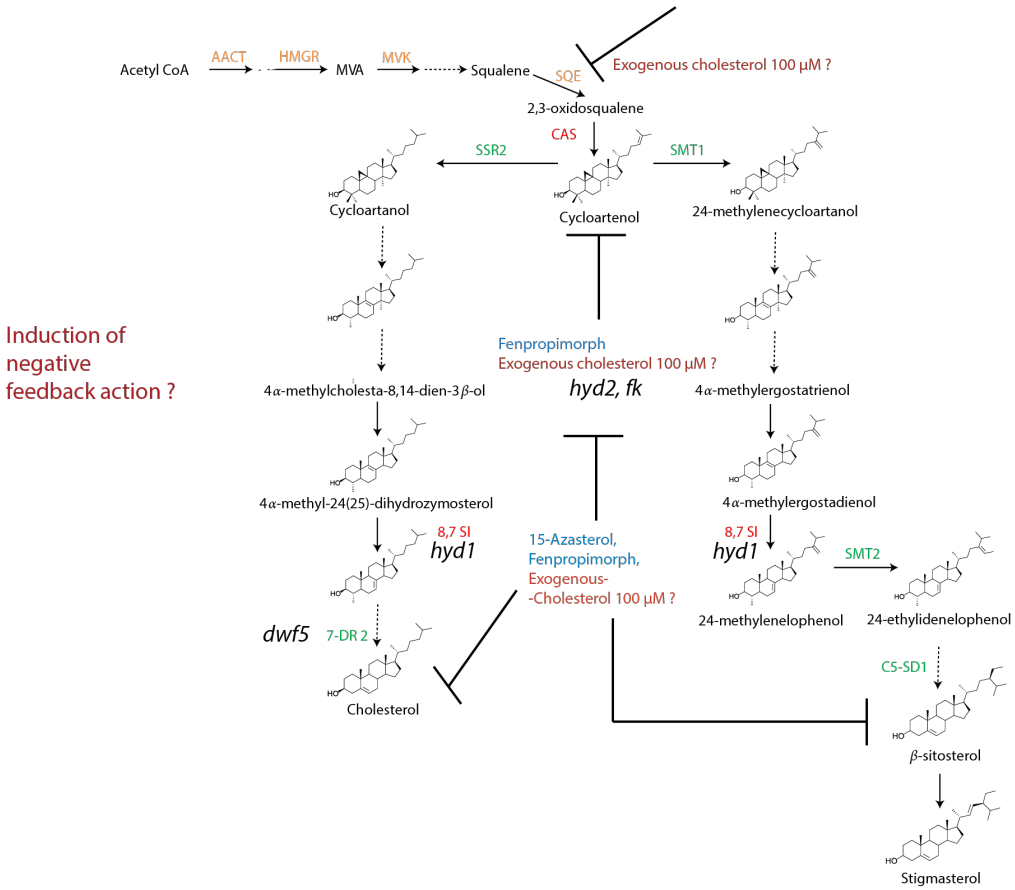

Figure 3. Proposed pathway of negative feed back action of exogenous sterol applied at a concentration of 100 µM.

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APPENDIX

HYDROPONIC SYSTEM FOR ARABIDOPSIS EXTENDED TO CROP PLANTS

Lawrence Griffing and Krishna Kumar

Abstract

When using Arabidopsis grown hydroponically for gene and drug discovery, a method for translating this approach to crop (and weed) species needs articulation and investigation. In this review, we describe existing inexpensive, frequently aseptic, hydroponic systems for Arabidopsis and compare them to other hydro- ponic methods for gene and drug discovery in crop plants. Besides gene and drug discovery, an important use of hydroponic analysis is for understanding growth in controlled, enclosed systems, such as during spaceflight and in simulated extra- terrestrial environments. When done initially with Arabidopsis, will these results apply to the growth of other species? We highlight the strengths and weaknesses of existing translational hydroponic approaches whereby results with Arabidopsis extend to other plant species. We find that the existing or slightly modified hydroponic approaches used in Arabidopsis research extend well to crop plants that grow upright about 40 cm in height, e.g., monocots, such as rice, and dicots, such as soy- bean. However, other, taller species such as maize, or vining species such as tomato, require extensive modification to provide larger enclosures and root stabilization.

Keywords: translational research, drug discovery, herbicide discovery, gene discovery, bioregenerative systems, speed breeding, fast generation cycling systems

1. Introduction

Arabidopsis thaliana (hereafter referred to as Arabidopsis) is a model plant system and, unlike most other plants, has a very large number of sequenced chemi- cally induced mutations and libraries of insertional mutations in genes of known and unknown function [1]. This genetic power of Arabidopsis makes it a continuing resource for studying the functions of genes under a variety of conditions. Often, these conditions are best-controlled using hydroponic systems to control nutrients or other abiotic (e.g., drugs, light, solute stress) or biotic (microbes) interactions in the rhizosphere. Furthermore, with tight control of rhizosphere conditions using hydroponics, other experiments on the shoots, leaves or flowers can proceed.

2. Hydroponic methods for Arabidopsis

Tocquin et al. [2] briefly review earlier approaches to Arabidopsis hydroponics. More recent studies have developed low cost, efficient systems, which are based on

the use of reusable and sometimes sterilizable plastic materials [3–7]. These systems differ in whether they offer (a) aseptic conditions (b) synchronous, rapid growth,
(c) growth to maturity and (d) low cost for set-up and maintenance. All of them grow the plants in simple, defined liquid media (usually a ½-strength Murashige and Skoog medium or nitrogen-supplemented ¼-strength Hoaglands solution). They also provide access to the rhizosphere for root phenotyping and drug delivery. As shown in **Figures 1**, **2**, the root system is readily available for imaging or for biochemical analysis. Efficient harvest of intact roots is difficult in soil-grown plants, whilst hydroponics provides clean, and potentially aseptic, harvest of roots. However, hydroponics depresses the formation of root hairs and produces develop- mental changes in other root tissues (reviewed in [8]).

Tocquin et al. [2] and Monte-Bello et al. [7] describe systems where the plants grow in agar-filled end-clipped mini-tubes placed either in the holes of an auto- clavable pipette tip holder [7], or in holes drilled into dark plastic sheets covering an opaque plastic bin [2], **Figure 1**. The setup by Tocquin et al. [2] is not aseptic and uses polyethylene plastic, which is not autoclavable, but does provide syn- chronous, rapid growth using optimized nutrients based on the modifications of Hoagland's medium [9]. Although not aseptic, the dark plastic sheet discourages the growth of mold and algae at the surface. The media is not circulating or arti- ficially aerated, known in the popular literature as Kratky-type hydroponics [10]. Groups of a dozen or so plants grow in a single tray and the equipment is scalable to larger plantings (trays) or to larger plants (see below). By lifting the plastic sheet, the roots are easily harvested, **Figure 1**. The cost is low, but the cut and drilled plastic sheets are not available commercially. Unlike the similar system [7], it provides media and space required to grow the plants to maturity. Arabidopsis has a shorter generation time in hydroponics than in soil [2], where single-pulse long day lighting induces flowering in 6–7 weeks with hydroponics and 8 weeks in soil [11]. On the other hand, the Monte-Bello setup provides only enough room to grow plants hydroponically to a 4-leaf stage (3–4 weeks), but under aseptic

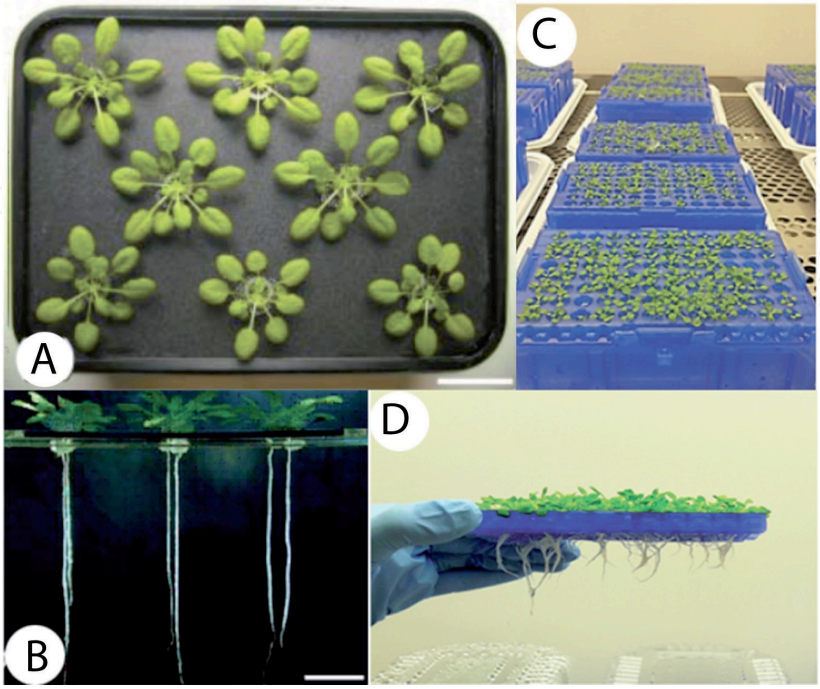

Figure 1.

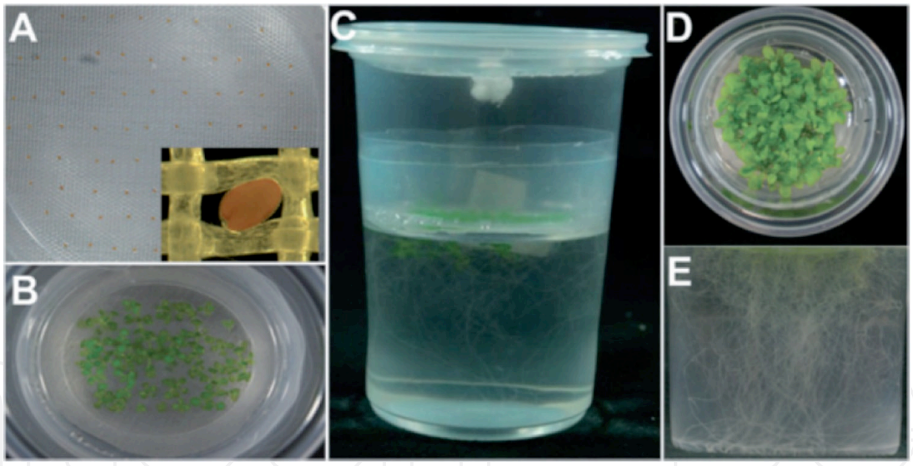

Figure 2.
Cup-based hydroponics for Arabidopsis. (A) Seeds sown on mesh, inset = magnified view of Arabidopsis seed on mesh. (B) Top view of 12-day-old seedlings. (C) Side view of 12-day-old seedlings. (D) Top view of 21-day-old seedlings. (E) Side view of 21-day-old seedlings [5].

conditions, **Figure 1**. It is unclear whether, at this stage, it is feasible to transfer plants to other, larger tube systems [4].

Other Arabidopsis hydroponic systems use an insert into a plastic box or cup [3, 5, 6, 12]. All produce synchronous growth to maturity. The plastic cup system
[5] is autoclavable when using cups of polypropylene. Covering the plants with an autoclavable lid or, in later stages, a tall cup, maintains sterile culture in early
stages of growth. The polypropylene cups are very cheap, because they are available commercially as single-use plastic containers. In this cup system, plants germinate on a screen (plus agar with medium) wedged between a smaller upper cup and larger lower cup, **Figure 2**. Lifting out the screen makes the root system available for analysis and harvest, as is also described in the non-sterile hydroponic culture of Arabidopsis on a supported nylon screen in a beaker [13].

Arteca and Arteca [12] and Nguyen et al. [6] use classic Magenta™ GA-7 boxes as the media chamber and float foam squares containing the plants on the surface of the media. These are modifications of one of the earliest hydroponic culture sys- tems, consisting of a water or nutrient reservoir, an air pump, tube, and a floating platform [14, 15]. Nguyen et al. [6] aseptically pre-germinated the plants on agar and then gently wedged them into foam holders. Robison et al. [3] report another version of this using rock wool and inexpensive food container boxes. More han- dling of the delicate plants occurs when there is transplantation of plants initially grown in agar. Germination directly on rock wool plugs (with a 0.15% w/v agar) is also possible [16]. Once in the foam or rock wool holders and open to the environment, plant growth is no longer aseptic. One procedure [6] also includes aeration of the media with a bubble stone. Others [12] show that there is no effect of bubble stone aeration on growth.

3. Closed and semi-closed systems for Arabidopsis

Some hydroponic systems are open systems that add new media and do not reuse or recycle old media [17]. Providing a continuous supply of defined nutrient or drug-containing solution makes open systems costly and does not take advantage of the ease with which hydroponic nutrients can be recycled or

that use flow, such as the nutrient film technique (NFT), deep flow technique (DFT) and aeroponics (misted nutrient solution sprayed on the roots), cycle a larger volume of nutrient medium. However, both cycled and uncycled hydroponics share the problem that the ionic balance or salinity within the nutrient medium can change over time [18, 19]. Computational approaches based on continuous read-out from ion-selective electrodes provide real-time optimization of hydroponic media [20-22]. Such optimization in closed systems require, as part the enclosure, addi- tives or scrubbers (such as ion exchange resins) to add or remove certain ions or nutrients.

Semi-closed and closed, aseptic systems for Arabidopsis growth are useful for
(1) drug discovery, (2) gene discovery, (3) plant-microbe interactions, and (4) growth in non-terrestrial environments. The advantage of closed or semi-closed hydroponic systems for discovery of drugs effective against plants is that, unlike the situation in animals where target animals are not available for ethical or logisti- cal reasons, it provides a method to evaluate the living target organism from early to late stages of growth [23]. If done on sufficient scale, it can be a form of high throughput in vivo screening. Whole organism screening covers important drug or herbicide properties such as uptake, efficacy, and breakdown. However, the fre- quent use of Arabidopsis, and other model organisms, e.g., duckweed (*Lemna* spp.) and the model grass, *Brachypodium distachyon*, as test species for drug discovery, particularly for herbicides, is problematic [24]. Model species may not have the same mechanisms for uptake, delivery, and metabolism of the drugs as agricultural weeds and crops. They have different life cycles and environmental preferences and constraints. Therefore, it is important to extend the work on closed and semi-closed systems using Arabidopsis to crop species and weed species, as described below.

This is also true for gene discovery. Identifying and preliminary mapping of multi-genic quantitative trait loci (QTLs) that produce desirable phenotypes is possible with recombinant inbred lines (RILs) and near isogenic lines (NILs) of Arabidopsis, if there is minimal environmental contribution to the character. The standard conditions of closed or semi-closed hydroponics seem ideally suited for these studies. When soil is used, it could be a source of irreproducibility. Growth conditions using soils produce irreproducible Arabidopsis leaf phenotypes in different labs, even controlling for many environmental variables and nutrient conditions [25]. Similar multi-lab reproducibility experiments with hydroponically grown Arabidopsis plants are not available, but provide an exciting opportunity for new study. Exploiting closed or semi-closed hydroponics tested with Arabidopsis could be an important step in speed breeding and the analysis of RILs or NILs of fast-cycling crop species [26], as described below.

Sequencing-based analysis of bacterial communities on plants reveals the diversity and complexity of the interaction of plants with the microflora of the rhizosphere [27]. Hydroponic approaches to analysis of Arabidopsis-microbe inter- actions provide a way to monitor how multiple bacterial species colonize the root or interact with each other to form these complex interactions [28]. These approaches require aseptic culture of Arabidopsis and controlled introduction of monocultures of bacteria into the media. Harris et al. [28] adapted the simple, inexpensive, closed hydroponic system described above [5] to analyze the colonization of Arabidopsis roots with *Pseudomonas*, *Arthrobacter*, *Curtobacterium*, and *Microbacterium* species.

Completely enclosed, but not necessarily hydroponic, systems were common for early studies on the growth of plants in extraterrestrial environments (see review, [29]). The cultivation system of choice is recirculating, enclosed hydroponics, how- ever, for future space flights that would include plants in a life support system [30]. The new recirculating enclosed system by NASA and

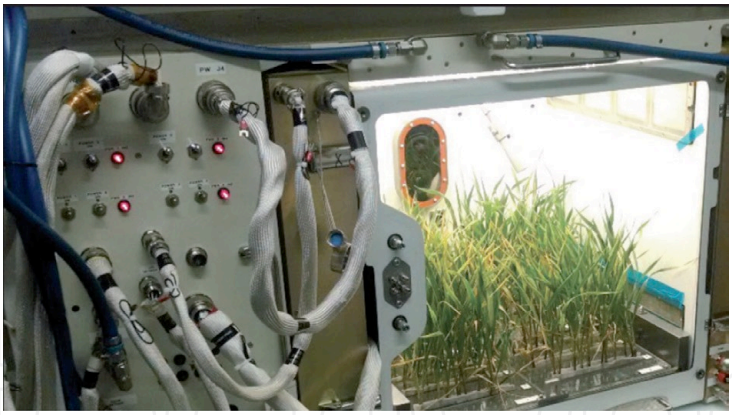

Figure 3.

Advanced plant habitat of NASA showing growing wheat plants in an enclosed chamber with an Arcelite substrate (https://www.nasa.gov/sites/de-fault/files/atoms/files/ad-vanced-plant-habitat.pdf).

The initial tests of the Plant Habitat-01 in the International Space Station will be studies on Arabidopsis. Future experiments will include durum wheat, as shown in **Figure 3**. Studies on soil-less bioregenerative life support systems funded by the European Space Agency use the nutrient film technique (NFT) of hydroponics [32], with the caveat that the implementation of such a technique in microgravity is yet to come. All of these life support systems are gas-tight enclosures that will monitor gases emitted by the plants, because the recycling of carbon dioxide and oxygen by plants or other photosynthetic organisms will be a necessity in long-term flights such as those to Mars.

4. Extending closed and semi-closed systems of Arabidopsis to crop species

A semi-closed hydroponic system that is very close to those described above for Arabidopsis is the single-tube hydroponics of Kuroda and Ikenaga [33]. As in the procedures for Arabidopsis used by Nguyen et al. [6] and Robison et al. [3], the plants initially germinate on an agar (gelrite) medium containing ½ strength Murashige and Skoog medium. They grow a variety of crop plants, i.e., rice, soybean, Azuki beans, and corn, instead of Arabidopsis. At 2 weeks, Kuroda and Ikenaga [33] transplant the intact germinated seedlings into 12 ml polypropylene tubes with two holes cut into the sides to allow the entry and exit of hydroponic medium (1/10 strength Murashige and Skoog medium). A covered outer tray contains the medium and a rack for the tubes in which the plants grow. The tube supports the plant during culture and contains the root ball of each plant, thereby facilitating removal for analysis without damaging the roots. The size of the culture tube is larger than that used by Tocquin et al. [2] for Arabidopsis because the seeds and new roots of crop plants are much larger, but the tray system for growth in hydroponics is very similar. For soybean, an additional prop supports the plant during growth. Rice and soybean plants grown in single tube hydroponics produce high viability seed with seed weights equal to or exceeding plants grown in the field. Single tube hydroponics facilitate analyzing and screening the T1 seeds from the transgenic plants with shorter generation times and small amounts of seeds.

The hydroponic system of Conn et al. [4] directly translates an Arabidopsis hydroponic culture system to crop plants. They use a system

kept the roots of separate plants free from tangling, thereby facilitating measure- ment and analysis. Although Arabidopsis was the main test plant for this system, wheat, cucumber, and tobacco also successfully grew.

Although both techniques [4, 33] require transplantation of newly germinated plants, which has the downside of more manipulation, transplantation may be desirable for crop plants with low germination rates and for studies on post-emer- gent drug treatments. It has the additional advantage of protecting the plant from water molds and other contaminants because the initial germination is aseptic.

It becomes apparent in studies that translate work on hydroponically grown Arabidopsis to crop plants that just the difference in physical size of the seeds and plants dictates some of the modifications. In contrast to Arabidopsis, experiments involving larger plants require root stabilization. When larger crops, such as *Zea mays*, grow in hydroponic conditions, lack of support for the root system can result in breakage and damage of the lateral root system [34]. When growing wheat varieties to test the effects of salinity, Munns and James [35] used quartz rock as
a stabilizing substrate in a hydroponic flow system. More complex, but definable, substrates may be necessary because they interact with nutrients and help deter- mine their availability, e.g., a defined clay substrate for corn [36, 37].

The generation time for soil-grown Arabidopsis decreases by 1 to 2 weeks when grown hydroponically on defined medium [2, 11]. Accelerated breeding programs for crops facilitate the production of RILs and NILs for gene discovery. In fast generation cycling systems, **Figure 4** [26], plants with long generation times, such as crop plants, are sped up using a variety of technologies. Speed breeding can produce generation times that are a third to a half the time [38, 39]. One of the technologies used in speed breeding is in vitro growth. With a neutral rhizosphere support medium, such as agar for Arabidopsis [40], speed breeding for Arabidopsis translates to speed breeding in wheat [41]. Besides achieving fast growth of the seedling, an important feature of many fast generation cycling systems is overcom- ing seed dormancy with early stage embryo culture in vitro, **Figure 4** [26, 42] or harvesting immature seed and drying it [38, 39]. With embryo culture, there will always be aseptic transplantation of agar-grown embryos, but for immature seeds, transplantation is not necessary.

Complete enclosure of the growing crop is one of the features of recent speed breeding technologies [38, 39, 43]. Soybeans grown hydroponically using NFT in the completely enclosed bioregenerative life support systems have a 110–133 day

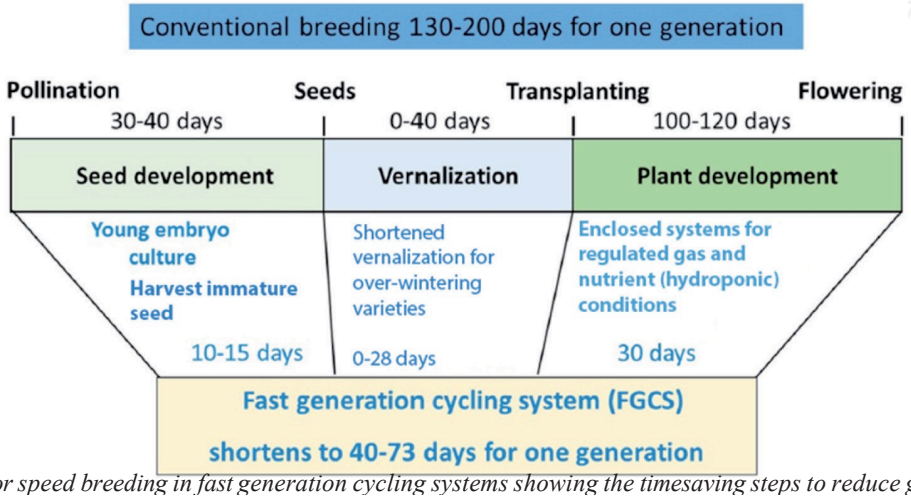

Figure 4.
Schedules for speed breeding in fast generation cycling systems showing the timesaving steps to reduce generation

generation time [32]. Soil grown soybeans have a generation time of 132 days, but growing them in an enclosed system with elevated carbon dioxide decreases the generation time to 70 days [43]. Combining hydroponics of crop species with other technologies of speed breeding might produce even shorter generation times or higher yields and seed viability.

Complete enclosure of the growing crop is also a feature of crops grown for space exploration. Because soybean is one of the species best suited for growth in space [44], its nitrogen fixing symbiosis with bacteria is of interest. As described above, hydroponic submersion systems inhibit root hair development, upon which the initial stages of nodule formation depend. Hence, it is not a surprise to see that hydroponic inoculation of soybean with its nodulation partner, *Bradyrhizobium japonicum*, does not improve nitrogen use efficiency [44]. However, other kinds of plant growth promoting microbes (PGPMs), including some of those tested in hydroponic systems with Arabidopsis [28], produce higher photosystem II efficiency in hydroponically-grown soybean plants [45]. This could be beneficial in speed breeding, which improves with improved photosynthesis achieved with elevated carbon dioxide [43]. Those plants grown to maturity in enclosed hydro- ponics and inoculated with PGPMs show stabilized microbial communities over time [46].

5. Conclusions: translational research on hydroponics from Arabidopsis to crops

As described in Woodward and Bartel [1], research on Arabidopsis can some- times directly translate into discoveries in crops. One example that they use is the expression of MYB12 in tomatoes, which derived from initial discoveries in Arabidopsis revealing increased production of flavonoids upon overexpression.

The overexpression of MYB12 in tomatoes produces so much flavonoids, the color of the fruit changes from red to orange [47]. However, this small mustard family plant has a growth habit and life cycle so different from most crop plants, can lessons learned from hydroponic studies on Arabidopsis be translated to crops?

The answer is: mostly. Most of the technical approaches used with Arabidopsis translate to crop plants with minor modification, except for those crops that are very large and need extra support for growth. The benefits of using Arabidopsis for investigating the different techniques of hydroponics are those that make it valued as a model organism, i.e., its size, well-characterized genome, and short generation time. In fact, given the depth of knowledge on gene function in Arabidopsis, current research on Arabidopsis hydroponics could apply more widely to studies on fast breeding crop plants for gene discovery, on target plants for herbicide and drug discovery, and on plants used for bioregenerative life sup- port systems in space.

Adoption of some the techniques used in Arabidopsis hydroponics could decrease the cost and size (important for space studies) of enclosed test systems without changing the viability and yield of the crop plants grown in those systems. For example, the effects of space travel are varied and complicated. However, most of the work done to date has focused on the microgravity component of space flight without the proper control of having a 1-g set of plants growing in the same space vessel [29]. Because Arabidopsis is small and well characterized, the initial tests for the design and implementation of these proper controls may be more feasible (and the data achieved more insightful) for Arabidopsis than for the crops identified as "the best" space plants, i.e., durum and

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