THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

INVESTIGATION OF CELL WALL UPTAKE, TRAFFICKING, AND ORGANIZATION IN *ARABIDOPSIS THALIANA* USING A METABOLICALLY INCORPORATED CLICK REPORTER

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ABSTRACT

Limited tools for imaging cell walls in plants have made it difficult to visualize cell wall synthesis and dynamics. It has been reported that the sugar analog, fucose alkyne (FucAl), may be incorporated into the matrix polysaccharide, rhamnogalacturonan I (RGI), and can be labeled using a copper-catalyzed click reaction. In order to study the uptake, processing, trafficking, and organization of labeled cell wall components, mutants in various steps in the molecular pathways thought to be involved in fucose metabolism and wall synthesis were assessed for defects in the amount (mean fluorescence intensity, MFI) and organization (anisotropy) of FucAl-related fluorescence, using spinning disk confocal microscopy. We found that mutations in genes related to the uptake and processing of sugars have differing effects on the incorporation of FucAl. Because the copper-catalyzed click labeling reaction is toxic to cells, a copper-free strain-promoted click reaction was used to incorporate another sugar analog, Kdo azide (KdoAz), to develop a tool to study the molecular pathways involved in matrix polysaccharide synthesis and dynamics *in vivo*. Recently developed and improved cyclic alkyne reporters provide opportunities to optimize this labeling technique. The utility of these metabolic biochemical labeling tools could have far-reaching applications in the study of the plant cell wall, increasing our understanding of cell wall formation and dynamics.

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ACKNOWLEDGEMENTS

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Chapter 1

Introduction

Although much is unknown about the primary cell walls of *Arabidopsis thaliana,* it is understood that they contain a dynamic array of a variety of polysaccharides. These include cellulose, hemicelluloses, and pectins (Liepman et al. 2010). Cellulose microfibrils are long and unbranched and are synthesized at the plasma membrane, where they form parallel chains and crystalline structures. Celluloses form hydrogen bonding crosslinks with hemicelluloses, which are branched polysaccharides that are secreted as soluble polymers and diffuse and unfold within the wall (Somerville et al. 2004). Similarly, pectins are secreted and diffuse into the cell wall to form a mesh-like matrix (Bashline et al. 2014). Primary cell walls have the unique ability to contribute both structure and function, demonstrating strength and stability while maintaining the flexibility and dynamic abilities needed to allow for growth and expansion. Plant cell walls grow and expand in part through the addition of new wall material. However, it is unknown exactly how the synthesis of new wall components, as well as the expansion of walls already present, are regulated (Cosgrove 2018). The uptake, trafficking, and organization of cell wall proteins and components is a vital factor affecting cell wall organization and dynamics (Barnes and Anderson 2018). It is not completely understood how exactly each of the components of the cell wall is specifically regulated (Ebine and Ueda 2015). Understanding the organization and dynamics of this large carbon-sink would allow us to harness the energy that plants store in the form of the cell wall linkages for other purposes, such as the production of renewable bioenergy and biomaterials.

Although cell wall organization and dynamics have been studied using various genetic, mechanical, and biochemical methodologies, the advent of recent imaging techniques have expanded tools that are available to study and image the cell wall (Voiniciuc et al. 2018). The difficulty in investigating the details of cell wall synthesis and dynamics comes, in part, from the fact that cell wall carbohydrates are made up of sugars not appropriate for genetically encoded fluorescent tags. At present, there has been success in using monoclonal antibodies and carbohydrate binding modules (CBMs) to bind to polysaccharide structures with high resolution and sensitivity (Lee et al. 2011). This technique, however, uses a defined epitope that will be on the order of three to eight sugars, which can be a challenge because of the limited availability of defined epitopes for immunogen preparation and probe characterization (Lee et al. 2011). While precisely imaging the arrangements of cell wall polysaccharides has not been easy, single sugar imaging probes offer promise in the future of cell wall imaging.

A copper-catalyzed click chemistry reaction has been used to label glycans in a variety of organisms, allowing these otherwise genetically incompatible polysaccharides to be imaged and studied (Hein and Fokin 2010). The copper-catalyzed azide-alkyne click chemistry (CuAAC) reaction is experimentally simple, and progresses in aqueous solution at room temperature without a need to protect from oxygen. It requires only small amounts of reactants and produces few to no byproducts (Wu et al. 2004). The reaction needs only the azide and alkyne reactants, as well as the copper catalyst and ascorbic acid reductant, to produce the triazole product that covalently links adducts to the azide and alkyne groups in a short amount of time and at ambient temperatures (Rostovtsev et al. 2002). The utility of this reaction in live systems is vast, and has been used to image glycans in a variety of living organisms, such as zebrafish (Clavadetscher et

al. 2016). The speed and ease at which this reaction takes place, along with the small reactant size, makes it a candidate for labeling the polysaccharides of the cell wall.

There is indirect biochemical evidence showing that the sugar analog, fucose alkyne (FucAl), is incorporated into the pectic polysaccharide, RGI, in seedlings of *Arabidopsis thaliana* and can be fluorescently labeled and imaged using the copper-catalyzed click chemistry reaction (Anderson et al. 2012). This sugar analog allows the cell to take up and incorporate a chemical reporter that can be used to study biological aspects of cell wall organization. The alkylated fucose monomer is the first such sugar to be found to be taken up by the plant and can be successfully labeled using an azido fluorophore, but a subset of additional click-compatible sugar analogs have more recently been shown to incorporate into the cell wall (Dumont et al. 2016; Hoogenboom and Berghuis 2016; Mcclosky et al. 2016; Zhu and Chen 2017). Indirect biochemical analysis of the labeled polysaccharide suggests that FucAl is incorporated into the matrix polysaccharide, rhamnogalacturonan I (RGI) (Anderson et al. 2012). However, much is still unknown about the specific location of the modified sugar and the mechanisms leading to its incorporation, which can be elucidated by using biochemical analyses and genetically targeting steps in the possible molecular pathway responsible for the uptake, trafficking, and organization processes affecting this FucAl-containing polysaccharide (Figure 1). The possibilities for studying the organization and dynamics of cell wall matrix polysaccharides are myriad, and determining where and how this precise chemical reporter is incorporated is the first step.

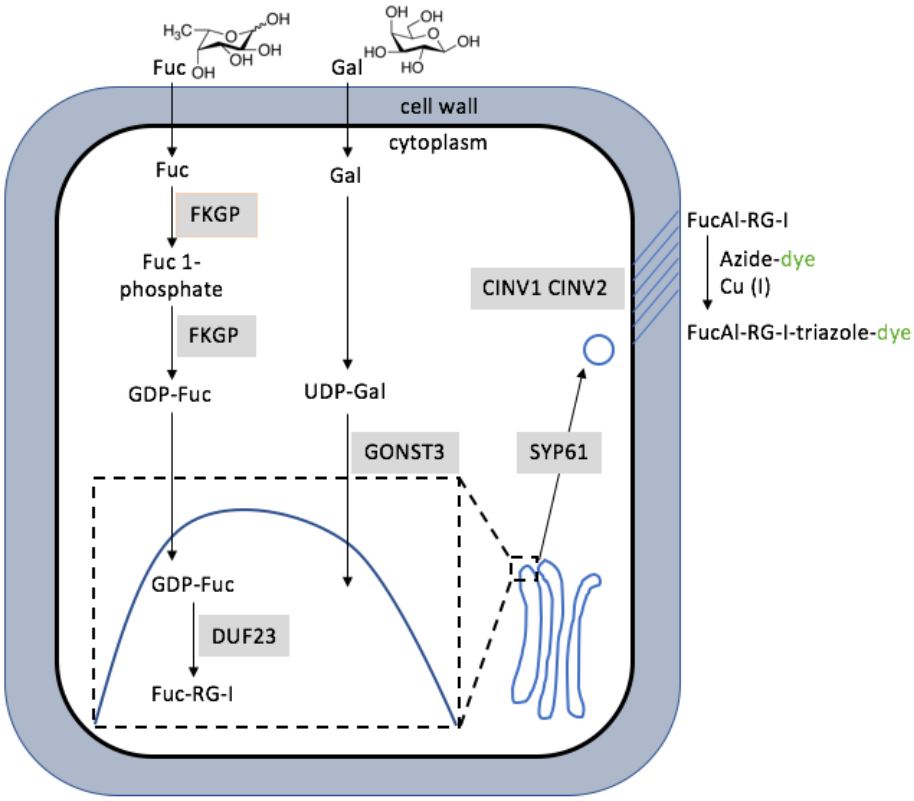

Figure 1: Select genes involved in the uptake, trafficking, and organization of fucose and galactose

Chapter 2

Methods

Experimental Design

In order to investigate the molecular mechanisms leading up to fucose alkyne incorporation into *Arabidopsis thaliana* cell walls, genetic mutants and chemical treatments were used. Mutants in various genes related to the uptake, trafficking, and incorporation of different sugars into the various components of the cell wall were obtained and tested for incorporation against wild type controls. In each experiment, mean fluorescence intensity (MFI) and anisotropy were measured to quantify the amount of fucose alkyne incorporated and the striations seen after incorporation and labeling, respectively.

Plant Growth Conditions

Arabidopsis thaliana wild type, Col-0, seeds were sterilized in 30% bleach for 20 minutes at 23°C and washed four times with sterile water. Seeds were incubated at 4°C for at least three days, then grown vertically on ½ MS [2.2 g/L Murashige and Skoog salts, 0.6 g/L MES-KOH pH 5.6, 1% (wt/vol) sucrose] plates for 5d at 23°C under constant light. Mutant and RNAi lines included *fkgp3* (Ian Wallace), *gonst3* (Jenny Mortimer), *duf23* (Henrik Scheller), *osm1* (Georgia Drakakaki), *cinv1 cinv2* (Alison Smith), and *fer4* (Gabriele Monshausen). For *cytosolic invertase* (*cinv1 cinv2*) experiments, mutant and Col seedlings were grown on ½ MS

0% sucrose plates, since this mutant is defective in sugar metabolism and can be partially rescued by exogenous sugar addition (Barratt et al. 2009; Barnes and Anderson 2018 Mar 23).

FucAl Sugar Analog Incorporation and Fluorescent Labeling

To incorporate fucose into the cell wall, five day old seedlings were transferred to 1.8mL liquid MS with 2.5µM fucose alkyne (Thermo Fisher C10264), and incubated for 4h in light at 23°C. Some experiments used altered incubation times, and are noted where applicable. The seedlings were then washed three times in ½ MS liquid without sucrose. The labeling reaction was carried out for 1h at 23°C in the dark in a separate tube containing 1.8µL of labeling solution [½ MS liquid containing 1mM $CuSO_4$, 1mM ascorbic acid, 0.1µM Alexa-488 azide] (Anderson et al. 2012). The seedlings were then washed three times with ½ MS liquid. This general protocol is illustrated in Figure 2.

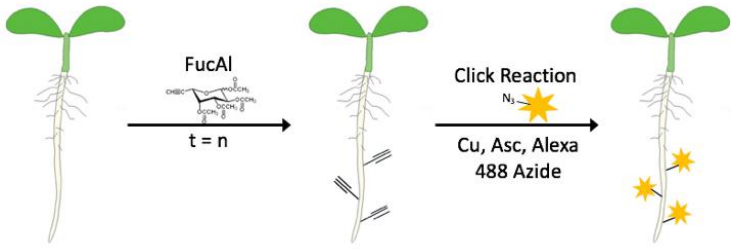

Figure 2: Labeling Protocol

An illustration of the sugar analog incorporation and copper-catalyzed labeling protocol.

Galactanase Digestion

To investigate if FucAl is incorporated into galactan side chains, five day old seedlings were transferred to 1.8mL liquid ½ MS containing 2.5µM FucAl, and incubated for 24h in light

at 23°C. Following the labeling reaction as described above, seedlings were then incubated with 10U/mL *endo-1,4-β-galactanase* (Megazyme, E-EGALN) in 100mM ammonium acetate pH 4.5 with shaking for 24h at 37°C. The seedlings were again washed three times in ½ MS liquid.

Kdo Azide Incorporation and SPAAC Labeling

The SPAAC labeling protocol was used to label matrix polysaccharides *in* vivo. Five day old seedlings were transferred to 1.8mL liquid MS with 25μM Kdo azide (Click Chemistry Tools, 1241-25), and incubated for 16h in light at 23°C. The seedlings were then washed three times in ½ MS liquid. For copper-catalyzed labeling, the labeling reaction was carried out similar to copper-catalyzed labeling of FucAl, using 0.1μM Alexa-488 alkyne instead of Alexa-488 azide. The seedlings were then washed three times with ½ MS liquid and imaged. For the strain promoted labeling reaction, seedlings incubated with 25μM Kdo azide for 16h in light at 23°C were then reacted with 10μM DIBO-488 alkyne (Thermo Fisher, C10405) for 1hr in the dark, washed, and imaged.

Microscopy and Image Analysis

Seedlings were then imaged using confocal microscopy. A Zeiss Cell Observer SD microscope equipped with a Yokogawa CSU-X1 spinning dish head was used with a 100x 1.4 NA oil-immersion objective. 488-nm and 561-nm lasers were controlled by Axiovsision 4.8 software. Z series were collected with a 200-nm step size and reassembled in ImageJ. To measure the amount of fucose alkyne incorporated, mean fluorescence intensity was calculated

in ImageJ by selecting the boundaries of 4-6 representative cells per seedling and measuring mean gray value for each cell. To quantify striations, anisotropy was measured using the ImageJ plugin, FibrilTool, on 4-6 cells per seedling, as described in Figure 3 (Boudaoud et al. 2014). Experiments were repeated three times ($n \geq 15$ seedlings per treatment) and all images across three experiments were taken with identical microscope settings. We used t-tests to test whether mutants were different from the experiment's paired wild type control.

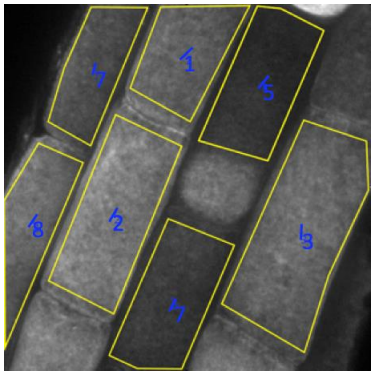

Figure 3: Image Analysis

An example of MFI and anisotropy measurements. 4-6 cells per seedling were measured for MFI (ImageJ, mean gray value) and anisotropy (ImageJ, FibrilTool). Values were averaged across at least three experiments.

FESEM Imaging

In order to visualize possible differences in matrix polysaccharides appearance at high magnifications, seedlings were treated with isoxaben and imaged using FESEM. This sample preparation protocol was adapted by William Barnes from two previous studies (Sugimoto et al. 2000; Xiao et al. 2016). Roots of five day old seedlings were first prepared using carborundum abrasion to create holes in cells. Roots were then washed in 2mL 20mM HEPES pH7 with 0.1% Tween for 1hr, with occasional swirling, followed by three 5min water washes. Seedlings were washed in 3mL 100% ethanol two times, and then kept in 100% ethanol for 30min. After being

transferred to foam capsules, samples were dried using the following program settings on a Leica Critical Point Drier: 19 cycle slow speed procedure with consistent stirring, cooling temperature 15°C, heating temperature 40°C, CO_2 influx speed slow, gas out speed 90% slow. Samples were mounted and sputtercoated (EMITECH K575X) for 3s with iridium under a 40mA current on a rotating stage. Images were collected by field emission scanning electron microscopy using a Zeiss Sigma VP-FESEM under 10kV and 80μA beam current conditions.

Cell Viability Assay

To test cell viability in different $CuSO_4$ and ascorbic acid concentrations, a pseudo reaction protocol was used, in which $CuSO_4$ (0-1mM) and ascorbic acid (0-10mM) were incubated together for 10min before seedlings were added and then incubated for 1hr rocking in the dark. To assess histidine as a protective ligand (Kennedy et al. 2011), a similar pseudo-reaction protocol was used, in which 0.05-1mM $CuSO_4$, 0.05-1mM Histidine, and 1mM ascorbic acid were incubated for 10min prior to being incubated with seedlings rocking in the dark for 1hr. Cell viability during different duration reactions was tested by incubating 0.1-1mM $CuSO_4$ and 1mM ascorbic acid for 10min, and then with seedlings for 5, 15, 30, and 60min. Following each of these treatments, seedlings were washed with ½ MS liquid and stained for cell viability using FDA and PI. Seedlings were labeled in 5μg/mL fluorescein diacetate (FDA) (Sigma, D6883-50MG) in ½ MS liquid for 30s, washed three times in ½ MS liquid, labeled in 3μg/mL Propidium Iodide (PI) (Life Technologies, P3566) in ½ MS liquid, and then washed three times. By estimating the number of cells positive for PI stain and negative for FDA stain, a Cell Viability Rating was given to each sample (1-10) and averaged across three experiments.

Chapter 3

Results

Investigations into the incorporation of FucAl

To investigate the molecular mechanisms that lead to the incorporation of the chemical reporter, Fucose Alkyne (FucAl), various steps thought to be involved in sugar processing and incorporation were tested for defects in the amount of FucAl incorporated and the organization of the FucAl during incorporation. The relative amount incorporated was measured from confocal micrographs by Mean Fluorescence Intensity (MFI) and organization into striations was measured by anisotropy. While much is unknown about the specific molecular pathways involved, Figure 4 illustrates a possible schematic, detailing select steps considered here.

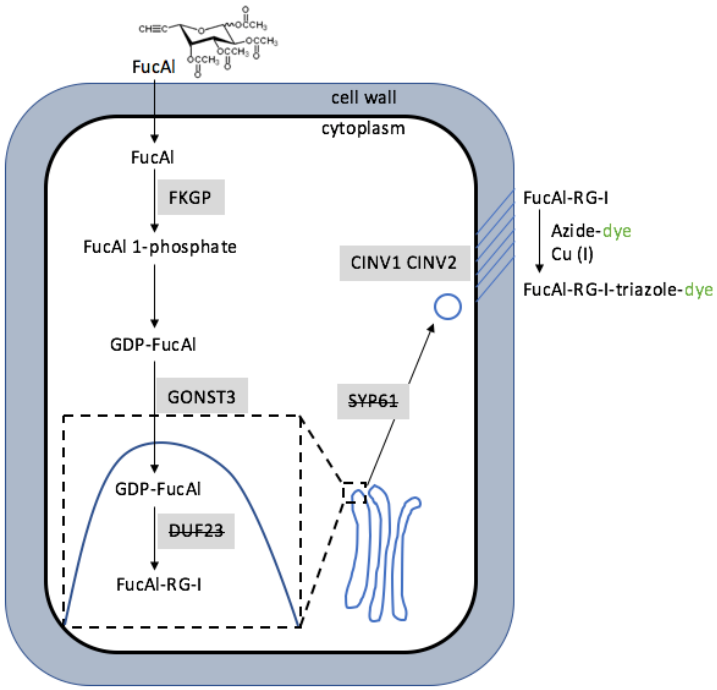

Figure 4: Molecular Schematic

A possible molecular schematic describing the uptake, processing, trafficking, and organization of Fucose Alkyne (FucAl) into the plant cell wall (adapted from Anderson and Wallace 2012). Mutations in the steps thought to be involved were tested for amount of FucAl incorporated (mean fluorescence intensity) and organization into striations (anisotropy) in order to investigate molecular contributors of pectin dynamics and organization.

To study how FucAl is taken up by the cell and incorporated into the components of the cell wall, multiple steps were investigated. One of the molecular targets involved in the plant cell's fucose salvage pathway that may be involved in the uptake of FucAl is the bifunctional enzyme L-FUCOSE KINASE/ GDP-L-FUCOSE PYROPHOSPHORYLASE (FKGP) (Kotake et al. 2008; Villalobos et al. 2015). After 4h incubation with FucAl, mutant *fkgp-3* lines had 34% decreased MFI compared to Columbia (Col) wild type lines ($p<0.0001$, t-test) (Figure 5). The *fkgp-3* mutants also had 30% decreased anisotropy compared to WT controls ($p<0.001$, t-test). These results indicate that FucAl may be taken up by the plant cell through the endogenous fucose salvage pathway. GOLGI NUCLEOTIDE SUGAR TRANSPORTER3 (GONST3) is a putative Golgi-localized nucleotide sugar transporter, and could be responsible for the uptake of FucAl into the Golgi Apparatus (Handford et al. 2004). Four independently generated RNAi

gonst3 knockdown lines with different predicted strengths were provided on a collaborative basis by Jenny Mortimer, Joint Bioenergy Institute, and tested for MFI and anisotropy (Figure 6). Each of the lines had significantly decreased MFI and anisotropy compared to controls, suggesting that GONST3 may contribute to the uptake of FucAl into the Golgi, or that FucAl is attached to a GONST3-dependent cell wall moiety, such as the galactan side chain of RGI (Mohnen 2008).

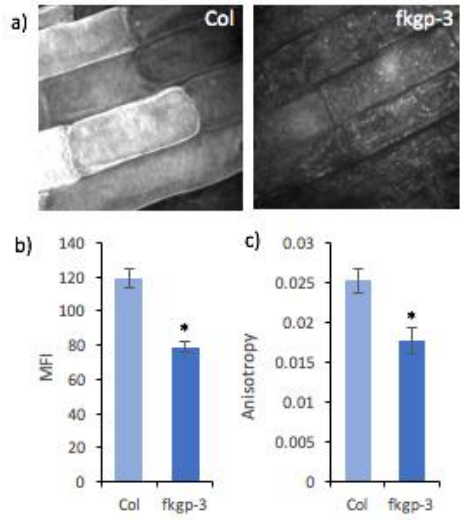

Figure 5: FucAl incorporation in *fkgp-3* mutants

Five day old Col (WT) and *fkgp-3* mutant seedlings were grown in 2.5µM FucAl solution for 4h. 8-bit max projection z-stacks were produced and mean fluorescence intensity (MFI) and anisotropy were measured from 4-6 cells/seedling ($n \geq 15$ seedlings). Units for MFI and anisotropy are arbitrary and are used for comparison only. (* $p < 0.05$, t-test)

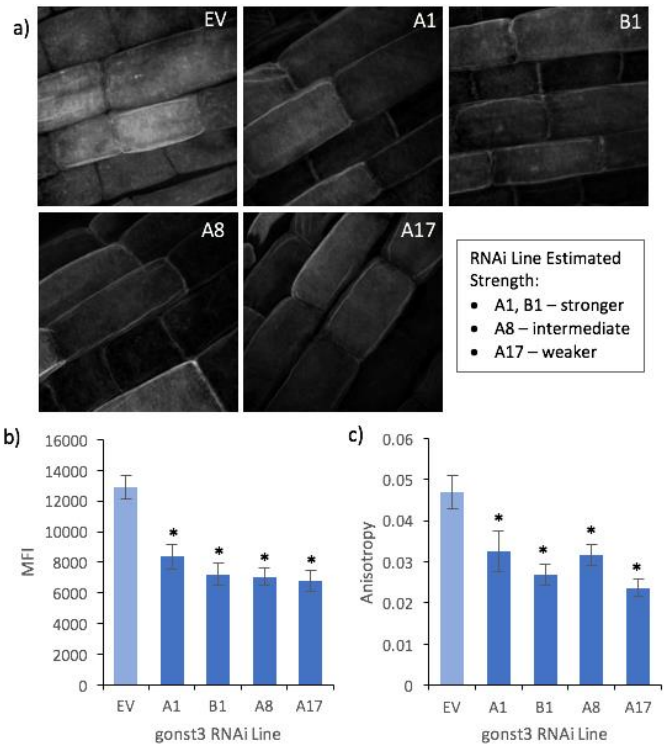

Figure 6: FucAl Incorporation in *gonst-3* knockdown

Five day old EV (empty vector) and 4 independently generated *gonst3* RNAi line seedlings were grown in 2.5μM FucAl solution for 4h. 16-bit max projection z-stacks were produced, background subtracted (pixel width = 10, sliding paraboloid), and mean fluorescence intensity (MFI) and anisotropy were measured from 4-6 cells/seedling ($n \geq 15$ seedlings). Units for MFI and anisotropy are arbitrary and are used for comparison only. (* $p < 0.05$, t-test)

Because DUF23 proteins have been implicated in the addition of galactose to side chains, and fucose is expected to be found in galactan side chains of RGI, *duf23* mutants may show defects in FucAl incorporation (Mohnen 2008; Hansen et al. 2012). After imaging following incorporation with FucAl, *duf23* mutant lines showed no significant change in MFI, and a 24% increase in anisotropy ($p<0.05$) when compared to control lines (Figure 7). This suggests that FucAl may not be incorporated into these galactan side chains of RGI, and could possibly be incorporated in place of another sugar, rather than where fucose is normally incorporated. However, when MFI was measured in WT seedlings incorporated with FucAl, click labeled, then treated with β-1,4-endogalactanase, FucAl-related fluorescence significantly decreased by 25% compared to DMSO control seedlings ($p<0.0001$, t-test) (Figure 8).

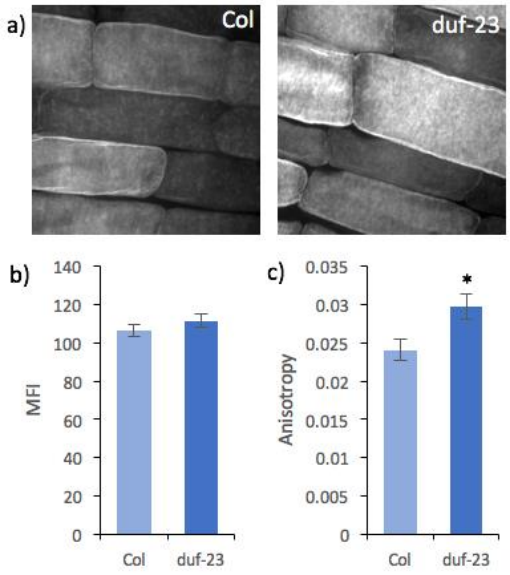

Figure 7: FucAl Incorporation in *duf23* mutants

Five-day old Col (WT) and *duf23* mutant seedlings were grown in 2.5µM FucAl solution for 4h. 8-bit max projection z-stacks were produced and mean fluorescence intensity (MFI) and anisotropy were measured from 4-6 cells/seedling (n ≥ 15 seedlings). Units for MFI and anisotropy are arbitrary and are used for comparison only. (* $p < 0.05$, t-test)

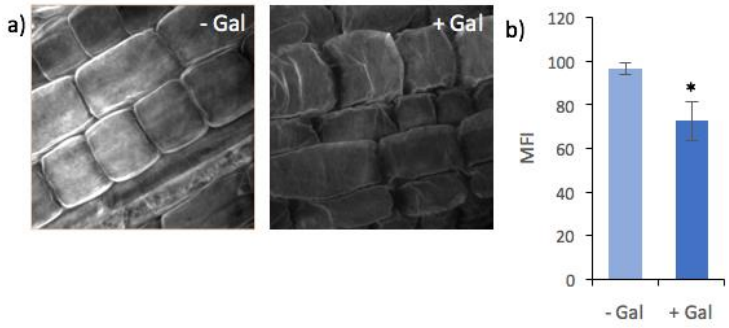

Figure 8: Galactanase treatment reduces fluorescence after FucAl incorporation and click labeling

5d old Col (WT) seedlings were grown in 2.5µM FucAl solution for 24h, followed by a 24h incubation with either β-1,4-endogalactanase or DMSO. 8-bit max projection z-stacks were produced and mean fluorescence intensity (MFI) was measured from 4-6 cells/seedling (n ≥ 15 seedlings). Units for MFI are arbitrary and are used for comparison only. (* $p < 0.0001$, t-test)

The trafficking and organization of this FucAl-labeled polysaccharide can be studied in a similar way, by targeting related genes and assessing the effect on amount incorporated and organization of integrated FucAl. SYNTAXIN OF PLANTS 61 (SYP61) is responsible for the exocytic trafficking to the plasma membrane of vesicles that contain proteins related to cell wall polysaccharides (Drakakaki et al. 2012). Neither the MFI nor the anisotropy measured in the

osm-1 mutant of SYP61 (Zhu et al. 2002) were significantly altered compared to WT controls, suggesting that the polysaccharide into which FucAl is incorporated is not trafficked in OSM-1-containing vesicles (Figure 9). Cytosolic invertase genes *CINV1* and *CINV2* are involved in the processing of glucose sugars and activity of cellulose synthase (CesA) complexes at the plasma membrane (Barratt et al. 2009). After FucAl incorporation in WT and *cinv1 cinv2* double mutant lines, measurements showed no difference in MFI, and a 31% decrease in anisotropy ($p<0.0001$) (Figure 10), suggesting that these cytosolic invertase genes are needed for the organization of FucAl, but not the rate of incorporation. To visualize further the effects of impaired cellulose synthesis on the appearance of recently deposited matrix, a protocol that preserved matrix polysaccharides was used to image seedlings treated with isoxaben at high magnifications using Field Emission Scanning Electron Microscopy (FESEM). Carborundum abrasion allowed seedlings to be prepped easily and efficiently, while matrix polysaccharides were preserved (Figure 11). However, insufficient data prevented us from drawing definitive conclusions about changes in the nanoscale patterning of the cell wall in isoxaben-treated seedlings.

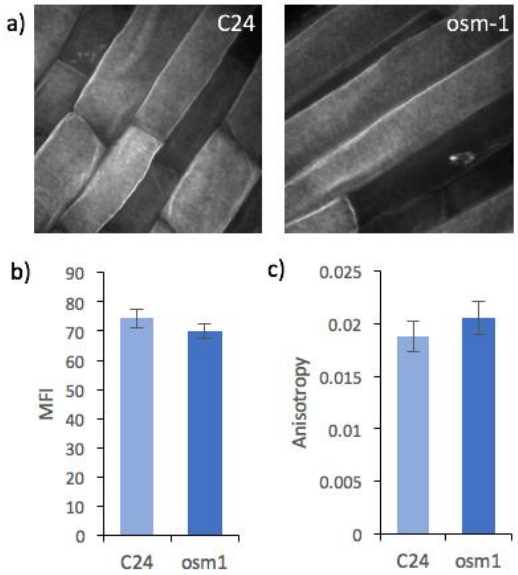

Figure 9: FucAl incorporation in *osm-1* mutants

Five day old C24 (WT) and *osm-1* mutant seedlings were grown in 2.5µM FucAl solution for 4h. 8-bit max projection z-stacks were produced and mean fluorescence intensity (MFI) and anisotropy were measured from 4-6 cells/seedling (n ≥ 15 seedlings). Units for MFI and anisotropy are arbitrary and are used for comparison only.

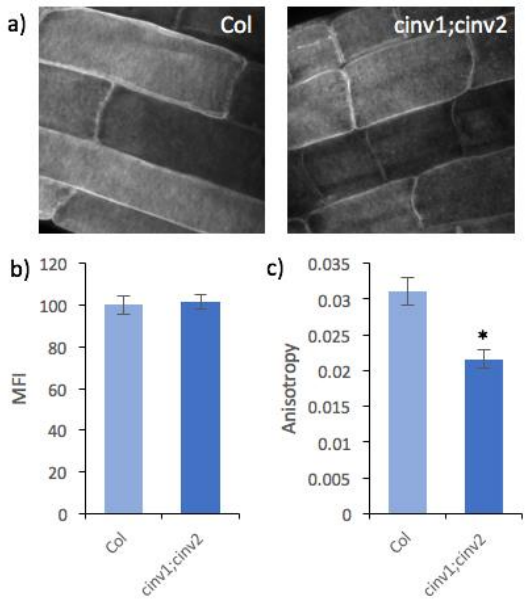

Figure 10: FucAl incorporation in *cinv1cinv2* mutants

Five day old Col (WT) and *cinv1cinv2* mutant seedlings were grown in 2.5µM FucAl solution for 4h. 8-bit max projection z-stacks were produced and mean fluorescence intensity (MFI) and anisotropy were measured from 4-6 cells/seedling (n ≥ 15 seedlings). Units for MFI and anisotropy are arbitrary and are used for comparison only. ($p < 0.0001$)

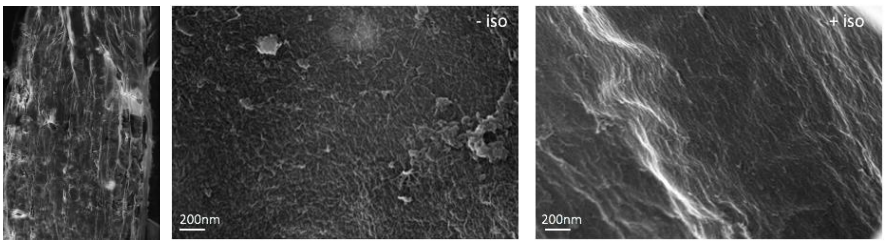

Figure 11: FESEM imaging of isoxaben-treated seedlings

Five day old Col (WT) seedlings were treated with 0 or 1μM isoxaben for 6.5h. Seedlings were then prepped using the carborundum abrasion protocol and imaged at 100kx magnification using FESEM.

The receptor tyrosine kinase, FERONIA, is involved in cell wall integrity sensing and regulating cell elongation (Shih et al. 2014; Feng et al. 2018; Lin et al., in submission). To investigate whether cell elongation is related to FucAl-labeled matrix deposition, *fer-4* mutant seedlings were tested for MFI and anisotropy following FucAl incorporation (Figure 12). Neither the MFI nor anisotropy were significantly altered in *fer-4* mutant lines when compared to wild type controls, suggesting that matrix deposition occurs independently of cell expansion.

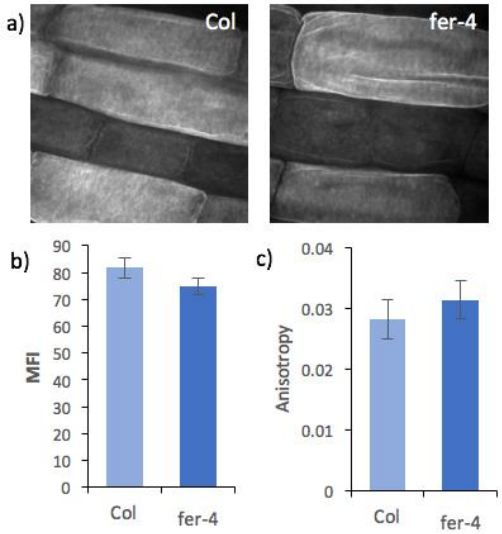

Figure 12: FucAl incorporation in *fer-4* mutants

5d old Col (WT) and *fer-4* mutant seedlings were grown in 2.5μM FucAl solution for 4h. 8-bit max projection z-stacks were produced and mean fluorescence intensity (MFI) and anisotropy were measured from 4-6 cells/seedling ($n \geq 15$ seedlings). Units for MFI and anisotropy are arbitrary and are used for comparison only.

In vivo labeling of matrix polysaccharides

Investigations into the optimization of the copper-catalyzed click reaction to be used in live cell imaging involved altering individual reaction parameters and measuring cell viability. Cell viability after incubation with various concentrations of $CuSO_4$ and ascorbic acid showed insufficient cell survival, with the highest viability occurring with 0.5mM $CuSO_4$ and 1mM ascorbic acid (Figure 13). Survival in this condition was estimated to be 4.37 compared to 9.29 in the 0mM $CuSO_4$ 0mM ascorbic acid control. Viability generally decreased with higher concentrations of $CuSO_4$ and ascorbic acid. Different reaction durations were then assessed for cell viability at different $CuSO_4$ concentrations (1mM, 0.5mM, 0.1mM) (Figure 14). Cell viability decreased as reaction duration increased at all copper concentrations. It has been reported that using a ligand molecule to chelate toxic copper in the reaction solution can mitigate low survival; for example, histidine can act as a ligand (Kennedy et al. 2011). Cell viability was tested after incubation with varying concentrations of copper and histidine at a 1:1 concentration ratio (Figure 15). Most reaction conditions did not significantly increase cell survival. Only the 0.05mM $CuSO_4$ and His condition showed a 42% increase in survival compared to the 0.05mM $CuSO_4$ and 0mM Histidine control ($p<0.0001$).

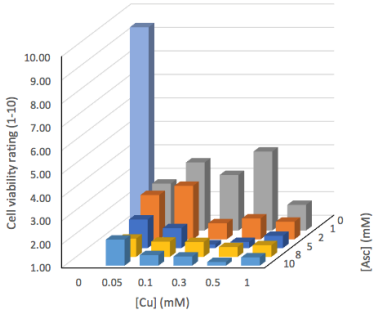

Figure 13: Cell viability and concentration of $CuSO_4$ and ascorbic acid

5d old Col (WT) seedlings were incubated with varying concentrations of $CuSO_4$ (0-1mM) and ascorbic acid (0-10mM) for one hour incubations. After PI and FDA staining, seedlings were given a cell viability rating (1-10) ($n \geq 17$ seedlings).

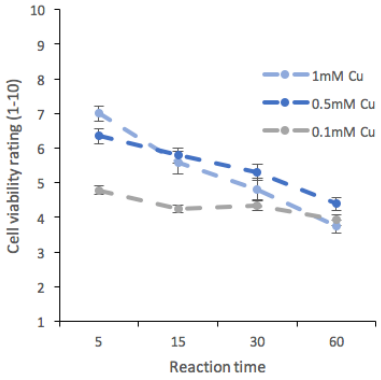

Figure 14: Cell viability and reaction length

5d old Col (WT) seedlings were incubated with varying concentrations of $CuSO_4$ and ascorbic acid (1:1) for varying durations. After PI and FDA staining, seedlings were given a cell viability rating (1-10) ($n \geq 27$ seedlings).

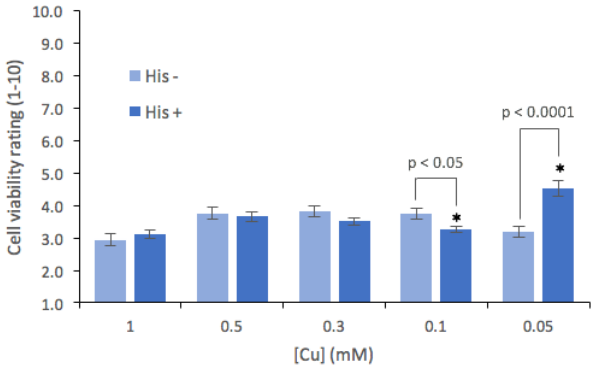

Figure 15: Cell Viability and histidine ligand

5d old Col (WT) seedlings were incubated with varying concentrations of $CuSO_4$ (0.05-1mM) and Histidine (1:1) for one hour incubations. After PI and FDA staining, seedlings were given a cell viability rating (1-10) ($n \geq 30$ seedlings).

A strain-promoted azide-alkyne cycloaddition reaction has been shown to be able to effectively label azide chemical reporters, without the use of a toxic copper catalyst (Baskin et al. 2007). Because this strain-promoted cyclic alkyne requires an azide sugar to react with, Kdo azide was used as a chemical reporter, which has been shown to be incorporated into the matrix polysaccharide, RGII, and is able to be labeled using the CuAAC reaction, similar to FucAl (Dumont et al. 2016). When incubated with seedlings for 16h, KdoAz was successfully labeled using Alexa-488 alkyne (Figure 16). Striations similar to FucAl incorporation and labeling patterns were seen, and minimal intracellular fluorescence was seen. KdoAz was then labeled

with 1μM and 10μM DIBO-488 (Figure 16). Cell walls appeared to show similar labeling with 10μM DIBO, but also showed some intracellular fluorescence, as well as fluorescence of what appeared to be artifacts above the cell wall surface. Labeling using 1μM DIBO was still visible, though less pronounced than labeling with 10μM DIBO.

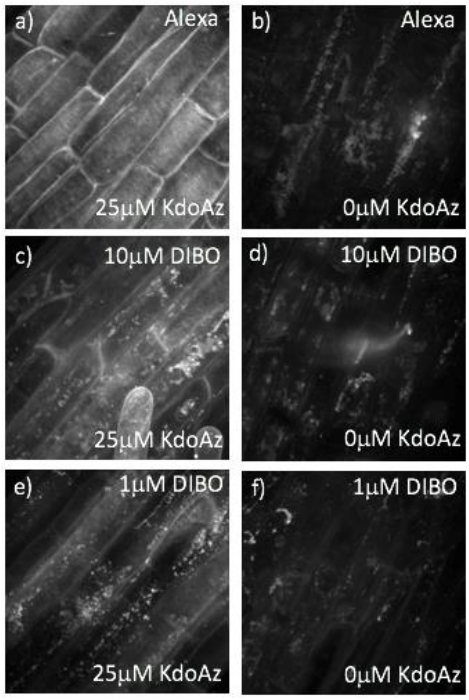

Figure 16: KdoAz incorporation and labeling

5d old Col (WT) seedlings were incubated with 25μM KdoAz for 16h, then labeled with a) 0.1μM Alexa-488 alkyne, b) 10μM DIBO-488, or c) 1μM DIBO-488. 8-bit max projection z-stacks were produced.

Chapter 4

Discussion

This study used the metabolically incorporated sugar analog, FucAl, to fluorescently label matrix polysaccharides in the plant cell wall. Genetic analysis was used to target various steps in the molecular pathway thought to be involved in the uptake, processing, trafficking, and organization of the FucAl sugar. This click-compatible molecular probe allowed us to investigate these molecular mechanisms responsible for incorporation of sugars into matrix polysaccharides, but also study the potential use of click chemistry to visualize the plant cell wall *in vivo*.

Investigations into the incorporation of FucAl

Previous research provides evidence suggesting that FucAl is metabolically taken up via the fucose salvage pathway (Anderson et al. 2012). The L-fucose salvage pathway enzyme, L-FUCOSE KINASE/ GDP-L-FUCOSE PYROPHOSPHORYLASE (FKGP), is a key molecular player in the uptake of fucose in solution (Villalobos et al. 2015). The fact that *fkgp-3* mutant seedlings showed significant defects in the amount and organization of FucAl as imaged in the cell wall, further supports the idea that FucAl is taken up by the cell by the endogenous fucose salvage pathway. These results also provide an example of FucAl being treated as endogenous fucose, rather than being mistaken as another sugar. Conversely, the Gogli-localized sugar transporter mutant, *gonst3* showed mixed results when tested for FucAl incorporation and organization. GONST3 is part of a family of nucleotide sugar transporters shown to be involved in the uptake of GDP-bound sugars into the Golgi (Handford et al. 2004). Each of the independently generated RNAi lines generated were tested for incorporation and organization of

the sugar analog, and all were shown to have significantly decreased MFI and anisotropy. These results suggest that GONST3 might play a role in the uptake of GDP-FucAl into the Golgi Apparatus. Interestingly, since GONST3 has been shown to be a galactose sugar transporter, it is possible that FucAl may be incorporated in place of another sugar, rather than in the natural location for fucose (Mortimer, unpublished). Alternatively, GONST3 might be required for the synthesis of the galactan side chains of RGI, where fucose is thought to be added. Data obtained comparing *gonst3* RNAi lines to WT controls involved background subtraction (10 pixel, sliding paraboloid) from max projection z-stacks. This image analysis protocol provided more conclusive results, distinguishing FucAl-related fluorescence from background fluorescence, and giving a better representation of relative values for comparison.

A paper published in 2012 identified DUF23 as an enzyme responsible for the synthesis of β-1,4-galactan, found in the matrix polysaccharide, RGI (Hansen et al. 2012). These galactan side chains of RGI have been shown to contain fucose (Mohnen 2008). Previous investigations into the use of FucAl as a metabolic labeling tool have provided indirect biochemical data supporting the fact that FucAl may be incorporated into RGI (Anderson et al. 2012). However, after measuring MFI and anisotropy in a *duf23* mutant line incorporated with FucAl, no significant decrease was seen, suggesting that FucAl may not be incorporated into the fucose-containing side chains of RGI synthesized by DUF23, or that there are other players involved in the synthesis of these fucose-containing side chains. *duf23* mutant lines did exhibit a significant increase in anisotropy, which could be explained by the FibrilTool software used to measure striations. Many times, larger lines contribute disproportionately to the value calculated, even if those lines are due to cell folding as a result of cell death and mounting. The smaller striations seen from FucAl incorporation may be overshadowed by larger folds, and could explain the

increase seen in the mutant line. Conversely, seedlings incorporated with FucAl and then treated with β-1,4-endogalactanase had significantly decreased MFI, indicating that FucAl-related fluorescence is contained in the cleaved side chains, agreeing with previous investigations into the location of FucAl (Anderson et al. 2012). These results together suggest that the side chains containing FucAl may be influenced by other galactan biosynthesis genes in addition to *DUF23,* such as the GALACTAN SYNTHASE (GALS) genes (Liwanag et al. 2012; Laursen et al. 2018) or that FucAl is located in other galactan side chains, not synthesized by DUF23. Galactanase treatment may also have other effects, including destabilizing interactions of the entire RGI polysaccharide with the cell wall, solubilizing more than just the side chain in question.

To investigate the trafficking and organization of FucAl-labeled polysaccharides, *osm1* mutant seedlings were tested for MFI and anisotropy following FucAl incorporation. A study that isolated the SYP61 trans-Golgi network compartment was able to characterize the proteomic contents of the vesicle, which includes many proteins of then unknown function (Drakakaki et al. 2012). One of the notable proteins found to be localized to this compartment is ECHIDNA, a mutant of which has been shown to have defects in cell elongation, suggesting that there may be consequences for the trafficking of cell wall components to the cell wall (Gendre et al. 2013). However, *osm1* mutant seedlings had no defect in the amount or organization of FucAl incorporated. This may mean that FucAl-labeled polysaccharides are trafficked via some other mechanism, independent of SYP61 TGN compartments.

Cytosolic invertases (cinv) are important for the entry of carbon into the cellular metabolism of plants, specifically UDP-glucose, which is used in cellulose synthesis. *cinv1 cinv2* double mutants show severely reduced growth rates, due to a lack of substrate for cell wall synthesis (Barratt et al. 2009). These mutants have defective cesA activity and decreased

cellulose synthesis (Barnes and Anderson 2018 Mar 23). *cinv1 cinv2* double mutant seedlings showed no decrease in FucAl-related fluorescence, but did have significantly decreased anisotropy, suggesting that FucAl is being brought to the cell wall, but does not have the same organization once incorporated. This could be attributable to changes in cellulose organization in *cinv1 cinv2* mutants (Barnes and Anderson 2018 Mar 23). Experiments measuring MFI and anisotropy in wild type seedlings treated with isoxaben, which inhibits cellulose synthesis, showed similar results (McCloskey et al., unpublished). These results together suggest that FucAl labeled polysaccharides depend on cellulose to adapt the normal striation patterning seed in negative controls. It may also be inferred that while the *cinv1 cinv2* mutant lines affect cellulose synthesis, other polysaccharide trafficking is not affected, indicating that either cellulose specifically is unable to be synthesized along with other polysaccharides, or they are trafficked via different mechanisms. In an effort to visualize further the effects of impaired cellulose synthesis on the appearance of recently deposited matrix, an updated protocol was used to image matrix polysaccharides at high magnifications. Because defective cellulose synthesis causes a decrease in the striation patterning of FucAl incorporated into the cell wall, matrix polysaccharides may appear differently under these high magnifications using FESEM imaging. Further research is needed to conclusively determine the effect on matrix appearance.

To explore the question of whether or not cell expansion is related to matrix polysaccharide incorporation, *feronia* mutant seedlings were tested for MFI and anisotropy following FucAl incorporation. FERONIA is a receptor tyrosine kinase involved in cell wall integrity sensing and activating compensatory pathways for cell expansion (Lin et al. 2018). Mutant knockouts of *fer-4* show impaired growth phenotypes consistent with impaired mechanical development (Shih et al. 2014). However, FucAl-related fluorescence was unaffected

in *fer-4* mutants under normal conditions. FERONIA is required for cell viability under salt stress (Feng et al. 2018). It would be interesting to evaluate FucAl-labeled matrix polysaccharides in these conditions, as the stress signaling in response to salt stress may have an effect on FucAl labeling.

In vivo labeling of matrix polysaccharides

While genetically encoded fluorescent proteins have shaped the way proteins are studied, similar tools for the study of cell wall polysaccharides has lagged behind. Because of the size and nature of cell wall polysaccharides, another technique is needed to fluorescently visualize cell wall dynamics. Click chemistry provides vast opportunities to label and study a variety of biomolecules. Specifically, the copper catalyzed azide alkyne click reaction provides a unique opportunity to label the polysaccharides in the plant cell wall through metabolic incorporation of sugar analogs that can then be labeled using the CuAAC reaction (Anderson et al. 2012). However, because of the slow reaction kinetics, a copper catalyst must be used, which at concentrations needed for sufficient labeling, is toxic to cells (Drazkiewicz et al. 2004). Ascorbic acid is used as a reducing agent for the copper catalyst, and subsequently participates in side reactions that produce hydrogen peroxide (Hong et al. 2009). However, at higher concentrations ascorbic acid is able to act as an antioxidant, possibly reducing the number of free radicals as a result of the click reaction (Yen et al. 2002). When different concentrations of copper and ascorbic acid were incubated with seedlings, cell viability was not completely rescued in any specific condition. At the copper concentrations that would be needed for effective labeling, no amount of ascorbic acid was able to lessen the effects of copper toxicity, indicating that a

copper-chelating molecule may be needed. Ligands have been used to mediate copper related toxicity as a result of the CuAAC reaction, including THPTA, BTTES, BTTAA, and BTTP (Besanceney-Webler et al. 2011). Seedlings incorporated with FucAl and labeled using BTTP as a ligand resulted in a relative increase in cell viability, but not at copper concentrations necessary for effective labeling (McCloskey et al., unpublished). Histidine has been shown to mitigate copper toxicity in the presence of ascorbic acid by forming a complex and not allowing copper to participate in detrimental side reactions (Kennedy et al. 2011). However, cell viability in seedlings treated with ascorbic acid, copper, and histidine only showed a significant increase in viability at lower copper concentrations, not sufficient for labeling. The use of histidine may have an increased rescue effect when used at a 2:1 ratio with copper, rather than the 1:1 ratio that was tested. This suggests that histidine may still be a candidate for a chelating ligand, and should be looked into further for optimization of the CuAAC reaction to be used *in vivo*. The last variable that was tested for optimization of the CuAAC reaction was reaction length. Because of the toxicity of the copper catalyst, cell viability decreased as time incubated with copper and ascorbic acid increased. However, at all copper concentrations tested, cell viability was not completely rescued even with short incubation times that may not sufficiently label cell walls.

Initial attempts for cell wall labeling using the copper-free strain promoted azide-alkyne cycloaddition reaction (SPAAC) were promising. Imaging of glycosylated proteins on the surface of live cells has been successful in zebrafish, utilizing a similar reaction (Laughlin et al. 2008). Because this strain promoted reaction requires a cyclic alkyne fluorophore, it would not be suited for labeling of FucAl. An azide sugar analog is necessary, one of which was described and found to incorporated into the pectic polysaccharide, RGII (Dumont et al. 2016). While it cannot be assumed KdoAz is incorporated through the same molecular pathway that FucAl is,

this sugar analog, if labeled in live cells would provide a unique technique for visualizing matrix polysaccharides *in vivo*. Experiments using the SPAAC reaction seemed to show successful labeling of KdoAz. However, there appeared to be some intracellular fluorescence, possibly as a result of either fluorophore leaking into the cells, or live cell components' autofluorescence. There also appeared to be some labeling above the surface of the cells, which is likely an artifact that may have somehow reacted with the fluorophore or shows strong autofluorescence. These labeling results, while not initially as robust as copper-catalyzed labeling, show strong potential, and indicate that this strain-promoted reaction may lead to successful and strong *in vivo* labeling of matrix polysaccharides.

FucAl as a metabolic fluorescent reporter can be used to investigate the uptake, processing, trafficking, and organization of matrix polysaccharides in the plant cell wall. Multiple steps leading up to the incorporation of FucAl were able to be characterized because of the specificity of reporter incorporation. In future studies, genetic and biochemical analyses could further our understanding of the specific mechanisms responsible for FucAl incorporation, providing a unique tool for the investigation of matrix polysaccharide processing and dynamics. The use of a similar click labeled sugar analog was explored, using a copper-free strain promoted reaction that can be applied to *in vivo* studies. Recently developed and improved cyclic alkyne reporters provide opportunities to optimize this labeling technique. The utility of this biochemical labeling technique could have far-reaching applications in the study of the plant cell wall, increasing our understanding of cell wall formation and dynamics.

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