

Chapter 2

Using Chemical Genomics to Study Cell Wall Formation and Cell Growth in *Arabidopsis thaliana* and *Penium margaritaceum*

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Abstract

The cell wall is directly involved in cell growth, and its ability to loosen and rearrange allows for cell expansion through the existing turgor pressure. Thus, information on cell wall deposition and rearrangement can provide insights into the overall plant growth. This chapter describes two methods that can be used to evaluate cell expansion (1) in the model plant *Arabidopsis thaliana* and (2) the model alga *Penium margaritaceum*. These methods are further used to screen for small molecules that induce cell growth phenotypic changes affecting cell wall. Identification of such small molecules is beneficial due to their posttranslational mechanism of action that can be controlled in a temporal and spatial manner. Chemical genomics has the ability to overcome issues of genetic redundancy and lethality, which can hinder traditional genetic methods. The identification of small molecules in these screens will provide useful information on plant cell wall biology and overall plant growth.

Key words *Penium margaritaceum*, *Arabidopsis thaliana*, Chemical genomics, Cell wall biology

1 Introduction. Cell Wall and Cell Growth

The cell wall is a dynamic network of polysaccharides and proteins that constitute the extracellular matrix of the plant cell. It provides structural support and serves as a protective barrier for the protoplast against biotic and abiotic stresses [1]. The cell wall also plays a vital role during plant cell growth as its tensile strength serves as the structural regulator of turgor-driven expansion. Plant cell expansion typically occurs in one of two ways: *diffuse* expansion, where new cell wall materials are deposited and incorporated into the cell wall uniformly along the expanding cell and *polar* expansion, where growth is focused at a specific point or front of the cell [2]. In both types, changes in cell wall polymer arrangement lead to a weakening of the wall architecture, which permits the existing, constant turgor pressure to expand into the weakened cell wall via a process known as “creep” [1–4]. In pollen tubes, the rates of

growth correlate with rates of cell wall deposition, suggesting a dynamic model of continuous cell wall loosening, rearrangement, and polysaccharide deposition which allows turgor-driven cell expansion and reorientation for cell growth [5, 6]. In fact, the amount of cell wall material deposited can predict with up to 90 % accuracy the degree of pollen cell expansion [5].

The generation of plants with altered cell wall and expansion characteristics has been very valuable in the dissecting mechanisms of plant cell growth. Derived from either genetic mutations or application of pharmacological agents, wall associated structures or events may be specifically “tested” to elucidate their roles in the expansion process.

1.1 Using Chemical Genomic Approaches to Study Cell Wall Modifications During Cell Growth

Chemical genomics is the use of small molecules rather than genetic mutations to perturb, study, and control the cellular and physiological function of proteins [7]. This approach is similar to that employed in the screening of chemicals for pharmacological drug and herbicide applications [8].

As a result of the altered cell wall function in plant cell growth and development, mutations in biosynthetic genes can be lethal [9, 10]. Chemical genomic screens can be performed at specific developmental stages using sublethal concentrations of small molecules that overcome lethality limitations [7, 8]. Additionally, redundancies that are observed in gene products involved in cell wall biosynthesis and metabolism [11, 12] and phenotypes of knockout mutations may be difficult to detect. Small molecules can be highly specific and be used to distinguish a single protein from a large family of proteins, or they can target proteins with conserved active sites, thus addressing redundancy. Despite these advantages, fundamental challenges in plant chemical biology remain, including target identification and off-target effects [8]. The protocols described below illustrate chemical genomic screens for plant and algal cell wall and growth modifications.

1.2 Model Organisms to Study Cell Wall Development and Cell Growth: *Arabidopsis thaliana* and *Penium margaritaceum*

The small flowering plant, *Arabidopsis thaliana* (Brassicaceae, Angiospermae), is commonly used as a model organism in cell expansion and cell wall deposition studies [13]. Many *Arabidopsis* mutants with altered cell wall properties have already been characterized [14], providing a useful toolset for future chemical genomic screens.

Penium margaritaceum (Peniaceae, Zygnematales, Streptophyta) is a green alga that has recently attracted attention as another efficacious model organism for cell wall studies. *Penium* is single celled, easy to grow, and produces only a simplified primary cell wall predominately composed of cellulose and pectic polysaccharides [15]. The alga also has a simple cylindrical shape that makes genetic or experimental alterations to cell wall/cell expansion easy to monitor and quantitative growth measurements easy to

Table 1
Examples of chemicals affecting cell wall composition and cell growth

Chemical	Effect	References
Isoxaben	Cellulose biosynthesis, targeting CESA3 and CESA6	Scheible et al. [20] and Desprez et al. [21]
BFA	Disruption of Golgi-mediated trafficking, alters cell wall secretion	Nebenführ et al. [28] and Hörmanseder et al. [29]
Oryzalin	Depolymerizes microtubules, alters cell wall texture	Morejohn et al. [30] and Chan et al. [31]
Morlin	Alters microtubule organization and CESA velocity	DeBolt et al. [22]
Chemical A	Inhibits UDP-glucose transfer activity in Golgi-enriched fractions	Zabotina et al. [27]
DCB	Inhibits cellulose biosynthesis	Montezinos et al. [23]
CGA	Inhibits cellulose biosynthesis	Peng et al. [26]
Cobtorin	Disrupts microtubule/cellulose relationship	Yoneda et al. [24, 25]

calculate. The rapid growth of *Penium* also allows for acquisition of abundant cell wall material that may be subsequently used for specific biochemical analyses [16]. Additionally, live *Penium* may be labeled with monoclonal antibodies (mAbs), placed back in culture, and subsequently monitored for growth dynamics [15, 17]. Finally, a new protocol for transforming *Penium* has recently been developed that allows for the generation of cell lines that are useful for the study of cell wall mechanics including knockouts, overexpressed lines, and ones expressing fluorescent protein fusions [18].

Here, we describe the protocol for using chemical genetics with these two model organisms to identify potentially important pharmacological inhibitors that may be used to dissect the processes of plant cell wall expansion. The notable similarities in cell wall biochemistry found throughout the plant kingdom [16] allow for reasonable and valuable translation of chemical hits from these model systems to plants of agricultural importance.

1.3 Examples of Small Molecules Affecting Cell Wall Deposition

Chemical genomics has been a powerful tool in enhancing our understanding of cell wall biology. An arsenal of bioactive molecules have been identified and have had significant impacts in cell wall studies [19]. These include (Table 1): *isoxaben* (N-[3-(1-ethyl-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide), which inhibits incorporation of UDP-Glucose into cellulosic regions of the cell wall and has played an instrumental role in identifying members of cellulose synthase complexes (CESA), CESA3 and CESA6 [20, 21]. The inhibition specificity of isoxaben for only two members of the cellulose synthase superfamily also illustrates the power of small molecules in overcoming functional

redundancy. *Morlin* (7-ethoxy-4-methyl chromen-2-one) was identified in a screen for swollen organ morphology, such as roots. It affects microtubule organization in the cell cortex and the velocity of CESA movement in the plasma membrane, possibly targeting the interaction between the CESA complex and the microtubules [22]. The herbicide, *DCB* (2,6-dichlorobenzonitrile), is a cellulose biosynthesis inhibitor that arrests movement of CESA complexes at the plasma membrane [23]. *Cobtorin* (4-[(2-chlorophenyl)-methoxy]-1-nitrobenzene) also alters the relationship between cortical microtubules and the production of cellulose microfibrils [24, 25]. Yoneda et al. [25] used cobtorin in showing that pectin plays a critical role in the deposition and maintenance of cellulose microfibril orientation. *CGA* (1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1 λ 4,2,4,6-thiatriazin-3-amine) also inhibits cellulose biosynthesis [26]. *Chemical A* (methyl 3-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl) benzoate) inhibits the transfer of UDP-glucose in Golgi membranes [27] and consequently affects downstream deposition of cell wall components. In addition, *Brefeldin A* (BFA; 4-Dihydroxy-2-(6-hydroxy-1-heptenyl)-4-cyclopentanecrotonic acid) blocks Golgi-dependent trafficking and has been shown to inhibit secretion of cell wall components [28, 29]. *Oryzalin* (4-(dipropylamino)-3,5-dinitrobenzenesulfonamide) targets tubulin and alters cellulose crystallinity [30–32].

1.4 High-Throughput Chemical Screening for Growth Defects in *Arabidopsis thaliana* and *Penium margaritaceum*

An effective strategy for the identification of novel inhibitors of cell wall deposition is the assessment of the effects of large arrays of small molecules on the phenotype of specific cell wall/growth mutants. Forward chemical genomic screens can be performed by employing a chemical library (*see Note 1*) of small molecules that yield a specific phenotype such as root swelling [22] in specific mutant or wild type plants. In addition, chemical screens can be based directly on biochemically determined cell wall modifications [27, 33]. In this chapter, we describe a screen for *Arabidopsis thaliana* mutants based on hypocotyl length using the herbicide, *isoxaben*.

In the *Arabidopsis*-based procedure, changes in growth are assessed by measuring hypocotyl lengths, a suitable indicator of cell wall perturbation. This approach benefits from the ease of measurement compared to biochemical methods. Resistance to the herbicide isoxaben is observed in the *ixr1* and *ixr2* mutants which carry mutations in cellulose synthase 3 (CESA3) and cellulose synthase 6 (CES6) [20, 21].

This chapter also introduces the methodology to assess the effects of small molecule inhibitors on *Penium margaritaceum* during cell wall expansion and to detect cell wall structural and biochemical changes. To demonstrate this, BFA was chosen to induce changes in *Penium* cell growth. The protocols below provide examples of chemical screens that can be adjusted to the specific needs of individual research projects.

2 Materials

2.1 *Arabidopsis thaliana*

Screening for changes in hypocotyl length in Arabidopsis thaliana after chemical application

To prevent contamination, aseptic laboratory materials and double-distilled or deionized water should be used during germination.

2.1.1 Growth Materials for *Arabidopsis thaliana*

1. Sterilization solution: 30 % (v/v) sodium chlorate (bleach) in ethanol (absolute) with 30 μ L Triton X-100 (Sigma) per 50 mL of solution.
2. 1.5 mL Eppendorf tubes (Fisher, #05-402).
3. 0.5 $\times$ *Arabidopsis* growth medium (AGM): 2.3 g/L Murashige minimal organics medium (Sigma, #M6899), 10 g/L sucrose (Fisher), and 8 g/L Phytigel (Sigma, #P8169) (*see Note 2*). Autoclave for 30 min at 120 °C and store at room temperature (RT).
4. Plant growth incubator set at 24 °C with a 16 h light cycle.
5. Aluminum foil.
6. Seed stock for screening phenotype of interest, this includes both wild type and mutant seeds. In this example we are using *Arabidopsis thaliana* Columbia (Col-0) wild type seeds and *ixr1-1* mutant seeds.
7. Parafilm.
8. 1 μ L inoculation loop (Globe scientific, #2801).
9. 6 well plate (Fisher 08-772-1B).
10. Chemical library of interest to be screened in 5 mg/mL stock solution in dimethylsulfoxide (DMSO) (Sigma, #D8418).
11. Flatbed scanner.
12. 4 % v/v formaldehyde solution in water.
13. Gelrite (Research Products International Corp, G35020).
14. A clear plate or lid for holding Gelrite.
15. Vortex Genie 2 (Fisher, #NC9864336).

2.2 *Penium margaritaceum*

Using small cell molecules to perturb the wall in Penium margaritaceum

Sterile medium and aseptic techniques should be employed during all procedures.

2.2.1 Growing *Penium*

1. *Penium margaritaceum*, a Skidmore college isolate (Skd-8).
2. Sterile polystyrene 25 cm² cell culture flask (Corning Incorporated, #430372).
3. 1.5 mL Eppendorf tubes (Fisher, #05-402).
4. Microcentrifuge tubes (Eppendorf, #5417C).

5. 2 μL pipette and 200 μL pipette (Gilson).
6. MWC (Modified Woods Hole Medium) [34]. To make MWC combine stock solutions 1–17 (1 mL each), 0.115 g/L (final volume) TES dry buffer (Sigma, #T5691-100G), 1.5 % Soil–Water Supernatant (Carolina Biological Supply Company, Burlington, #15-3790) and make up to 1 L with deionized water. Autoclave at 120 °C for 20 min.

(a) *Macronutrients (stock solutions):*

1. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ —36.80 g/L.
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ —37.00 g/L.
3. NaHCO_3 —12.60 g/L.
4. $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ —11.40 g/L.
5. NaNO_3 —85.00 g/L.
6. $\text{Na}_2\text{O}_3\text{Si} \cdot 9\text{H}_2\text{O}$ —28.40 g/L.

(b) *Vitamin stocks:*

7. Thiamine HCl—0.1 g/L.
8. Biotin—0.0005 g/L.
9. Cyanocobalamin—0.0005 g/L.

(c) *Combined trace elements:*

10. EDTANa_2 —4.36 g/L.
11. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ —3.15 g/L.
12. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ —0.01 g/L.
13. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ —0.022 g/L.
14. $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ —0.01 g/L.
15. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ —0.18 g/L.
16. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ —0.006 g/L.
17. H_3BO_3 —1.00 g/L.

2.2.2 Chemical Treatment and Microscopy

1. 7- to 14-day-old *Penium margaritaceum* culture.
2. Chemicals to be screened.
3. MWC solution.
4. DMSO as control (*see Note 3*).
5. Carnation Instant nonfat milk.
6. JIM5 or LM18 monoclonal primary antibodies (Plant Probes, Leeds, UK) (*see Note 4*).
7. Fluorescein isothiocyanate (FITC)-Goat Anti-Rat secondary antibody (Invitrogen, #62-9511).
8. LSM 700 confocal laser scanning microscope (Carl Zeiss, Germany), Olympus Fluoview 1200 (Olympus, Center Valley, PA, USA), or other high resolution confocal microscope.

9. Disposable transfer pipettes (Fisher Scientific, #S30467-1).
10. Microscope cover glasses 22×40 mm (VWR international, #16004-306).
11. Microscope slides, Superfrost Plus (VWR).

3 Methods

3.1 *Arabidopsis thaliana*

3.1.1 Seed Sterilization

Perform work with sterile seeds and plates in a laminar flow hood. All procedures may be carried out at room temperature, unless otherwise specified.

1. Add 500 μL of sterilization solution to 50 mg of *Arabidopsis* seeds in a 1.5-mL microcentrifuge tube.
2. Shake on speed 3 or lower for 10 min in a Vortex Genie.
3. In a laminar flow hood, remove sterilization solution and rinse three times with 100 % ethanol, allow to air dry.
4. Store sterilized seeds at 4 °C.

3.1.2 Growth and Chemical Treatment of *Arabidopsis thaliana* Seedlings

1. Prepare 5 mL of 0.5 AGM/phytagel and add a starting volume of 25 μL of the 5 mg/mL chemical stock. Dispense 5 mL of the AGM supplemented with chemicals into the wells of a 6 well plate, and use DMSO as a control. Here 10 nM of isoxaben is used as treatment. Pipette repeatedly to mix the chemical thoroughly within the media. Allow media to solidify (*see Note 5*).
2. Dispense the required number of seeds onto the sterile side of a parafilm slice.
3. Use the needle tip of a sterile 1 μL inoculation loop to transfer the seeds onto the media in the 6 well plate. Between 15 and 20 seeds are recommended per well, positioned in a straight line 2 cm from the top of the well (*see Note 6*). In the presented example we will be using both Col-0 and *ixr1-1* seeds, each being allocated a well per chemical treatment and control.
4. Cover completed screening plates with aluminum foil and leave in a 4 °C refrigerator for 48 h to cold vernalize and ensure uniform germination (*see Note 7*).
5. After cold vernalization, place plates in a 24 °C growth chamber. Expose to light for 3 h to induce germination and cover with foil to etiolate and allow for hypocotyl expansion while growing vertically (*see Note 8*).
6. Image seedlings at 5, 7, and 10 days, this can be easily done using a flatbed scanner. Use a resolution of at least 300 dpi when scanning to allow for accurate measurements (*see Note 9*).
7. On the tenth day fix the seedlings using 400 μL of 4 % v/v formaldehyde per well, and allow to rest for at least 2 h.

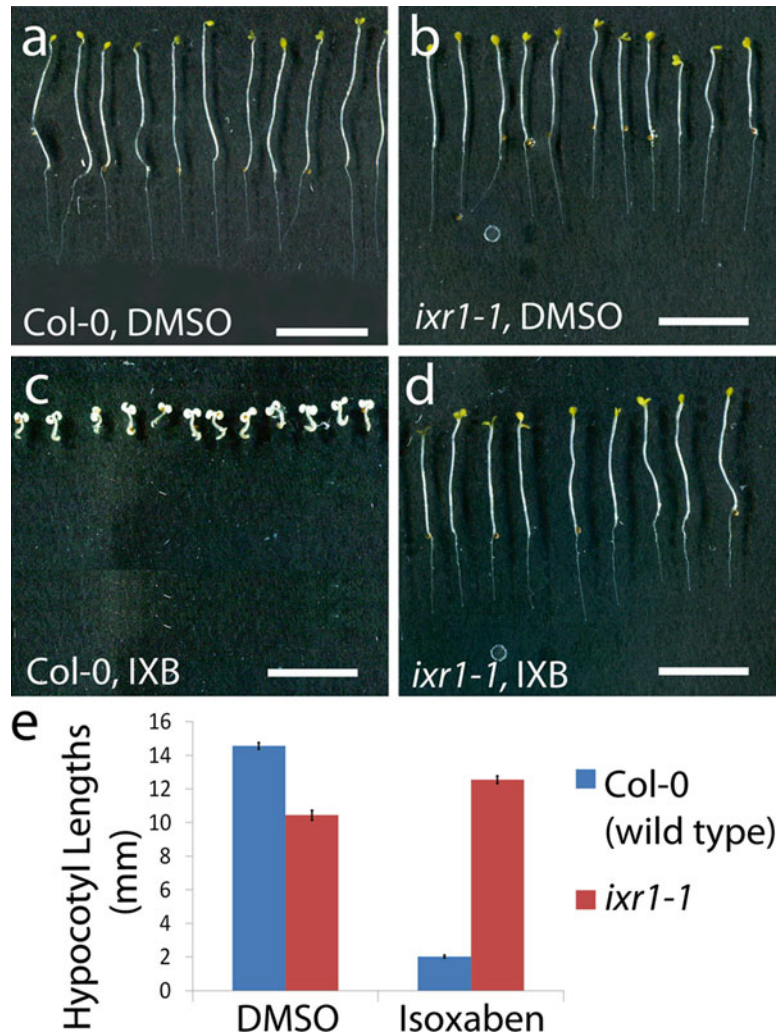


Fig. 1 Hypocotyl growth in the presence of *isoxaben*. Results of *isoxaben* screen against *Arabidopsis thaliana* wild type Columbia (Col-0) (a, c) and the Cellulose Synthase (CESA3) mutant *ixr1-1* (b, d). The mutant *ixr1-1* shows resistance to 10 nM *isoxaben* compared to Col-0 (d). Quantification of hypocotyl length (e) reveals no growth difference for the mutant, whereas Col-0 exhibits a sevenfold reduction. Two way ANOVA of the growth effects between the two genotypes. $p < 10^{-15}$. Scale = 10 mm

8. Make a solution of 1 % Gelrite in ddH₂O, microwave and pour into a clear plate, and let solidify.
9. Transfer fixed seedlings on the top of a 1 % Gelrite agar bed in a clear plate, and align them in parallel for subsequent imaging and accurate measurements. Figure 1 demonstrates the results of the example using the mutant *ixr1-1* and the cellulose inhibitor *isoxaben*.

3.1.3 Analysis of Hypocotyl Lengths Using ImageJ

1. Measure the seedlings lengths from your scanned photographs using ImageJ [35]. Set scale (Analyze>Set Scale) by using a ruler scanned at the same resolution as the scanned seedlings.
2. Use the segmented line tool to track the plant hypocotyl.
3. Press “Ctrl M” to measure.
4. Transfer measurements to an Excel worksheet, and perform a two-way ANOVA using the statistical package R [36], or other software package of your choice. Compare each genetic background’s response to the treatment.

3.2 *Penium margaritaceum*

3.2.1 *Penium* Propagation

Perform *Penium* culture transfers (i.e., establishing subcultures) in a laminar flow hood. All the procedures may be carried out at RT unless otherwise specified. Subcultures are performed weekly or as needed. Seven- to fourteen-day-old cultures should be used for screening.

1. Add 30 mL of sterile MWC to a 25 cm² cell culture flask.
2. Add 3 mL of 7- to 14-day-old *Penium* cell culture to the flask; disperse the cells by gentle shaking of the flask.
3. Incubate the freshly subcultured *Penium* in a growth chamber at 18–24 °C with a 16:8 h light:dark (L:D) cycle under cool white fluorescent light (74 μmol/m² s Photosynthetic Photon Flux).

3.2.2 Harvest of *Penium* Cells

1. Add 1 mL of 7- to 14-day-old *Penium* cell culture to autoclaved Eppendorf tubes (shake the cell culture flask by hand until all the *Penium* is uniformly suspended in MWC) (*see Note 10*).
2. Centrifuge at 1,500×*g* for 1 min to pellet the cells.
3. Remove supernatant by pipetting the supernatant out, then add 1 mL of *Penium* cell culture and vortex for 10 s to mix the cells.
4. Repeat **steps 1–3** three times (*see Note 11*).
5. Remove supernatant from harvested cells by pipetting, then add 1 mL of MWC.
6. Centrifuge at 1,500×*g* for 1 min to pellet the cells. Repeat **steps 5** and **6** twice to remove any of the extracellular polymeric substance (EPS) that may be secreted while in culture.
7. Resuspend the cell pellet in 300 μL of MWC supplemented with desired small molecules
8. Calculate cell density with a hemacytometer [37].

3.2.3 Chemical Treatment

1. Make chemical treatment solutions in sterile Eppendorf tubes, using selected chemical stock solutions diluted in MWC growth medium. Here, we use 25 μM BFA as the working solution.

2. After harvesting cells from the procedure employed above (**steps 1-8**, Subheading **3.2.2**), adjust the final volume to 300 μL with the chemical treatment solution, and incubate for 24 h or other preferred times under the same growth conditions described earlier.

3.2.4 *Penium Cell Wall Immunolabeling*

After chemical incubation, live cell labeling can be initiated.

1. Centrifuge the treated cells at $1,500 \times g$ for 1 min, and discard supernatant.
2. Add 300 μL of blocking solution which consists of 1 % Carnation Instant Nonfat Milk in MWC. Mix by vortexing and let sit for 20 min. This constitutes the primary blocking step of the labeling process.
3. Centrifuge the treated cells at $1,500 \times g$ for 1 min, and discard supernatant. Add 300 μL of MWC, vortex cells, recentrifuge, and remove supernatant. Repeat this step twice more. This constitutes the wash.
4. Centrifuge the treated cells at $1,500 \times g$ for 1 min, and discard supernatant. Resuspend the pellet in primary antibody solution (1:10 dilution of JIM5 or LM18 in MWC).
5. Mix by vortexing or inverting (*see Note 12*).
6. Incubate for 90 min at RT (*see Note 13*).
7. Wash three times with fresh MWC—*see step 3*.
8. Add 300 μL of blocking solution (prepared as described in **step 2**).
9. Centrifuge the treated cells at $1,500 \times g$ for 1 min, and discard supernatant. Resuspend pellet in the secondary antibody solution consisting anti-rat FITC or tetramethylrhodamine (TRITC) (1:50 dilution in MWC) for 90 min at RT. Gentle shaking during this period is recommended.
10. Wash the cells three times with fresh MWC and proceed with microscope imaging or with a secondary chemical treatment.
11. For a postlabeling chemical treatment, return the cells into the same chemical treatment solution, and incubate for additional time periods as described earlier. Aliquots of cells may be taken from the treatment solution and viewed at various periods. Unlabeled zones represent new areas of cell wall.
12. For recovery experiments, treated cells are washed three times with MWC. The pellet is resuspended in fresh MWC and cultured as described earlier. Aliquots of cells may be examined at different time periods thereafter to examine cell recovery.

3.2.5 *Microscopy Visualization*

1. Transfer 15 μL of cell suspension onto a microscope slide using a disposable transfer pipette and add cover glass of appropriate thickness for the microscope (*see Note 14*).

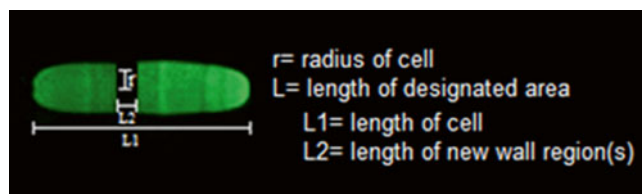


Fig. 2 Quantitative parameters for *Penium margaritaceum* growth. To quantify the effect of chemical treatments on *Penium* growth the length of the cells (L_1) and length of new wall regions (L_2) are measured and compared between treated cells and nontreated cells (control)

2. For chlorophyll autofluorescence imaging, one can use the FITC 488 excitation with a filter that allows for emission signals over 580 nm. Alternatively, a Texas Red filter set may be used for chlorophyll autofluorescence.

3.2.6 Calculating Cell Growth Measurements During Treatment

For each of the chemical treatments, perform the following quantitative measurements using Image J and the confocal images acquired as described earlier:

1. Measure the corresponding length of a designated area (L) as shown in Fig. 2. L_1 corresponds to the overall length of the cell. L_2 corresponds to the length of newly deposited cell wall as shown by nonfluorescent zones produced postinitial JIM5 labeling.
2. Calculate the Surface area (SA) of a cell and the specific area of new cell wall after polysaccharide labeling using the formula: $SA = 2(\pi \times r^2) + (2\pi \times r) \times L$, where r = radius of cell (8.5 μm) (Fig. 5).

3.2.7 Expected Results. Phenotypic Changes in *Penium* Growth After BFA Treatment

The chemical treatment (25 μM BFA) used in this experiment causes representative phenotypic changes shown in Fig. 3.

3.3 *Penium* Growth Changes After BFA Treatment

The length (L_1) of treated cells decreased significantly under 25 μM BFA ($p=0.0067$). In addition, new cell wall regions were affected under BFA, suggesting an effect on cell division and growth of new cells. New cell wall regions (L_2) were shorter and almost nonexistent in 25 μM BFA ($p=0.00018$) (Fig. 4).

Quantitative analysis of the surface area (SA) showed a significant reduction in the newly formed cell wall area of BFA treated cells compared to the control ($p=0.00043$) (Fig. 5). This establishes the effect of BFA on cell division, likely due to perturbation of Golgi-dependent pathways involved in secretion of cell wall components.

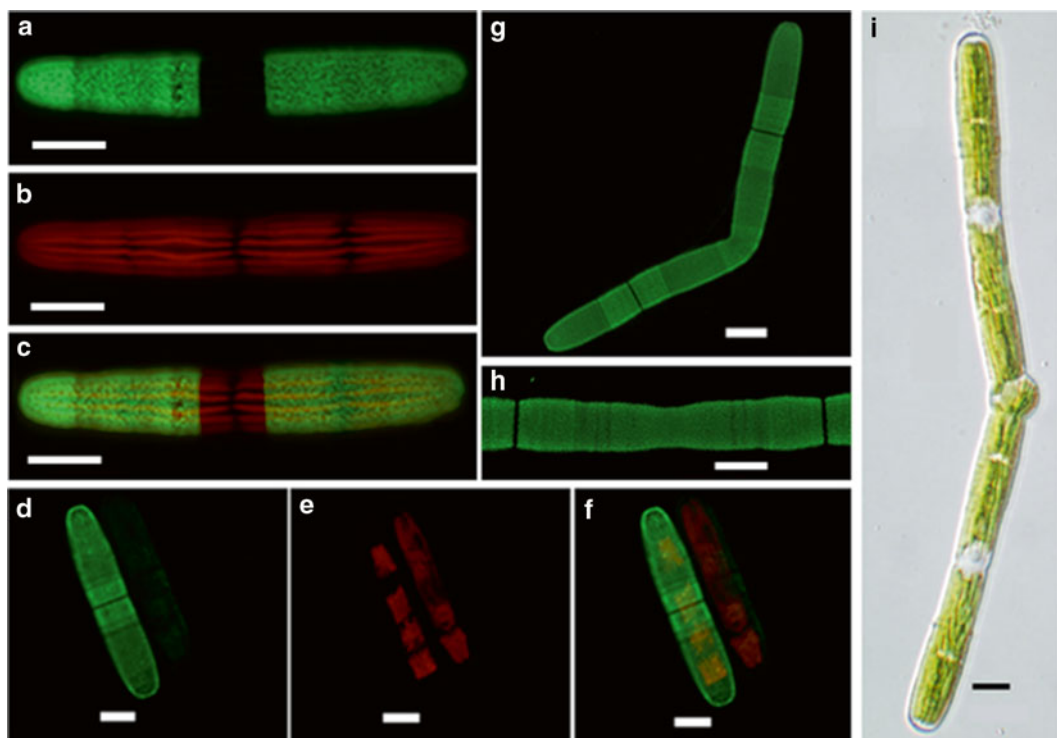


Fig. 3 Effects of BFA treatment on *Penium* growth. Panels **a–c** show typical cell morphology of control treated cells after 24 h labeling. JIM 5 labeling shows immunostaining of homogalacturonan (**a**). Panel **b** shows chlorophyll autofluorescence. **c** is an overlay of **a** and **b**. Scale bar = 20 μ m. Typically BFA treatment caused reduction of cell growth as shown in panels **d–f**. In addition, other cellular effects are observed: inhibition of cell division and cytokinesis effect (**g**, **h**). Highly irregular swollen isthmus (**i**). The panels **g**, **d**, **h** represent JIM5 labeling. Panel **e** represents chlorophyll autofluorescence. **i** represents brightfield image. Scale bar = 20 μ m

4 Notes

1. Chemical libraries are commercially available from chemical companies such as ChemBridge or Maybridge. Targeted libraries can be assembled searching for structural similarity to known active compounds or diverse chemicals selected for the same biological activity [38, 39].
2. Phytigel generally shows more consistent, reproducible results in chemical screens because of its increased purity over phytoagar, however best results are obtained in media containing sucrose.
3. DMSO was used as a control at the same dilution as the corresponding chemical. All the other procedures (antibody labeling, washing, and visualization) remain the same.
4. JIM5 binds to a relatively low methyl esterified homogalacturonan found in the walls of diverse plant species. LM19 recognizes the homogalacturonan domain of pectic polysaccharides [40, 41].

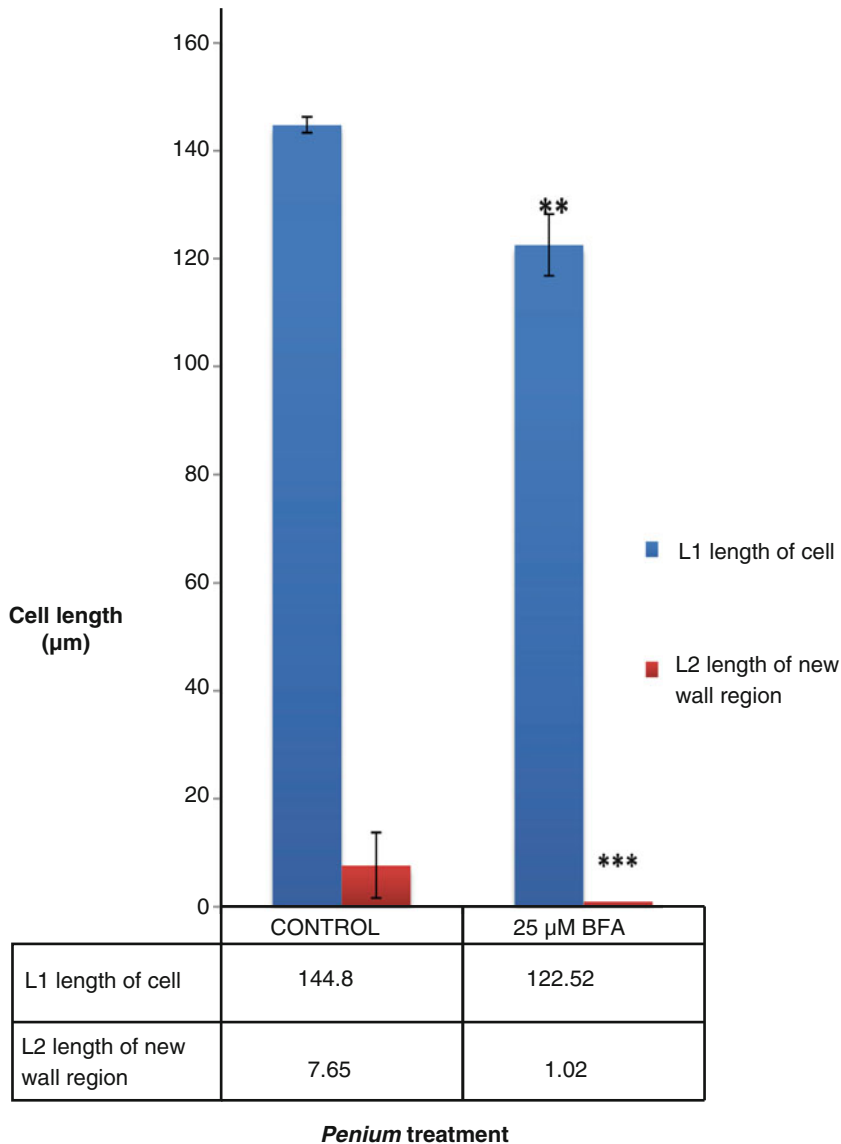


Fig. 4 Cell length analysis of BFA treated *Penium* cells. Calculated length of designated areas (L1 and L2) of *Penium margaritaceum* in control and 25 μ M BFA. L1 length of whole cell, L2 length of new wall regions. ** $p < 0.01$, and *** $p < 0.001$

5. Start with a dilution 1:200 of the original stock (5 mg/mL) and decrease the concentration if plants are not germinating or are too heavily affected. Alternatively, increase concentration if no effect is seen.
6. Perform secondary and tertiary screens to increase the sample number and observe several dilutions in order to confirm the original results.
7. Increase the vernalization time to more than 48 h if seeds are not germinating uniformly.

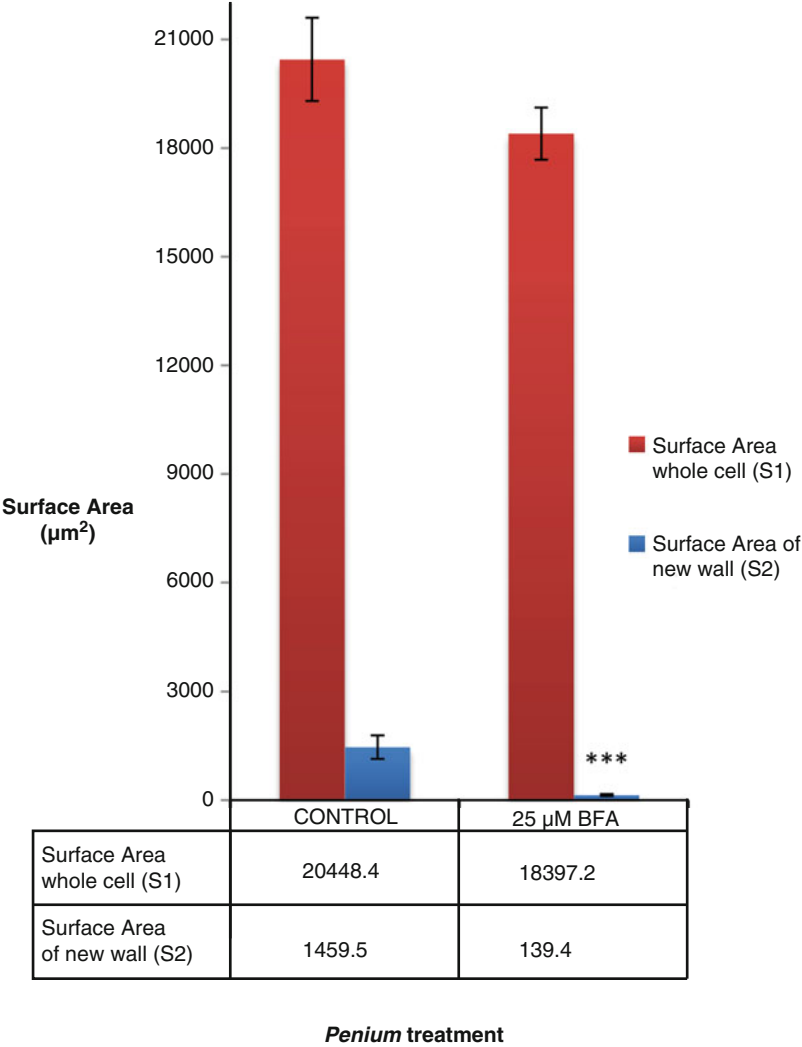


Fig. 5 Calculation of surface areas (SA) for the whole cell, and the new cell wall in *Penium margaritaceum* under 25 µM BFA treatment. *** $p < 0.001$

8. In this protocol we are exploring the effects of isoxaben on hypocotyl expansion; however, the same procedure can be followed for observing changes in root growth by growing the plants under light illumination.
9. Other methods can be used for imaging; scanning is a straightforward approach with high reproducibility.
10. This method can be adapted to 96-well plate high-throughput screens. *Penium* is convenient for microplate assays of pharmacological agents.
11. Harvest 3 mL of *Penium* culture by sequential centrifugation in a 1.5 mL Eppendorf tube.
12. The resuspension of the cell pellet should be slow and uniform.

13. It is recommended that the tubes be gently shaken during this period and/or occasionally vortexed every 30 min.
14. Add the cover glass gently to avoid cell damage.

5 Concluding Remarks

The protocols described here outline simple and straightforward chemical genomics-based methods for the study of plant cell growth via cell wall perturbation. In the *Arabidopsis thaliana* experiment we demonstrated the *ixr1-1* mutant's resistance to the chemical isoxaben, measuring hypocotyl length as a phenotype. We further demonstrated that the same concept can be used for *Penium margaritaceum*, a unicellular alga that serves as an analogous model for higher plant cell wall deposition and cell growth. By applying BFA, a disrupter of Golgi mediated trafficking and cell wall component secretion, cell wall deposition, and cell length were decreased. These detailed protocols may be adjusted for specific research projects to identify new chemical probes and their targets. Valuable insights can be obtained for the relationship of cell wall and cell growth. This in turn can be applied for crop improvement of agronomically important plants.

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