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Supplementary Materials for

Visualization of cellulose synthases in *Arabidopsis* secondary cell walls

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This PDF file includes:

Other supplementary material for this manuscript includes the following:

Materials and Methods:

Construction of the *proCESA7::YFP::CESA7* vector.

CESA7 cDNA was amplified with primers 5′-CACCGAAGCTAGCGCCGGTCTTGTC-3′ and 5′- TCAGCAGTTGATGCCACACTTGGA-3′ and cloned into a *pENTR-D/TOPO* vector (Life Technologies). Confirmed *pENTR-CESA7* plasmids where then used for Gateway-Clonase insertion into *pUBN::YFP:DEST* (*19*) to generate *proUBQ10::YFP::CESA7*. The *proUBQ10* of the *proUBQ10::YFP::CESA7* vector was then excised using *PmeI* and *XhoI* restriction enzymes. *CESA7* native promoter was then cloned using primers 5′-GTTTAAAC GGCTCCAACGTTTTCAGTTT-3′ 5′-CTCGAGCGGTGATCAATGAGAGACGA-3′ which contains the matching flanking restriction sites for *PmeI* and *XhoI* respectively, and ligated into the *PmeI* and *XhoI* digested *proUBQ10::YFP::CESA7* vector to generate the *proCESA7::YFP::CESA7*.

Generation of plant lines.

Seeds of *cesa7* (*irx3-4,* SALK_029940C) were obtained from the Arabidopsis Research Center and crossed with lines containing *proCaMV35s::VND7::VP16::GR* (*20*). F_2s homozygous seeds for both lines were isolated and were then transformed with either *CESA7pro::YFP::CESA7* or *UBQ1pro::mRFP::TUB6* using the floral dip method. T_2's of these lines were then crossed and F_2's genotyped and screened to identify lines homozygous for *irx3-4* and containing *proCESA7::YFP::CESA7, proUBQ10::mRFP::TUB6* and *proCaMV35s::VND7::VP16::GR*.

Seeds of *proCESA3::GFP::CESA3* in *cesa3(je5)* mutant background were a gracious gift from Samantha Vernhettes.

All plants were grown in 18 hrs of light at 21°C, and 6 of dark at 18 °C.

Seedling growth and induction.

Seeds were surface sterilized with 20% bleach and 0.1% Triton (Sigma) for 5 min, and then washed three times with sterilized distilled water. Seeds were sown on plates containing germination media (1× Murashige-Skoog (MS), 1% Sucrose, 1x Gamborg's Vitamin mix, 0.05% MES, 0.8% agar at pH 5.8). Plates were wrapped in aluminum foil and placed at 4°C for 2 days, before being moved into a growth chamber at 21 °C in a vertical position and grown for 3 days. Plates were then removed from the chamber and under sterile conditions, 10 ml of 10 μM dexamethasone (Sigma) in sterilized distilled deionized water was added on the plates for VND7::GR induction and control treatments (for *proCESA3::GFP::CESA3* in *cesa3(je5)* seedlings). Plates were then rewrapped in aluminum foil and grown in the dark in a chamber for an additional 12 h (earliest stages of secondary cell wall formation) before imaging began. Imaging was carried out between 12-24 hours following induction. The timing of induction varied slightly among cells in each

seedling, so stage of development was defined based on microtubule banding and secondary cell wall features. At the earliest stages, microtubules were dispersed, although bundled microtubule bands were observed; at mid-development, the microtubules outlined the spiral wall thickenings; late in development, the plasma membrane was deformed around the secondary cell wall thickening.

Drug treatments

Oryzalin (Sigma) was dissolved in DMSO for a stock concentration of 10 mM and used at a working concentration of 20 µM in 0.2% DMSO. Prior to induction, plates of 3-day-old seedlings were treated with 10 ml of 0.2% DMSO or 20 µM oryzalin for 6 hrs. Seedlings were then induced by adding 10 µl of dexamethasone stock solution (10 mM) into the media and gently shaking to mix the solution. Seedlings were then returned to the growth chamber under conditions described above. Samples were mounted in 20 µM oryzalin during imaging to ensure the drug was not washed away.

DCB treatment

2,6-dichlorobenzonitrile (DCB) (Sigma) was dissolved in DMSO for a stock concentration of 25 mM and used at a working concentration of 10 µM in 0.04% DMSO. Plates of 3-day-old seedlings were treated with 10 ml of 10 µM dexamethasone with either 0.04% DMSO (control) or 10 µM DCB (Sigma) in 0.04% DMSO. Seedlings were then returned to the growth chamber under conditions described above. Samples were mounted in 10 µM DCB during imaging to ensure the drug was not washed away.

Live cell imaging.

Seedlings were mounted between #1.5 45×40 and 24×24 mm coverslips with water and sealed with silicone vacuum grease.

Prior to using an optimized spinning disk confocal system as described below, imaging was performed on a standard spinning disk confocal set up (Leica DMI6000 inverted microscope (Leica), Perkin-Elmer UltraView spinning-disk system and a Hamamatsu 9100-02 CCD Camera and a 63X 1.34NA oil lens). YFP was imaged using a 514-nm laser and 540/30 emission filter. RFP was detected with a 561-nm laser and 595/50-nm emission filter. All images were captured using the Volocity 6.3 software package (Perkin Elmer). However, as shown by Fig. S2, this instrument set up did not provide the necessary detection and resolution to distinguish individual CSCs.

The majority of imaging was performed on a Leica DMI6000B inverted microscope equipped with adaptive focus control (Leica), Yokogawa CSU-X1 spinning disc head, Photometrics Evolve 512 Camera, mSAC Spherical Aberration Correction (Intelligent Imaging Innovations) and a 100X 1.4NA oil lens. The combination of a sensitive camera, adaptive focus control, objective lens with a high numerical aperture and spherical aberration correction were essential to visualize CESA7-containing CSC in secondary cell wall thickenings. Both GFP and YFP were imaged using a 488 laser, 488/561 dichroic mirror (Semrock) and a 525/50 emission filter (Semrock). RFP was imaged using a 561

laser (Coherent), 488/561 dichroic mirror (Semrock) and a 605/64 emission filter (Semrock). Photobleaching was achieved using Vector FRAP/photoactivation system (Intelligent Imaging Innovations). All images were captured using SlideBook6 software (Intelligent Imaging Innovations).

Image analysis.

Images were processed using ImageJ software. Background correction was performed using the "subtract background" tool with a rolling ball radius of 30 pixels. For images with noticeable x/y drift, a post-acquisition, recursive alignment plugin was used (StackReg plugin http://bigwww.epfl.ch/thevenaz/stackreg) to realign images. For images tracking CSCs at the plasma membrane, a post-acquisition frame-averaging plugin (running z-projector plugin http://valelab.ucsf.edu/~nico/IJplugins/Running_ZProjector.html) was employed to average 3 frames to highlight slow moving CSC complexes at the plasma membrane. Kymographs were generated using the Multiple Kymograph plugin with a line width of 3 pixels. Movies were generated using MtrackJ (http://www.imagescience.org/meijering/software/mtrackj/manual/).

Correlation analysis

Background correction was performed using the "subtract background" tool in ImageJ with a rolling ball radius of 30 pixels followed by a post-acquisition frame-averaging plugin (running z-projector plugin (http://valelab.ucsf.edu/~nico/IJplugins/Running_ZProjector.html). Areas where signal from intracellular compartments were not observable were selected and analyzed using the Coloc 2 correlation analysis plugin for ImageJ (http://fiji.sc/Coloc_2).

Image analysis for density measurements.

Images were processed using ImageJ software as described above. A more extensive background correction was performed using the "subtract background" tool with a rolling ball radius of 5 pixels. Kymographs were then generated and the plot profile tool was then used to measure CSC density, as described in Figure S3.

Transmission electron microscopy.

36-hr induced seedlings were high-pressure frozen using a Leica HPM 100 in A and B type sample holders (Ted Pella) using 1-hexadecene (Sigma) as a cryoprotectant. Samples were freeze-substituted in 2% osmium tetroxide (Electron Microscope Sciences) and 8% 2,2-dimethoxypropane (Sigma) in acetone for 5 days at -78°C, then slowly warmed to room temperature over 2 days. Samples were then slowly infiltrated with increasing concentrations of Spurr's resin over 4 days and embedded in polyethylene flat-bottom capsules. Samples were sectioned to approximately 70 nm thickness using a Leica UCT microtome and mounted on 75 mesh copper grids (Ted Pella) coated with 0.3% formvar (Electron Microscope Sciences) and then stained with 2% uranyl acetate in 70% methanol and Reynold's lead citrate. Samples were viewed using a Hitachi H7600

Transmission Electron Microscope equipped with an Advanced Microscopy Techniques CCD camera (Hamamatsu ORCA) at an accelerating voltage of 80kV.

Cellulose quantification

2 grams of surface sterilized seeds were sown on plates containing germination media and placed at 4°C for 2 days. Following, plates were transferred to growth chamber and grown under light for 5 days at 21°C. Seedlings were then treated with 10ml per plate of either: 0.01% Ethanol and 0.2% DMSO (uninduced control) or 10 µM dexamethasone and 0.2% DMSO (induced control) or 10 µM dexamethasone and 20 µM oyrzalin (induced oryzalin treated) and placed back in the growth chamber for 24hr. Aerial tissue was then harvested using a razor blade and placed in a 50ml falcon tube and submerged in liquid nitrogen. The samples were freeze-dried and ground on a Mini Mill (Thomas Wiley) to pass through a #40 mesh (0.425 mm). Tissue was then dried for 24hr at 50°C and 15mg of tissue was weighed into each tube. First, the Alcohol Insoluble Residue (AIR) was prepared as described in (*21*). The AIR was then subjected to a series of extractions in a procedure modified from the AIR fractionation method previously described (*21*). Specifically, the modification was the removal of the 1M potassium hydroxide extraction, as well as the chlorite extraction and post-chlorite 4M potassium hydroxide extractions. The resulting cellulose residue was then pre-dried in a vacuum centrifuge and finished in a 50°C oven overnight before the final weights were measured.

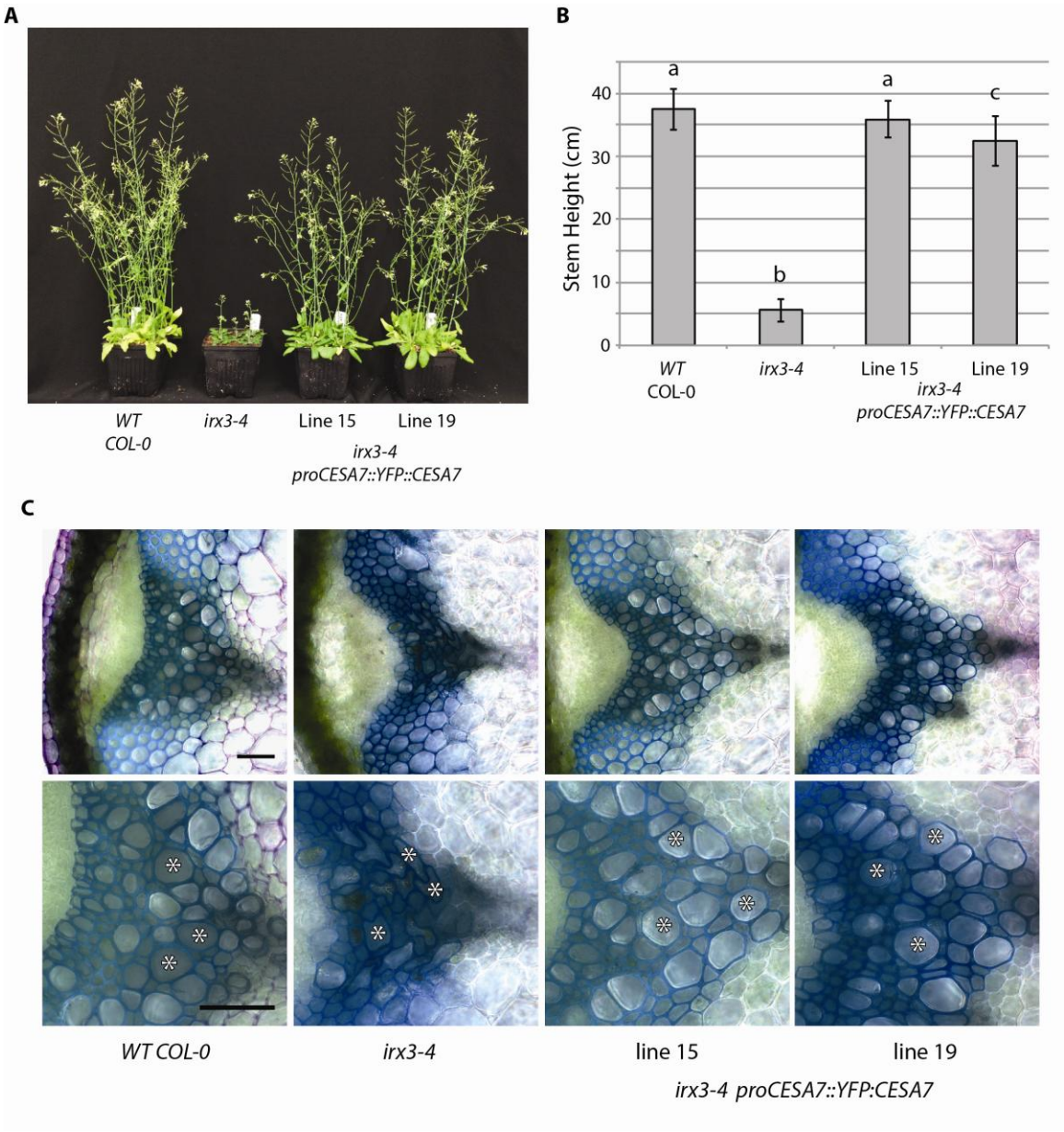

Fig. S1

The *cesa7/irx3-4 mutant* phenotype is complemented with the *proCESA7::YFP::CESA7* construct. (A) Growth habit of four-week-old Arabidopsis plants contrasting wild-type with *cesa7* mutant allele *irx3-4*, and two independent transformants where CESA7 is expressed under its native promoter in the *irx3-4* background. (B) Stem height of four-week-old Arabidopsis plants of the same genotypes. Means with different letters represent statistically significant differences (Tukey's pairwise comparison, $p<0.01$) among 25 plants for each line, error bars = standard deviation. (C) Cross sections of stems from the bottom 1 cm of four-week-old plants stained with 0.01% toluidine blue for 5 min. Bottom panels show higher magnification of xylem tracheary elements (examples labeled with asterisks). Note the collapsed, irregular xylem (*irx*) phenotype of the *cesa7/irx3-4* mutant. Scale bars: 50 μm (C).

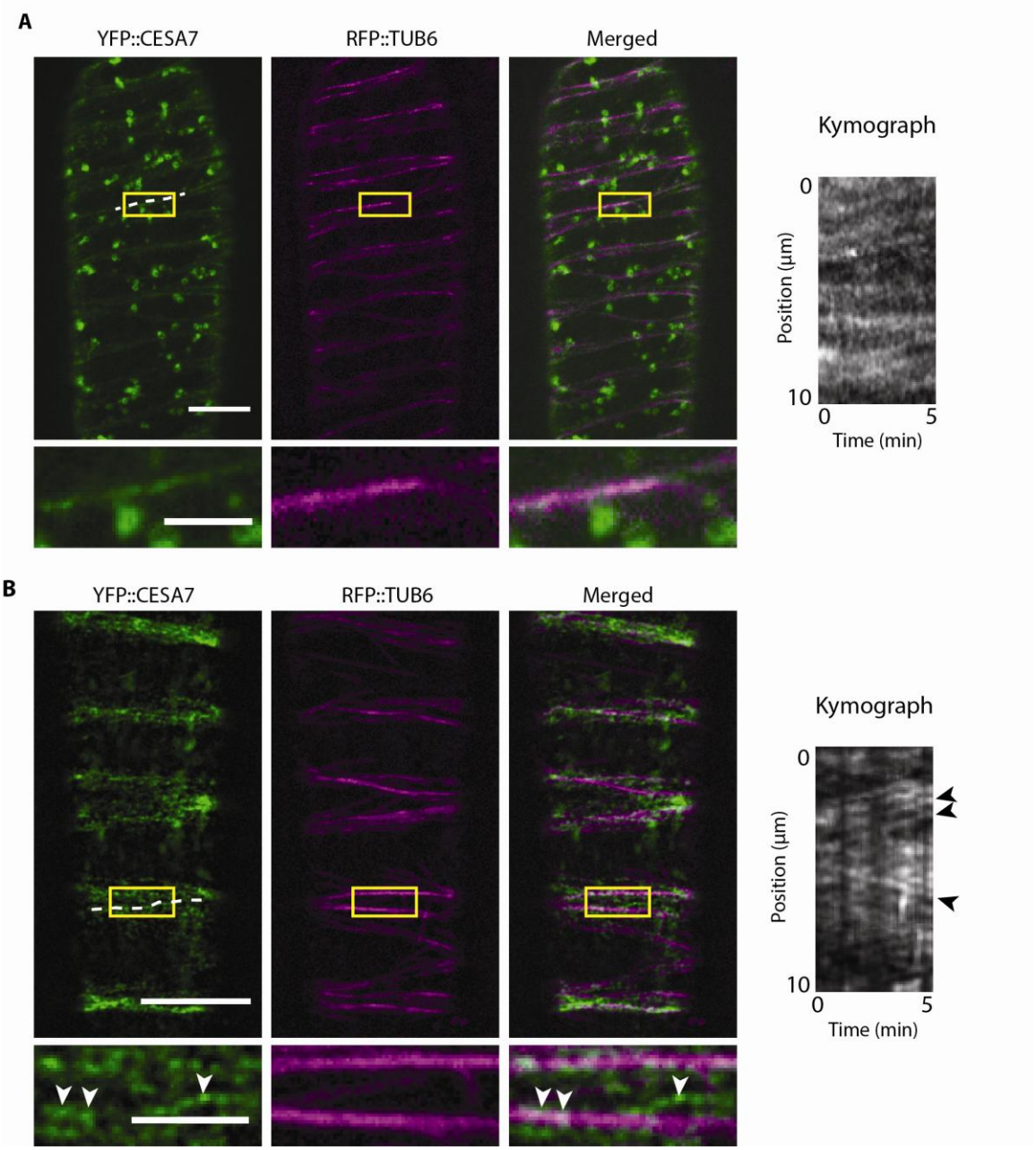

Fig. S2

Due to the high density of signal and curvature of the developing secondary cell wall, use of an optimized imaging system is required to visualize distinct secondary cell wall CSCs. (A) Images of YFP::CESA7 and RFP::TUB6 in VND7::GR-induced cells taken on a non-optimized spinning disk confocal system ("standard spinning disk confocal" described in the supplemental materials and methods). Zoom in (inset) reveals that individual complexes are not distinguishable due to the curvature of the plasma membrane around the secondary cell wall and concentration of signal in these domains, as highlighted by kymograph analysis along the dotted line. (B) Images of the induced cells taken on an optimized spinning disk confocal system (as described in the materials and methods). Zoom in (inset) reveals that distinct complexes can be observed (arrowheads) and tracked through kymograph analysis (arrowheads point to the same 3 complexes as in inset). Note vertical lines in kymograph in (B) are due to Z-drift. Scale bars: 10 µm, and 3 µm in inset.

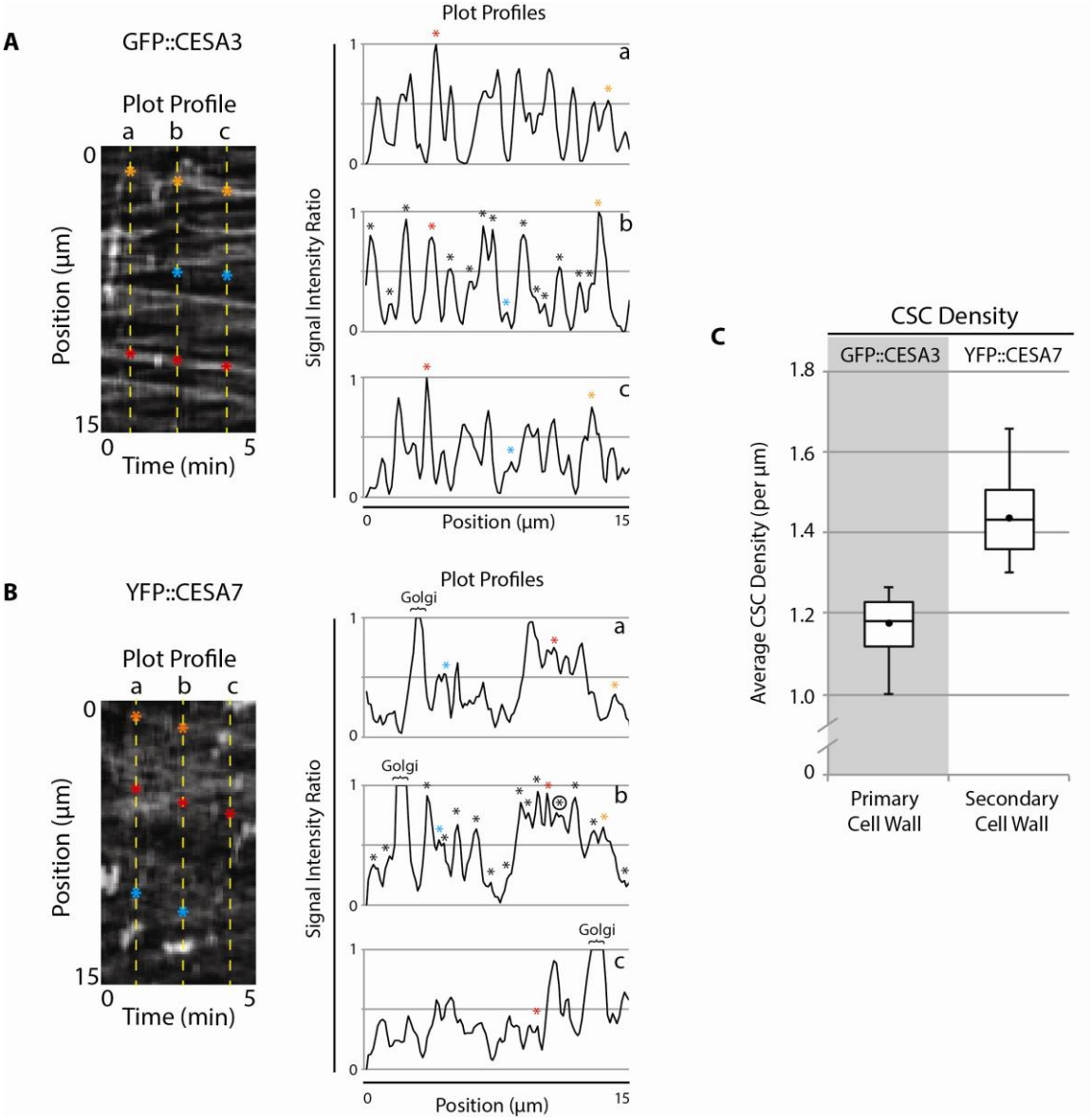

Fig. S3

Secondary cell wall CSCs have a higher density than primary cell wall CSCs. Densities of plasma membrane localized CSCs were measured by plot profile analysis of kymographs from tracks generated from time lapse movies processed with a strict image processing for background subtraction to minimize intracellular signals. Only peaks associated with linear tracks in the kymograph were counted. This was done by generating 3 plot profiles at the 3 time points. If a peak was visible in at least 2 profiles, and could be followed on the kymograph, it was counted as one CSC (marked with an asterisk in plot profile b). Colored asterisks highlight example peaks as they move through the kymograph. (A) Densities of primary cell wall CSCs. (B) Densities of secondary cell wall CSCs were measured as above. However, due to the higher density of signal, in some cases the point spread functions of the complexes overlapped, leading to wide peaks making it difficult to accurately count the CSCs present (circled asterisk). These peaks were only counted as a single CSC. Additionally, signal from intracellular Golgi and SmaCCs often obscured CSC lines, and were not counted in the analysis. (C). Box plot of average CSC densities per µm of track (10 tracks averaged per cell, 6 cells per stage). Primary wall density was 1.16 ± 0.1 CSCs per µm and secondary wall density was 1.45 ± 0.13 CSCs per µm. This quantification method is biased towards false negatives, thus this quantification of secondary cell wall CSC density is likely to underestimate true density. However, there is still a significant difference between primary and secondary cell wall CSC densities (*t*-test, $p<0.01$).

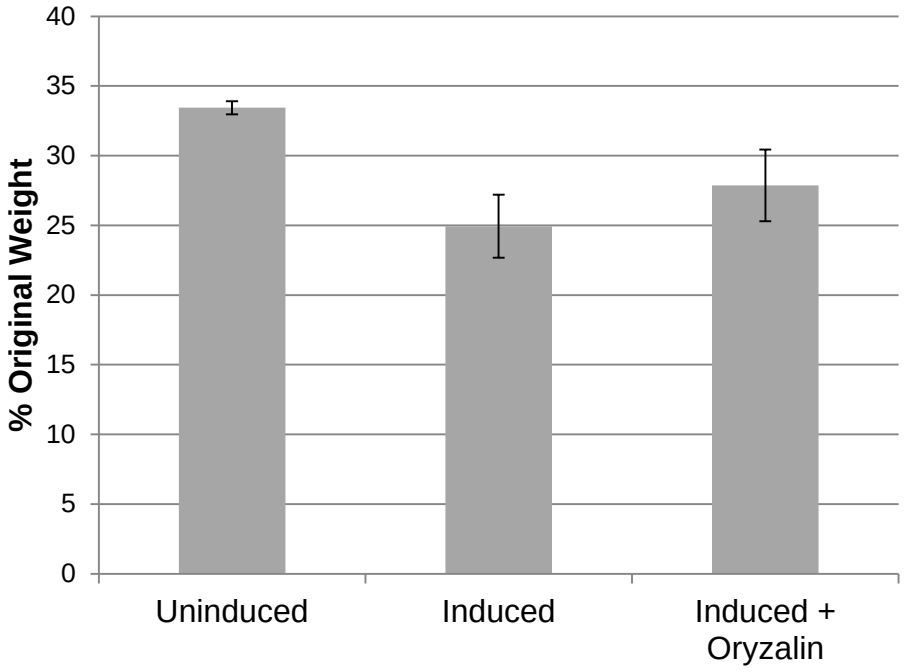

Fig. S4

Total cellulose content is not affected by depolymerization of microtubules by oryzalin treatment. Average total cellulose content as a percentage of starting dry weight of aerial tissue of 5-day old seedlings (± SD). 5-day old light grown seedlings were treated for 24 hrs with either: 0.01% ethanol and 0.2% DMSO (uninduced control) or 10 µM dexamethasone and 0.2% DMSO (induced control) or 10 µM dexamethasone and 20 µM oyrzalin (induced oryzalin treated) prior to freeze-drying. Three technical replicates of the pooled samples were processed for each treatment.

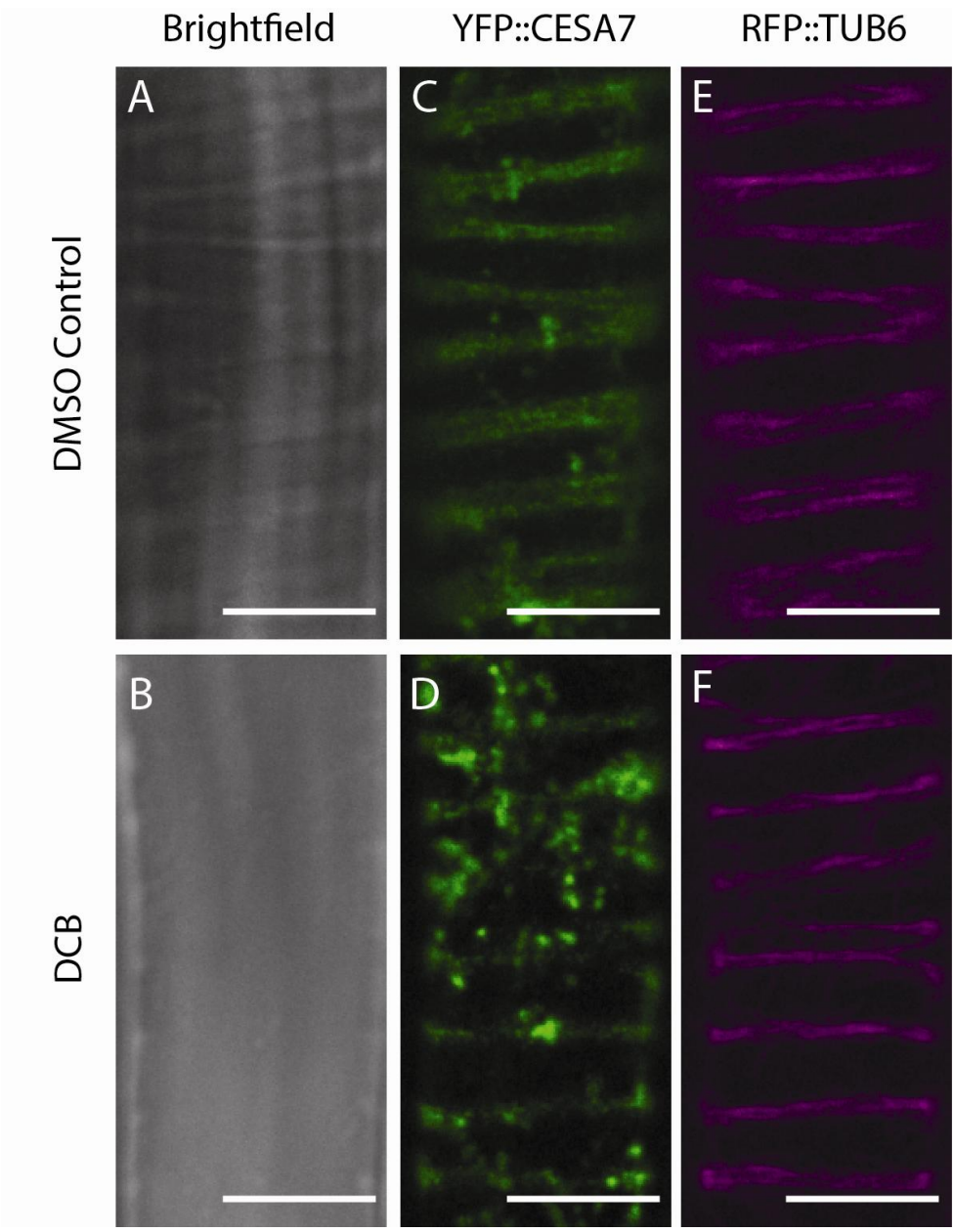

Fig. S5

Inhibition of cellulose production by 2,6-dichlorobenzonitrile (DCB) does not prevent formation of microtubule bundles or reorganization of YFP::CESA7 signal. Brightfield and single optical sections of YFP::CESA7, RFP::TUB6 in VND7::GR seedlings treated with 10 μM DEX and 0.04%DMSO (DMSO Control) or 10 μM DEX and 10 μM DCB for 16 hrs. Distinct secondary cell wall thickenings do not form in the presence of the cellulose synthesis inhibitor DCB (A and B). However, YFP::CESA7 (C and D) and microtubules (E and F) still organize into distinct bands in the presence of DCB. Scale bars: 10 μm.

VND7::VP16::GR						
Gene Symbol	AGI	Probe Set ID	p	FC (abs)	[+DEX], normalized	[-DEX], normalized
CESA1	AT4G32410	253428_at	0.86	-1.04	0.05	0.11
CESA3	AT5G05170	250827_at	0.98	-1.01	0.07	0.08
CESA6	AT5G64740	247251_at	0.91	-1.03	0.12	0.16
CESA4	**AT5G44030**	**249070_at**	**0**	**2.74**	**0.86**	**-0.6**
CESA7	**AT5G17420**	**246425_at**	**0.06**	**1.49**	**0.39**	**-0.18**
CESA8	**AT4G18780**	**254618_at**	**0.01**	**2.95**	**1.08**	**-0.48**
KOR1	AT5G49720	248573_at	0.58	1.11	0.17	0.01
KOR2	AT1G65610	264685_at	0.66	1.04	-0.05	-0.11
COB	AT5G60920	247552_at	0.83	-1.07	-0.29	-0.18
COBL1	AT3G02210	259122_at	0.77	1.02	0.04	0.01
COBL2	AT3G29810	245228_at	0.48	-1.1	-0.05	0.09
COBL4	**AT5G15630**	**246512_at**	**0**	**1.54**	**0.32**	**-0.3**
COBL5	AT5G60950	247604_at	0.44	-1.11	0	0.14
COBL6	AT1G09790	264710_at	0.47	1.05	0.06	-0.01
COBL7	AT4G16120	245339_at	0.18	1.1	0.06	-0.08
COBL8	AT3G16860	256763_at	0.87	-1.03	-0.05	-0.01
COBL9	AT5G49270	248652_at	0.57	1.04	0.05	-0.02
COBL10	AT3G20580	257082_at	1	-1	-0.01	-0.01
COBL11	AT4G27110	253924_at	0.69	1.03	0.05	0
KOB1	AT3G08550	258666_at	0.77	1.05	-0.11	-0.17
CSI	AT2G22125	263456_at	0.95	1.02	-0.06	-0.09

VP16::GR						
Gene Symbol	AGI	Probe Set ID	p	FC (abs)	[+DEX], normalized	[-DEX], normalized
CESA1	AT4G32410	253428_at	0.77	1.07	0.15	0.05
CESA3	AT5G05170	250827_at	0.79	1.06	0.04	-0.05
CESA6	AT5G64740	247251_at	0.57	1.19	0.08	-0.17
CESA4	AT5G44030	249070_at	0.98	1	0.01	0
CESA7	AT5G17420	246425_at	0.83	-1.02	0.01	0.04
CESA8	AT4G18780	254618_at	0.27	-1.16	-0.1	0.11
KOR1	AT5G49720	248573_at	0.86	1.03	-0.01	-0.05
KOR2	AT1G65610	264685_at	0.61	-1.04	-0.04	0.03
COB	AT5G60920	247552_at	0.88	-1.05	-0.42	-0.35
COBL1	AT3G02210	259122_at	0.66	-1.03	-0.02	0.03
COBL2	AT3G29810	245228_at	0.45	-1.06	-0.04	0.04
COBL4	AT5G15630	246512_at	0.51	-1.03	-0.04	0.01
COBL5	AT5G60950	247604_at	0.77	-1.04	-0.01	0.05
COBL6	AT1G09790	264710_at	0.95	-1	-0.03	-0.02
COBL7	AT4G16120	245339_at	0.68	1.03	0.02	-0.03
COBL8	AT3G16860	256763_at	0.52	1.13	0.25	0.07
COBL9	AT5G49270	248652_at	0.97	1	0.07	0.06
COBL10	AT3G20580	257082_at	0.86	1.01	0.05	0.04
COBL11	AT4G27110	253924_at	0.45	1.06	0.03	-0.05
KOB1	AT3G08550	258666_at	0.93	-1.01	-0.1	-0.08
CSI	AT2G22125	263456_at	0.65	1.14	-0.07	-0.26

Table S1

Secondary cell wall related genes and not primary cell wall related genes are upregulated during VND7::GR induction
Microarray data from Yamaguchi *et al*. (*2*) for selected cell wall-related genes in either Arabidopsis seedlings constitutively expressing the *VND7::VP16::GR* or *VP16::GR* (vector control) with or without 10 µM dexamethasone induction for 4 h. Seedlings were also treated with 10 µM cycloheximide, a protein synthesis inhibitor, to prevent the production of secondary transcription factors. No significant differences were observed in the expression of cellulose synthesis components or secondary wall regulators after DEX treatment in the empty vector (control, *VP16::GR*) control line, as compared to control treatments.

Stage of Development	YFP::CESA7 area coincident with MT Domains	MT domains coincident with YFP::CESA7 Area
Early	0.57 ± 0.11*	0.62 ± 0.17*
Mid-	0.79 ± 0.11	0.89 ± 0.08
Late	0.83 ± 0.04	0.88 ± 0.09

Table S2

CSC area coincident with microtubule domains. Proportion of total area occupied by YFP::CESA7 particles at the plasma membrane coincident with RFP::TUB6 labeled cortical microtubules, and the proportion of microtubule domains coinciding with CESA particles. Values are expressed as means of Mander's colocalization coefficents as previously described (*22*). (*) indicates means with statistically significant difference (Tukey's pairwise comparison, $p<0.01$)

Movie S1

Distribution and motility of YFP::CESA7 in the cell membrane of VND7::GR induced seedlings at mid-development of secondary cell wall formation.

Time lapse of VND7::GR induced cells with YFP::CESA7 and RFP::TUB6. Linear, bi-directional steady movement of YFP::CESA7 particles (arrowheads) are observed in tight domains of secondary cell wall formation. These domains are closely associated with underlying cortical RFP::TUB6 labeled microtubules. Movie acquired over 5 min at 5 sec intervals, and processed as described in the materials and methods. Scale bar: 10 μm.

Movie S2

Golgi and SmaCCs containing YFP::CESA7 rapidly move between and pause at domains of secondary cell wall formation.

Time lapse of VND7::GR induced cells with YFP::CESA7. Numerous intracellular compartments containing YFP::CESA7 are observed including Golgi (red arrows), Golgi-independent SmaCCs (blue arrows) and Golgi-associated SmaCCs (yellow arrows). Compartments are observed to move quickly between domains and pause at domains of secondary cell wall formation. Movie acquired over 5 min at 5 sec intervals. Scale bar: 10 μm.

Movie S3

Coordination between YFP::CESA7 containing Golgi and SmaCCs can be transient.

FRAP of a secondary cell wall thickening reveals a YFP::CESA7 containing Golgi body (yellow arrowhead) and closely associated SmaCC (red arrowhead) moving rapidly from the lower secondary cell wall band and approach and pause at the upper secondary cell wall domain. The Golgi body eventually moves on, while the SmaCC remains stationary, presumably representing an insertion event into the plasma membrane. Movie acquired over 7.5 min at 5 sec intervals. Scale bar: 2.5 μm.

Movie S4

Delivery of at least two YFP::CESA7 containing complexes by a SmaCC.

FRAP of a plasma membrane surrounding secondary cell wall thickening reveals YFP::CESA7 containing Golgi and closely associated SmaCC pause. The Golgi body eventually moves on while the SmaCC remains stationary and splits into two distinct particles with linear trajectories and steady velocities (arrowheads) presumably

representing an insertion event of at least two CSCs to the plasma membrane. Movie acquired over 7.5 min at 5 sec intervals. Scale bar: 2.5 μm.

Movie S5

Loss of secondary cell wall CSC organization at the plasma membrane due to near complete depolymerization of microtubules with oryzalin treatment. Cells were treated with 20 μM oryzalin for 6 hrs prior to induction with dexamethasone. Loss of cortical microtubules prior to and during induction results in YFP::CESA7 labeled complexes at the plasma membrane lose their tight annular banding pattern as observed in the DMSO control. Instead disorganized 'swarms' of particles are observed at the plasma membrane. Movie acquired over 5 min., images acquired every 5 sec intervals. Scale Bar: 10 μm.

Movie S6

Insertion events of secondary cell wall CSC complexes to the plasma membrane are unaffected by depolymerization of microtubules s by oryzalin treatment.

Time lapse movie of a FRAP experiment of oryzalin treated induced VND7::GR cells reveals that insertion of YFP::CESA7 labeled complexes at the plasma membrane (arrowheads) still occur without the presence of microtubules. Movie acquired over 7.5 min, images acquired every 5 sec Scale Bar: 5 μm