Novel tool to quantify cell wall porosity relates wall structure to cell growth and drug uptake

Xiaohui Liu, Jiazhou Li, Heyu Zhao, Boyang Liu, Thomas Gunther-Pomorski, Shaolin Chen, and Johannes Liesche

Corresponding Author(s): Johannes Liesche, Northwest A&F University

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November 14, 2018

Re: JCB manuscript #201810121

Dr. Johannes Liesche
Northwest A&F University
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China

Dear Dr. Liesche,

Thank you for submitting your manuscript entitled "In vivo quantification of cell wall porosity relates wall structure to cell growth and drug uptake". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

You will see that all three reviewers appreciate that your manuscript characterizes a simple and needed tool to measure porosity, an aspect of plant cell wall structure. An important additional experiment would be to test the ability of this technique to report changes in porosity in the opposite direction as the mutants being utilized, therefore rev 2's straightforward suggestion to use a chemical inhibitor is essential. Requested changes to the text made by all reviewers should be addressed, and we hope that you will be able to address all of the remaining reviewer comments in your revised manuscript.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

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Text limits: Character count for Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

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When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Dominique Bergmann, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

This ms describes an original technique to quantify in vivo cell wall porosity based on the quenching of the plasma membrane dye FM4-64 with the small molecules "black hole quencher 3", "malachite green" or "trypan blue" (TB). The authors show that the quenching efficiency depends on the accessibility of the quencher, which is reduced in yeast or bacteria, which have a cell wall, relative to HEK cells or GUVs. They test a few plant cell wall mutants and observe a reduction in quenching efficiency in root epidermis cells for some of them, indicating that the porosity of the walls is reduced in these mutants. There does not seem to be a correlation with cell wall thickness, although this is difficult to say given the small sample size and the fact that the cell wall mutants can affect other things than just well wall thickness. The authors try to establish a correlation between wall porosity (measured by the quenching efficiency of TB) and the ability of the roots to increase their growth upon drought stress. They observe that the porosity increases with the increase in cell length in the WT, that relative porosity is correlated with relative cell length. The authors conclude from this that increased porosity is a consequence rather a precondition for an increase in growth rate. Finally the authors show that the technique also works in yeast and thus could be used to identify compounds that facilitate the penetration of antifungal drugs into the cell wall.

There is certainly a need in the cell wall field to measure cell wall porosity in living cells, since it is thought to be critical for growth control and technological characteristics such as the degradability

of the cell wall. Therefore I appreciate very much such a simple technique to measure porosity. I have doubts however whether the ms fits the scope and meets the standards of JCB. For this I would have liked to see how the technique can be used for instance to obtain novel information on the role of cell wall changes in the control of plant cell expansion.

Minor remarks:
Typos: p3, line 78: "saccarification", p.6 line 158 "notes"
p.4. explain "GUV" in text

Reviewer #2 (Comments to the Authors (Required)):

In the manuscript "In vivo quantification of cell wall porosity relates wall structure to cell growth and drug uptake", Liu et al. outline a technical study aimed at developing an in vivo method to assess cell wall structural status. The method hinges on the quenching of the plasma membrane dye FM4-64 by black hole quencher 3, malachite green or trypan blue, which were chosen based on their sizes and spectral properties. The authors go on to show that quenching efficiency can be used to correlate cell wall structure/permeability and extensibility in Arabidopsis and permeability in yeast. Let me start by saying that I am enthusiastic about this manuscript and I do see a lot of value in what the authors have developed. The manuscript is well organized and written and the experiments are appropriate.

I do have one suggestion that I think is required to bolster the existing results. In the work comparing Arabidopsis cell wall mutants with wild type to assess cell wall porosity, the mutants that the authors have chosen all show a reduction in quenching efficiency. Since this is an important aspect of the paper, it would be useful here to have a contrasting effect either by mutation or chemical treatment (for example, by an cell wall synthesis inhibitor). I would favour using an inhibitor because it can be dosed and can be applied at different stages of growth. It would also provide a proof of principle that the technique could be used in chemical screening as the authors suggest (line 290). What was the reasoning behind using the specific mutants that were used? There are many cell wall mutants to choose from and it wasn't clear why these were the most appropriate.

Minor point: mur11 has been positionally cloned (Plant J. 2011(4):715-25).

Reviewer #3 (Comments to the Authors (Required)):

The manuscript by Liu et al., takes advantage of quenching effects compounds like tryphanblue, malachite green, BH3 have on the fluorescence of FM-464. The authors use this to investigate porosity of extracellular matrix in different cell types with a particular focus on plant cell walls. They use a combination of different imaging tools, mutants and analytical methods to try and turn the quenching effect into an analytical tool useful in different biological contexts (plants, yeast) and try to draw conclusions about changes in the mutant or treated cell walls.
While I do like the concept and see the potential of the assay for genetic screens I think there are a few issues. First of all I struggle to follow the story line in places, therefore I think the manuscript would benefit from clearer writing, highlighting of important points and a summary table correlating effects of mutants (specific changes in cell wall composition and structure) or treatments etc. on

quenching efficiency. This may also lead to me missing some points, which may address my following rather fundamental concerns about the work. An essential element to maximize the information value of the assay is a thorough understanding of what change is actually detected in their assay. Is it actual porosity or simply a change in structure and composition of the walls caused by the mutations / treatments, which could affect accessability for the quencher molecules based on something else (like charge of the cell wall matrix). Currently the authors show supportive qualitative, but not quantitative data that the cell walls have a different porosity /differ from controls. I think this is a serious limitation and they should try to find a way to turn this qualitative assay (yes there´s a difference / no there´s no difference) into something quantitative where they are confident that they are detecting quantitative changes in porosity and not something else.

Minor comments: Throughout the manuscript there is a large number of smaller and larger problems with the text / figure legends etc. I´ve listed below representative examples to show the authors what they need to check / correct throughout the manuscript:
- Abbreviations used need to be defined in the text when they are used the 1st time (example: line 103 GUV)
- Sup. Figure 1 is not described in main text
- Wording of figure legends need to be modified to meet scientific standards (examples figure 1 and supl. Figure S2: both descriptive / interpretative text)
- Supl. Figure 2 is mentioned with one very general sentence with limited information value in the main text. I think this needs to be expanded....
- Line 115: citation for Stern-Volmer equation is missing (Lehrer 1971)
- Figure 6 and 7 are interesting but at least figure 7 should be supplemental
- The authors do not discuss possible effects the different charges of the quencher molecules could have on interactions between the quencher molecules and the cell wall matrix. While I think figure 1 contains a nice graphical illustration of the different quencher molecules, it doesn´t really provide info regarding charges of the quencher molecules.
- The statement in line 225 is a bit trivial and devalues the work the authors have done. I suggest rephrasing.

Point-by-point response to the reviewers' comments

We thank both reviewers for their positive response to our original manuscript and helpful comments.

Reviewer #1

There does not seem to be a correlation with cell wall thickness, although this is difficult to say given the small sample size and the fact that the cell wall mutants can affect other things than just well wall thickness.

We agree that, considering the sample number and nature of samples, the conclusion from this experiment should be formulated more careful. We changed the respective part in the discussion (line 250 to 253). In light of the new results showing a significant correlation of cellulose fibril spacing and quenching efficiency, we chose not to conduct additional experiments on cell wall thickness.

I have doubts however whether the ms fits the scope and meets the standards of JCB. For this I would have liked to see how the technique can be used for instance to obtain novel information on the role of cell wall changes in the control of plant cell expansion.
We imagine this method to become one of the standard methods for evaluating the effect of certain genes/proteins on cell wall structure. Indeed, in co-author Chen Shaolin's lab this method is now being used to evaluate the genetic basis of the effect of environmental dynamics on cellulose biosynthesis. Furthermore, while the porosity change was indicated here to not be a precondition for root cell extension growth, this might be different in other cells, for example root hair growth. We extended the respective section in the discussion (Line 311-314).

Minor remarks:
Typos: p3, line 78: "saccarification", p.6 line 158 "notes"

This mistake has been corrected.

p.4. explain "GUV" in text
Explanation was added (line 108).

Reviewer #2:

I do have one suggestion that I think is required to bolster the existing results. In the work comparing Arabidopsis cell wall mutants with wild type to assess cell wall porosity, the mutants that the authors have chosen all show a reduction in quenching efficiency. Since this is an important aspect of the paper, it would be useful here to have a contrasting effect either by mutation or chemical treatment (for example, by an cell wall synthesis inhibitor). I would favour using an inhibitor because it can be dosed and can be applied at different stages of growth. It would also provide a proof of principle that the technique could be used in chemical screening as the authors suggest (line 290).

In the original manuscript we already documented the effect of the simulated drought treatment, which was shown to increase quenching efficiency. In addition, we realized the idea provided here of including cell wall inhibitors. Two inhibitors (Isoxaben and DCBN) were tested and both increased quenching efficiency of all three quencher. Taken together the results make a strong case for the assay having a useful dynamic range in the positive as well as negative direction. Figure 3, as well as results, methods and discussion sections were updated.

What was the reasoning behind using the specific mutants that were used? There are many cell wall mutants to choose from and it wasn't clear why these were the most appropriate.

Indeed, the selection of mutants was mainly based on our literature study and subsequent efforts to obtain seeds of homozygous plants. The only criteria for selecting mutants was that they affect different wall components. We added this information to the methods section. (lines 367f)

Minor point: mur11 has been positionally cloned (Plant J. 2011(4):715-25).

Thank you for making us aware of this. We updated Table 1 and included the reference.

Reviewer #3:

While I do like the concept and see the potential of the assay for genetic screens I think there are a few issues. First of all I struggle to follow the story line in places, therefore I think the manuscript would benefit from clearer writing, highlighting of important points and a summary table correlating effects of mutants (specific changes in cell wall composition and structure) or treatments etc. on quenching efficiency.

Considering the positive comments that other reviewers made about writing and structure of the manuscript, we did not make major changes. We assume that the lack of evidence for how exactly quenching efficiency relates to cell wall structure in the original version caused some confusion. This should be alleviated in the revised version, where we included new experiments that show the significant correlation of quenching efficiency and cellulose fibril density. Now the structure (1) showing the quenching effect, 2) proving its relation to cell wall structure and 3) application example) should be clearer.

This may also lead to me missing some points, which may address my following rather fundamental concerns about the work. An essential element to maximize the information value of the assay is a thorough understanding of what change is actually detected in their assay. Is it actual porosity or simply a change in structure and composition of the walls caused by the mutations / treatments, which could affect accessability for the quencher molecules based on something else (like charge of the cell wall matrix). Currently the authors show supportive qualitative, but not quantitative data that the cell walls have a different porosity /differ from controls. I think this is a serious limitation and they should try to find a way to turn this qualitative assay (yes there´s a difference / no there´s no difference) into something quantitative where they are confident that they are detecting quantitative changes in porosity and not something else.

We agree with the reviewer that not providing stronger prove was a clear shortcoming of the manuscript. Accordingly, it was the main point we strived to address in the revision. After experimenting with TEM, SEM and AFM, we finally found a different, more efficient way of conducting a quantitative analysis of the relationship of cell wall structure and quenching efficiency. We stained cellulose fibril with a fluorescent dye, then did 3D confocal imaging combined with image deconvolution. This yielded a high enough resolution to observe differences in the pattern of cellulose fibril organization as well as cellulose fibril density. After analysing all plants that we also determined the quenching efficiency for (including the newly added plants that were exposed to cell wall synthesis inhibitors), we could perform a correlation analysis. Indeed, we found a significant correlation of quenching efficiency and cellulose fibril density. These results provide a quantitative basis for the conclusion that the quenching assay can measure changes in cell wall porosity.

These new results are presented in the revised Fig. 3, and integrated in the methods, results and discussion sections.

Minor comments: Throughout the manuscript there is a large number of smaller and larger problems with the text / figure legends etc. I´ve listed below representative examples to show the authors what they need to check / correct throughout the manuscript:

We apologize for these mistakes which have now been corrected.

- Abbreviations used need to be defined in the text when they are used the 1st time (example: line 103 GUV)

This has been corrected.

- Sup. Figure 1 is not described in main text

The reference to Suppl. Fig. S1 was in the methods section. The numbering of the supplemental figures was corrected (i.e. Fig. S1 became S5).

- Wording of figure legends need to be modified to meet scientific standards (examples figure 1 and supl. Figure S2: both descriptive / interpretative text)

All figure legends were revised to adhere to the JCB format. Interpretative text was removed from the figure legends of figures 1, 3 and S2.

- Supl. Figure 2 is mentioned with one very general sentence with limited information value in the main text. I think this needs to be expanded....

Details of the experiment presented in Suppl. Fig. S2 (now S1) were integrated in the main text. Lines 95 to 97.

- Line 115: citation for Stern-Volmer equation is missing (Lehrer 1971)

The reference was added at that position. (Line 122)

- Figure 6 and 7 are interesting but at least figure 7 should be supplemental

Since the limit of supplemental figures was already reached, we chose to delete Figure 7 entirely. We agree with the reviewer that its information content is too limited for the main text and, furthermore, the differences are sufficiently explained in the discussion section.

- The authors do not discuss possible effects the different charges of the quencher molecules could have on interactions between the quencher molecules and the cell wall matrix. While I think figure 1 contains a nice graphical illustration of the different quencher molecules, it doesn´t really provide info regarding charges of the quencher molecules.

Following the Reviewer's suggestion, we have included charges in Fig. 1. There is no indication that they influence quenching efficiency as tested here (e.g. BHQ and MG have similar charge but very different quenching efficiency). The results section (Line 145-150) and the discussion section (line 266-269) were updated accordingly.

- The statement in line 225 is a bit trivial and devalues the work the authors have done. I suggest rephrasing.

Following the Reviewer's suggestion, the sentence was deleted as the characteristics of this assay are described at other positions of the discussion. For example, its simplicity is stressed with regard to its potential usefulness for high-content screening (line 315f).

January 22, 2019

RE: JCB Manuscript #201810121R

Dr. Johannes Liesche
Northwest A&F University
College of Life Sciences
Nongling Rd 1
Yangling 712100
China

Dear Dr. Liesche:

Thank you for submitting your revised manuscript entitled "In vivo quantification of cell wall porosity relates wall structure to cell growth and drug uptake". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, http://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

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2) Figures limits: Tools may have up to 10 main text figures.

3) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

4) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (either in the figure legend itself or in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.). Also, if you used parametric tests, please indicate if the data distribution was tested for normality (and if so, how). If not, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

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6) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions in the text for readers who may not have access to referenced manuscripts.

7) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

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h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

9) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

10) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Tools may have up to 5 supplemental display items (figures and tables). Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

11) eTOC summary: A ~40-50-word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person.

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13) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

14) A separate author contribution section following the Acknowledgments. All authors should be mentioned and designated by their full names. We encourage use of the CRediT nomenclature.

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Please upload the following materials to our online submission system. These items are required prior to acceptance. If you have any questions, contact JCB's Managing Editor, Lindsey Hollander (lhollander@rockefeller.edu).

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-- Cover images: If you have any striking images related to this story, we would be happy to consider them for inclusion on the journal cover. Submitted images may also be chosen for highlighting on the journal table of contents or JCB homepage carousel. Images should be uploaded as TIFF or EPS files and must be at least 300 dpi resolution.

It is JCB policy that if requested, original data images must be made available to the editors. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original data images prior to final submission.

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Thank you for your attention to these final processing requirements. Please revise and format the manuscript and upload materials within 7 days.

Please contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Dominique Bergmann, PhD
Monitoring Editor

Andrea L. Marat, PhD
Scientific Editor

Journal of Cell Biology

Reviewer #2 (Comments to the Authors (Required)):

The suggested changes have been addressed and the manuscript is acceptable.

Reviewer #3 (Comments to the Authors (Required)):

I think the additional experiments performed and modifications made to the manuscript have improved it nicely. The authors have satisfied my concerns...

~~In vivo quantification of~~Novel tool to quantify cell wall porosity relates wall structure to cell growth and drug uptake

Xiaohui Liu[1, 2], Jiazhou Li[1, 2], Heyu Zhao[1, 2], Boyang Liu[1, 2], Thomas Günther Pomorski[3,4], Shaolin Chen[1, 2], Johannes Liesche[1, 2]

[1] College of Life Sciences, Northwest A&F University, 712100 Yangling, China

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[4] Department of Molecular Biochemistry, Faculty of Chemistry and Biochemistry, Ruhr University Bochum, 44780 Bochum, Germany

Running title

A quenching tool to measure cell wall porosity

Abstract

Even though cell walls have essential functions for bacteria, fungi and plants, tools to investigate their dynamic structure in living cells have been missing. Here, it is shown that changes in the intensity of the plasma membrane dye FM4-64 in response to extracellular quenchers depend on the nano-scale porosity of cell walls. The correlation of quenching efficiency and cell wall porosity is supported by tests on various cell types, application of differently sized quenchers and comparison of results with confocal, electron and atomic force microscopy images.

The quenching assay was used to investigate how changes in cell wall porosity affect the capability for extension growth in the model plant *Arabidopsis thaliana*. Results suggest that increased porosity is not a precondition but a result of cell extension, thereby providing new insight on the mechanism plant organ growth. Furthermore, it was shown that higher cell wall porosity can facilitate the action of anti-fungal drugs in *Saccharomyces cerevisiae*, presumably by facilitating uptake.

Summary

Cell wall porosity of fungi and plants could not be determined *in vivo* previously. Application of a novel method links dynamic changes in wall porosity to stress-induced cell elongation in plant roots and uptake of anti-fungal drugs in yeast cells.

Introduction

Bacterial, fungal and plant cells depend on cell walls for mechanical support, the determination of cell shape and size, and a multitude of additional functions that are essential for the life of these organisms (Cosgrove 2000; Mattei et al. 2010; Winstel et al. 2013). Bacterial cell walls are a primary target for antibiotics (Young 2016; Brown and Wright 2016), just like the fungal cell wall is a target for the treatment of fungal infections (Burnham-Marusich et al. 2018). Plant cell walls form the starting material for many commercial products (Klemm et al. 2005) and are the main feedstock to produce second generation biofuels (Carroll and Somerville 2009).

Cell walls vary in complexity from the single-polymer of gram-positive bacteria to the specialized network of different polysaccharides and proteins in plants (Meeske et al. 2015; Gow et al. 2017; Burton et al. 2010). The complexity, together with the nanometer-scale dimensions and the wall's sensitivity to sample preparation processes, have limited the investigation of cell wall structure, especially in plant cell walls. The precise arrangement of the different cell wall components within a plant cell wall and the mechanism of dynamic changes in wall structure remain unknown (Cosgrove 2016). In addition, the lack of simple tools to probe cell wall structure prevents a detailed functional characterization of the many genes that have been implicated in wall synthesis and remodeling (Somerville 2004; Schneider and Persson 2015; Taylor-Teeples et al. 2015).

One parameter of cell wall structure is the porosity, which describes the capacity for molecular movement within the wall and is related to the spacing between wall polysaccharides. Porosity correlates with cell wall digestibility and saccharification efficiency, as used for biofuel production (Himmel et al. 2007; Ding et al. 2012; Tavares et al., 2015). In rice, cell wall porosity was linked to mesophyll conductance for CO_2, showing that it can even limit the photosynthetic capacity (Ellsworth et al. 2018). Furthermore, porosity influences the kinetics and capacity of a plant's leaf water uptake (Boanares et al. 2018). In bacteria, porosity could be linked to cell growth (Huang et al. 2008; Turner et al. 2013). In plants, however, no consensus on the relationship of cell wall structure and cell wall

extensibility could be reached so far (Bidhendi and Geitmann, 2016; Cosgrove 2016), which reflects our inability to follow dynamic changes in wall structure.

Currently available methods for determining the porosity of cell walls have significant shortcomings (Adani et al. 2011). Transmission electron microscopy, which could be used to visualize areas of different density within cell walls at nanometer resolution, requires fixation and dehydration of the sample, potentially introducing artifacts. The less invasive cryo-electron microscopy has been used to visualize spaces between cellulose fibrils, but its resolution appears limited to 20 nm (Derksen et al. 2011, Zheng et al. 2017). A higher resolution and quantitative data of pore size distributions and pore surface area can be obtained by gas adsorption, but also requires harsh sample treatment (Adani et al., 2011). To assess the actual capacity for molecular movement within the wall, especially that of living cells, approaches based on fluorescence spectroscopy or microscopy have been developed. De Nobel (1990) assessed the relative porosity of yeast cell walls by spectroscopically measuring the chemically-induced release of UV-absorbing cellular compounds. This assay could show differences between fungal species but impacts cell function and cannot be applied to complex tissues. Donaldson et al. (2015) used fluorescence quenching to quantify the porosity of dewatered wood, but their method is restricted to secondary cell walls as it depends on lignin autofluorescence.

In the present study, we aimed to establish a method to quantify cell wall porosity in different cell types, including those with primary cell walls, *in vivo*. It was hypothesized that access of a small, freely diffusing extracellular molecule to the plasma membrane depends on the structure of the extracellular matrix. The hypothesis was tested and confirmed by measuring the quenching effect of an extracellular quencher on the membrane-specific dye FM4-64 in lipid vesicles as well as mammalian, bacterial, fungal and plant cells.

While this method is relevant for various applications, including assessing the saccharification potential of cellulosic biomass, its value was demonstrated here by investigating the relationship of cell wall structure and extension growth in the model plant *Arabidopsis thaliana*, as well as the effect of cell wall structure on the uptake of anti-fungal drugs in the yeast model *Saccharomyces cerevisiae*.

Results

Selection of quenchers for FM4-64

The requirements towards quenchers for the membrane-specific dye FM4-64 are i) spectral overlap between dye emission and quencher absorption, ii) a dynamic quenching mechanism, iii) membrane impermeability. Of the commonly used quenchers, Black Hole Quencher 3 (BHQ3), Malachite Green (MG) and Trypan Blue (TB) have adequate sizes to investigate the nanoscale structure of cell walls (Fig. 1A). TB acts as a dynamic quencher as demonstrated by the shortening of the fluorescence lifetime of the Bodipy FL fluorophore in the presence of TB (Fig. 1B). A similar behavior has been shown previously for MG (Rolinski et al. 1999), while BHQ3 can act as dynamic as well as static quencher (Crisalli and Kool 2011). Dynamic quenching capacity was furthermore indicated by quenching efficiency correlating with the spectral overlap of quencher absorption and dye emission (Suppl. Fig. S1). TB quenched the fluorescent dye Basic Fuchsin, whose emission spectrum overlaps the TB absorption spectrum almost completely, with much higher efficiency than Bodipy FL, whose emission spectrum only overlaps about 20% (Suppl. Fig. S1). TB did not show measurable quenching of Calcofluor White (CW), whose emission peak is completely separated from the peak of TB absorption (Suppl. Fig. S1). The spectral overlap of quencher absorption with the emission of FM4-64 in the yeast plasma membrane was found to be between 20% for TB and 45% for BHQ3 (Fig. 1C), yielding a useful degree of quenching (see below). The inability to cross membranes has been previously demonstrated for BHQ3 (Zhang et al. 2014), MG (Wilhelm et al. 2015) and TB (Strober 2015). While all quenchers absorb excitation light at high concentrations, minimal absorption was observed at the concentrations used here (Suppl. Fig. S1).

Quenching of plasma membrane-localized FM4-64 is influenced by the extracellular matrix

Despite the limited overlap with the excitation spectrum of TB (Fig. 1C), fluorescence emission of FM4-64 incorporated in giant unilamellar vesicles (GUVs) gradually decreased in the presence of TB (Fig. 2A, B), demonstrating its potential in this experimental system. To test how quenching is influenced by the extracellular matrix, we compared quenching efficiency and quenchable fraction in GUVs, Human embryonic kidney 293 (HEK), *Escherichia coli* and *S. cerevisiae* cells labeled with FM4-64. The amphiphilic nature of FM–4-64 means that it has a high affinity to the nonpolar phospholipid bilayer, while its charged group prevents the dye molecule from crossing the membrane (Griffing 2008; Wu et al. 2009). Importantly, FM4-64 does not bind to cell walls. Plasmolysis experiments on onion epidermis cells showed FM4-64 to be exclusively present in the plasma

membrane (Suppl. Fig. S2). Since FM4-64 can be internalized via endocytosis, measurements were restricted to 5 min after application of the dye, during which time only the plasma membrane is labeled (Vida and Emr 1995; Bolte et al. 2004).

In all cases, addition of TB resulted in quenching of FM4-64 fluorescence (Fig. 2C-E). To estimate the quenching efficiency and accessibility of FM4-64 to TB, fluorescence quenching data were analysed by the Stern-Volmer equation (Equation 1) and by the modified Stern-Volmer equation (Equation 2) (Lehrer 1971). In HEK cells, quenching efficiency and quenchable fraction were similar to GUVs (Fig. 2F-I). In the bacterial and yeast cells, efficiency and quenchable fraction were significantly lower (Fig. 2F-I). The results demonstrate a lower accessibility of the plasma-membrane localized fluorophore in cells containing a cell wall.

Relationship of quenching efficiency and cell wall structure

After establishing that cell walls affect the quenching of plasma membrane-localized FM4-64, the relationship of cell wall structure and quenching efficiency was further investigated. Quenching experiments were performed on the root elongation zone of seedlings of Arabidopsis plants treated with chemicals known to affect cell wall structure as well as mutants with published cell wall phenotypes. The mutants have reduced amounts, or lack, one or more polysaccharide component of the cell wall (Table 1). Chemicals included the cellulose synthesis inhibitors 2, 6-dichlorobenzonitrile (DCBN) and isoxaben and the growth inducing polyethylene glycol (PEG; also used to simulate drought stress below). Experiments were conducted on epidermal cells, since these define organ morphology (Savaldi-Goldstein et al. 2007) and are accessible to dyes. In each experiment, FM4-64 staining was performed for less than 10 min to ensure that only the plasma membrane was labeled. It should be noted that the quenching assay is not only applicable to roots, but also works on other plant tissues for example maize leaves (Suppl. Fig. S3).

Differences were observed between the three different quenchers (Fig. 3). In each plant, MG yielded the highest quenching efficiency and BHQ3 the lowest, with TB falling in between (Fig. 3B). All mutants showed a reduction in quenching efficiency with TB and BHQ3 compared to wild-type plants, although the reduction was only significant for *cesa3*^{S211A}, *rol1* and *xxt1xxt2* (Fig. 3B). In contrast, quenching with MG did not show decreased efficiency in the *xxt1xxt2* mutant and even increased in the *cesa3*^{S211A} mutant (Fig. 3B). Exposure of roots to the three chemicals increased quenching efficiency

with all quenchers with the same order of efficiency MG > TB > BHQ3 as observed in the cell wall mutants (Fig. 3B). These results suggest that quenching efficiency depends on quencher size as the smaller MG can quench more efficiently and is not hindered by the structural changes in the *xxt1xxt2* and *cesa3*^{S211A} mutants that affect penetration of the bigger BHQ3 and TB (see Fig. 1 for size comparison of quenchers).

To further investigate how quenching efficiency relates to cell wall structure, we evaluated cellulose spacing of wild-type, mutants and chemical-treated plants by staining the roots with the cellulose-specific dye Pontamine Fast Scarlet 4B (S4B) (Anderson et al. 2010; Liesche et al. 2013). With the intensity of cellulose fibrils normalized among all images, differences in cellulose fibril density become apparent (Fig. 3C). These were quantified as histogram mean values of 2D projections of image stacks acquired throughout the cell wall. Lower mean values indicate a higher abundance of black pixels, i.e. spaces between cellulose fibrils. Plants treated with the cellulose synthesis inhibitors DCBN or isoxaben showed lower cellulose density compared to control plants and, accordingly, reduced mean values (Fig. 3C). Cell wall mutants, such as *rol1* showed an increased mean value and visible differences in the pattern of cellulose distribution (Fig. 3C). Histogram mean value was found to significantly correlate with quenching efficiency for all three quenchers (Fig. 3D).

The correlation of quenching efficiency with cellulose spacing was further corroborated by Atomic force microscopy (AFM) imaging of epidermal cells corresponding to those used in the quenching experiments. AFM images of the *cesa3*^{S211A} mutant indicate a change in spacing of cellulose compared to wild-type (Fig. 4). Whereas in the wild-type wall, thick cellulose bundles with relatively large spaces in between were evident (Fig. 4A), a tight network of thin cellulose fibrils was observed in the *cesa3*^{S211A} mutant (Fig. 4B). A similar difference has been observed in the epidermal cell walls of wild-type and the *xxt1xxt2* mutant (Xiao et al. 2016). Whether a high number of small pores in the *cesa3*^{S211A} mutant is responsible for the significantly increased MG quenching efficiency cannot be confirmed or excluded based on the AFM images.

In contrast, no indication for a correlation of cell wall thicknesses, measured on transmission electron microscopy (TEM) images~~,~~ and quenching efficiency was found (Fig. 5). The walls of mutants *mur11* and *xxt1 xxt2* were significantly thicker than those of wild-type plants, whereas walls of *rol1* were significantly thinner (Fig. 5). ~~These results do not correlate with quenching efficiencies (Fig. 5A).~~ For example, *rol1* had a lower quenching efficiency but a thinner wall. The *cesa3*^{S211A} mutant showed

strong differences in quenching efficiency, but its wall thickness was not significantly different from wild-type. The results strongly suggest~~s~~ that quenching efficiency depends on cell wall porosity, and can, therefore, be used as indicator for this parameter.

It should be noted that, for TB quenching, changes in the quenchable fraction were also tested but did not show significant differences (Suppl. Fig. S4), which is why only quenching efficiencies are considered in the following. Furthermore, only TB was used in follow-up experiments as it offered the highest dynamic range of the three quenchers based on the experiments conducted on cell wall mutants.

Correlation of cell wall porosity and cell length in plants

The mutant plants described above, as well as additional mutants that were tested (Table 1), all had significantly reduced root lengths (Suppl. Fig. S4), indicating that reduced cell wall porosity might correlate with reduced wall extensibility and cell elongation, a connection that has been debated for a long time in the plant science community (Bidhendi~~Bindhendi~~ and Geitman 2016; Cosgrove 2016). To test this hypothesis, we induced cell extension by exposing wild-type and mutant plants to simulated drought stress, namely growth on 10 or 20% (w/v) PEG, and determined quenching efficiencies (Fig. 6).

In wild-type plants, quenching efficiency increased upon PEG treatment (Fig. 4C), indicating an increase in wall porosity. As expected cell length and root length increased with PEG concentration (Fig. 6D, E). Regarding the cell wall mutants, a PEG-induced increase in quenching efficiency was observed in the *cesa3*^{S211A} and the *mur10* mutants, as well as an increase in cell and root length (Fig. 6A-E). A significant reduction of quenching efficiency was observed in the *mur1*, *mur11* and *rol1* mutants (Fig. 6C). For all plants that showed a reduction or no significant change in quenching efficiency, no significant PEG-induced increase in cell length was observed (Fig. 6A, D). However, in case of *mur1* and *mur11* root length still increased (Fig. 6B, E).

A correlation analysis was conducted using the data from wild-type and mutants under control and PEG-treatment conditions to test if quenching efficiency scales with cell length and root length. The analysis of absolute values (Suppl. Fig. S4) showed a significant correlation of cell length and quenching efficiency (R=0.45, P=0.0272, n=24) (Fig. 6F). Even higher significance was observed when testing correlation of relative quenching efficiency and relative cell length (R=0.565, P=0.0127, n=16) (Fig. 6G). A correlation of quenching efficiency with root length was only found when

comparing relative changes (R=0.436, P=0.035, n=16). As expected, cell length showed a very strong correlation with root length (R=0.677, P=0.0003, n=24) (Fig. 6H). The results support the notion of cell wall porosity being related to wall elasticity and, thereby, cell elongation. However, rather than being a pre-condition for cell elongation, increased porosity seems to be a consequence.

Cell wall porosity and drug uptake in yeast

To further illustrate the potential of the quenching assay, it was used to test if cell wall porosity influences the efficiency of antifungal drugs with a target inside the cell. Previously, lipid bilayers such as the plasma membrane in yeast or outer membrane of gram-negative bacteria have been seen as the decisive barrier for the uptake of anti-bacterial or anti-fungal drugs (Lambert 2002; Mishra et al. 2007). However, it was reported that disruption of the cell wall sensitizes the yeast model *S. cerevisiae* and the infectious *Candida glabrata* to the anti-malarial drug chloroquine (Islahudin et al. 2013) indicating that cell wall structure can influence drug uptake. Here, experiments were carried out on *S. cerevisiae*, which has a similar cell wall to the infectious *Candida* strains (Gow et al. 2017).

Several agents known to affect yeast cell wall structure (Okada et al. 2016) were tested for their influence on cell wall porosity using the quenching assay. Cells treated with CW, 2-Deoxyglucose (2-DG), a 42°C heat shock and sodiumdodecylsulfate (SDS) were found to have a higher FM4-64 quenching efficiency compared to untreated cells, indicating an increased cell wall porosity (Fig. 7A). The influence of CW, 2-DG, SDS, as well as of two known antifungal drugs with intracellular target, Amphotericin B and Voriconazole, on cell viability was tested. Amphotericin B and Voriconazole target the membrane integrity and ergosterol synthesis, respectively (ASDCD 2018). At the minimal efficient concentrations, where separate application of these compounds reduced viability after 24 h by about 50% (Fig. 7B), combinations of wall-modifying agents with Amphotericin B or Voriconazole further reduced viability, indicating a synergistic effect (Fig. 7B). Quantification of this effect showed that the efficiency of concomitant application of Amphotericin B and Voriconazole only increased by 15% compared to separate applications of the two drugs (Fig. 7C). All combinations of porosity-increasing chemicals with either Amphotericin B or Voriconazole led to an increase in treatment efficiency of at least 40% (Fig. 7C). While there was no linear correlation between quenching efficiency and the synergistic effect (Fig. 7C), these results do indicate a link between cell wall structure and efficiency of drug uptake.

To check if the cell wall-modifying chemicals at the concentrations used for the quenching assay visibly compromise the cell wall or if they merely cause changes in the internal structure, cells were studied by field emission scanning electron microscopy (Fig. 7). Only the appearance of 2-DG-treated cells showed strong deviation from control cells (Fig. 7F). The large indentations indicate that treatment led to major defects in the cell wall, whereas CW- and SDS-treatment did not affect appearance (Fig. 7D-~~E,~~ G).

Discussion

The quenching assay presented here is useful for quantifying the accessibility of the extracellular quencher molecule to the plasma membrane. Furthermore, the results demonstrate that this accessibility changes according to the structure of the extracellular matrix, i.e., the cell wall. Thereby, the assay constitutes a new quantitative method to probe the structure of cell walls *in vivo*.

Which structural feature(s) of the cell wall determine the quenching efficiency? At least four parameters can be considered (Fig. 8). Firstly, quenching efficiency could depend on the distance between the plasma membrane and the outer boundary of the extracellular matrix. No correlation of cell wall thickness and quenching efficiency was observed for Arabidopsis seedlings, although the relatively low sample number should be considered. Nevertheless, the result is in line with the fact that quenching is limited to about 5 nm distance (Zu et al. 2017).

Secondly, quenching efficiency could depend on the contact area of plasma membrane and open apoplastic space. This is unlikely, because the quenching efficiency showed large differences between wild-type plants and some of the cell wall mutants, while the quenchable fraction, i.e. the amount of membrane dye that can be accessed by the quencher, did not significantly change. Furthermore, no visible differences were found between the plasma membrane-adjacent wall region of wild-type and *xxt1 xxt2* mutant cells (Xiao et al. 2016).

Thirdly, the quenching efficiency might depend on the spacing between wall components, i.e. the molecular diffusion efficiency inside the cell wall. The comparison of quenching efficiency measurements with TEM and AFM images of the cell walls of wild-type and mutant plants supports this hypothesis. The walls of *cesa3*^{S211A} and *xxt1 xxt2* mutants, which had a decreased quenching efficiency, both have a denser appearance than the respective walls wild-type plants (Xiao et al., 2016 and Fig. 4). Importantly, quenching efficiency was influenced by the size of the quencher with MG

showing better quenching than the larger BHQ3 and TB. TB is longer but narrower than BHQ3, which might cause the better quenching of TB compared to BHQ3 that was observed here. Charge is unlikely to influence quenching as the negative charge of the cell wall (Crasnier et al. 1985) would be expected to facilitate access of the positively charged BHQ3, but not the negatively charged TB.

Fourthly, quenching efficiency might depend the molecular diffusion efficiency inside the wall, like in the third hypothesis, but this would depend on the binding of quencher molecules to cell wall components instead of the availability of spaces between components. TB was previously proposed to have a moderate ability to bind beta glucans and xyloglucan (Liesche et al. 2015). However, TB quenching efficiency in the different cell wall mutants does not support this observation. For example, in the *xxt1 xxt2* mutant, which lacks xyloglucans (Cavalier et al. 2008, Park and Cosgrove 2012), quenching efficiency was lower than in wild-type plants. If TB diffusion was influenced by xyloglucan binding, then quenching efficiency should increase in the absence of these binding sites. Quenching efficiency was also significantly reduced in the *rol1* mutant, even though this mutant has the same levels of xyloglucan and other glucans as wild-type plants (Diet et al. 2006).

In conclusion, it is most likely that the primary factor determining quenching efficiency is the spacing between cell wall components, especially between cellulose fibrils. That means that it would also be a good tool to test cell wall digestibility, which has previously been linked to cell wall porosity (Adani et al. 2011; Ding et al. 2012), in the same set of mutants analyzed here or in other plants for which the quenching measurements are made. This could be highly relevant for testing, maybe even as part of a breeding program, for biomass usability (Dixon 2013). In this respect the results also suggest that reducing certain cell wall components, at least in the primary wall, cannot be expected to be a good strategy for increasing digestibility, because decreased wall porosity might be a general response. This could explain why genetically modifying plants with increased expression of cell wall modifying enzymes sometimes does not have a beneficial effect for digestibility (Tavares 2015).

The potential of the quenching assay was illustrated by using it to investigate changes in wall porosity during drought-induced cell elongation in the model plant ~~*A. thaliana*~~Arabidopsis. Root elongation is an important part of a plant's drought response, as it enables access to residual water in the soil. It is achieved through a combination of higher cell division rates and cell elongation (Comas et al. 2013). Cell elongation depends on remodeling of the cell wall, and drought-induced wall remodeling is known to be enacted by enzymes that modify wall polysaccharides, especially expansins, xyloglucan

endotransglucosylases/hydrolases and pectin esterases (Tenhaken 2015, Lampugnani et al. 2018). The question of how cell wall structure relates to wall mechanics and action of cell wall-loosening agents is seen as the "grand challenge" in the field of cell wall biology (Cosgrove 2016). Results of the quenching assay provide some insight on this, by demonstrating that cell wall porosity changes during wall remodeling. The occurrence of stress-induced cell elongation in mutants with strongly decreased wall porosity indicated that increased porosity is not a pre-condition for elongation but a consequence. This was supported by the higher significance of correlation of quenching efficiency and cell length relative to non-stressed conditions compared to the correlation of absolute values of the two parameters.

The results suggest the following to happen: stress-induced loosening of the cell wall is accompanied by an increased physical distance between wall components or the removal of certain materials between load-bearing components. In the cell-wall mutants that are not able to remodel their cell walls in response to stress, cell wall-modifying enzymes can be present in the wall, but the lack/reduction of certain wall components prevents their action. These results could be extended by analyzing plants with altered amounts of cell wall modifying enzymes, coupled with a detailed chemical analysis of cell wall composition and crosslinking. Facilitated by the non-invasiveness the assay could thereby help to find the genetic basis of the adaptation of cell wall structure to environmental conditions. In addition, facilitated by the simplicity of the quenching assay, key genes controlling cell wall remodeling could be identified via forward genetics screening or through a genome wide association study.

The quenching assay presented here could also be used for chemical screens that aim to identify compounds that alter the cell walls of bacteria or fungi, and thus have relevance for biomedical and biotechnology research. This was illustrated here by testing porosity of yeast cells in the presence of cell wall-modifying agents and linking these results to uptake of common antifungal drugs with intra-cellular targets. While results clearly demonstrate a synergistic effect due to modification of cell wall structure, it is not clear if there is a correlation with porosity or if the effect is due to other effect. For example, it might be possible that destabilizing the cell wall affects the plasma membrane and facilitates uptake or action through this. Investigation on a larger scale would be needed to determine the full potential of cell wall-modifying agents for anti-fungal drug treatment.

Material and methods

Materials

The lipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids (Alabaster, Albama, USA). N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide (FM4-64), Bodipy FL and CW were purchased from ThermoFisher Scientific (Waltham, MA, USA). TB was purchased from Merck Millipore (Darmstadt, Germany), Basic Fuchsin from SigmaAldrich (St. Louis, Missouri, USA) and SDS, MG, 2-DG, PEG (MW 8000), isoxaben and DCNB from Solarbio (Beijing, China). BHQ3 was purchased from LGC Biosearch (Petaluma, CA, USA). Phosphate-buffered saline (PBS) contained 130 mM NaCl, 2.6 mM KCl, 7 mM Na_2HPO_4, 1.2 mM KH_2PO_4, and was adjusted to pH 7.4. Phosphate buffer (PB) with pH 5.8 was ~~adjusted~~obtained by mixing ~~by~~ 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 with the volume ratio~~n~~ of 23:2 and then diluted~~ent~~to a ~~the~~ concentration to 40 µM.~~,~~ PB with pH 6.8 ~~was adjusted~~obtained by mixing ~~by~~ the two ~~kinds of~~ stock solutions ~~with the volume ration of~~with a ratio of 51:49. Tyrodes balanced salt solution (TBSS) contained 136 mM NaCl, 2.6 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 5.56 mM D-Glucose, and 5 mM HEPES, and was adjusted to pH 7.4.

Electroformation of giant unilamellar vesicles

GUVs were produced by an electrophysical method (Angelova et al. 1992) as described in the following. DOPC was dissolved in chloroform to a final concentration of 0.5 mg/mL in a volume of 50 µL in pointy Schott glass tubes. Five µL of the mixture was placed in small drops on both electrodes and the chloroform dissipated by vacuum application for 15 min. The electroformation chamber was filled with 300 µL sterile-filtered 300 mM sucrose solution. Vesicle formation was achieved by applying an AC voltage, 20 mV and 10 Hz, for 3 h followed by 1 h at 2 V and 4 Hz. The GUVs (20 µL) were transferred on a microscope slide, and the same amount of PBS or quencher solution was added. GUVs were allowed to settle on the slide for 3 min. The quality of GUVs was checked using phase contrast microscopy.

Cell cultures and plant cultivation

E. coli (DH5α) cells were grown at 37°C in Luria-Bertani liquid medium for 4 h. Bacteria were collected from l mL medium, washed with 1 mL PBS and incubated with 100 µL PBS-based FM4-64

solution for 5 min. After washing with 1 mL PBS, cells were mixed with 10 µL quencher solutions on the slide and immediately analyzed under the microscope.

Yeast (*S. cerevisiae*) cells of the strain BY4742 were cultured in yeast extract peptone dextrose medium (1% yeast extract, 2% peptone, 2% glucose/dextrose, water) at 30°C For labeling with FM4-64, cells in 3 mL medium were grown to mid-logarithmic phase (OD600 ~ 0.6–0.8), washed with PBS and suspended in 200 µL PBS-based FM4-64 solution for 3 min, followed by washing and imaging. Cells were used within 10 min. For the induction of changes in the yeast cell wall, 5 µg/mL CW, 0.02% (w/v) 2-DG or 0.04 mg/mL SDS were added to the medium 2 h before quenching experiments were conducted.

~~Human embryonic kidney 293 (~~HEK~~)~~ cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. For labeling with FM4-64, cells were trypsinized using 0.25% trypsin-EDTA for 5 to 10 min and resuspended in TBSS. Cells were used for quenching assays within 1 h.

Arabidopsis ~~*(Arabidopsis thaliana)*~~ ecotype Colombia (Col-0), and the homozygous mutants *kor* (N298), *mur1* (N6244), *mur10* (N8578), *mur11* (N8579), *xxt1 xxt2* (N16349), *rol1* (N16373) (all obtained from the Nottingham Arabidopsis Stock Center) and CESA3^{S211A} (Chen et al., 2016) were used in this study. Mutants were selected to represent different types of changes in cell wall composition and structure. After surface sterilization with 10% (v/v) sodium hypochlorite for 10 min ~~and~~ followed ~~with~~ by vernalization for at least 3 days at 4 °C, seedlings were grown on half-strength Murashige and Skoog (MS) plates containing 0.22% (w/v)~~,~~ MS salts (Phyto Technology Laboratories, China), 1% (w/v) sucrose and 0.8% (w/v) agar powder (Solarbio, China), pH 5.8, under 16 h light/8 h dark in a growth chamber at 22°C. For drought treatment 3-d-old seedlings were transplanted from normal half-strength MS plates to plates containing either 10% or 20% (w/v) PEG for 4 days. For treatment with the cellulose synthesis inhibitors DCBN (Desprez et al., 2002) and isoxaben (Tateno et al., 2016), 6-d-old Columbia seedlings grown on half-strength MS plates were transferred to plates containing 5 nM isoxaben or 0.5 µM DCBN. After one day incubation in the growth chamber with 16 h light/8 h dark cycle at 22°C, seedlings were used for the quenching assay and cell wall structure analysis.

Quenching assay and imaging

FM4-64, from a 1 mM DMSO stock, was used at final a concentration of 50 µM for staining of GUVs, yeast and HEK cells, and at a concentration of 20 µM for staining plant cells. Stock solutions with different concentrations of BHQ3~~, MG~~ and TB were made by dissolving the quenchers in PBS, MG was dissolved ~~by~~in PB. From these quencher solutions, 20 µL were added to the GUVs/cells and 50 µL were added to the plant seedlings on the microscope slide to final concentrations ranging from 1 to 100 µM. After addition of quencher, samples were imaged immediately using a wide-field fluorescence microscope (DMi8, Leica Microsystems, Germany) for GUV experiments or a confocal microscope (Andor Revolution XD, Leica SP8 or a point scanning confocal system equipped with CLSD-2SS Dual-Channel PMT Module (Thorlabs, USA) on a Leica DMi8 microscope body. ~~Thorlabs confocal module~~) for experiments involving cells and plants. ~~1.49 N.A. 100×~~oil immersion objectives with N. A. 1.3 to 1.49 were used for GUV, bacteria, yeast and HEK cell imaging and a 1.10 N.A. 40×water immersion objective ~~were~~was used for plant cells. Imaging parameters for FM4-64 were excitation at 530 to 550 nm and emission detection at 560 to 620 nm on the wide-field system, and excitation at 514 nm, 532 nm or 543 nm and emission detection ~~detected under a TR-F~~at 570 to 616 or 550 to 610 nm ~~at 560 to 620 nm~~ on the confocal microscopes. Software supplied by the microscope manufacturers was used for image acquisition.

For each sample, the quenching assay was repeated at least three times, including the entire concentration range. As there was no significant variation between the data from different replicates, all data from each sample were pooled and analyzed together. While different intensity values were obtained from experiments performed using different confocal microscopes, there were no significant differences in quenching efficiency or quenchable fraction.

Absorption and Emission spectrum measurements

Absorption ~~and emission~~ scans were performed with a ~~Tecan~~ Infinite 200 Pro fluorescence spectrometer (Tecan, Switzerland) with the PBS-based solution at the concentration of 100 µM. CW, Basic Fuchsin and Bodipy FL were all used at a concentration of 10 µg mL^{-1} and mixed with TB right before conducting the measurement. The optimal excitation and emission parameters used in these tests were determined in separate wavelength scans for each dye.

Image analysis and calculation of quenching efficiency

All image analysis was performed in ImageJ (Schindelin et al. 2012). In order to correct for background, signal in an area of each image where no GUVs or cells were present was measured. The black level of the image was assigned to this value and the remaining signal values were redistributed between 1 and 255 using the brightness & contrast tool. Then, fluorescence intensity was measured by determining the average intensity of a 3 µm × ~~x~~ 5 µm region of interest (ROI) overlaid on the peripheral staining of each GUV or cell (Suppl. Fig. S5). The ROI size was reduced to 1 µm × ~~x~~ 2 µm for *S. cerevisiae* cells and 0.5 µm × ~~x~~ 0.5 µm for bacteria. In plant cells, ROI width was kept constant, while ROI length varied in relation to the in-focus area of plasma membrane (Suppl. Fig. S5).

Intensity values were transferred to Excel (Microsoft, WA, USA) and quenching efficiency and fraction of quenchable fluorophores were calculated according to the equations presented below. Quenching processes are generally described by the Stern-Volmer equation, which specifies the quenching efficiency,

$$\frac{F_0}{F} = 1 + K[Q] \qquad \text{Eq. 1}$$

where F_0 and F are the fluorescence intensity in the absence and presence of quencher at the concentration Q, respectively, and K is the quenching coefficient. In addition to quenching efficiency, the fraction of quencher-accessible fluorophores is used to characterize a quenching process. In order to determine the quenchable fraction of fluorophores, the Stern-Volmer equation (Eq. 1) can be modified to quantify the portion of fluorophores that are accessible for quenching, f_a, according to Lehrer (1971),

$$\frac{F_0}{\Delta F} = \frac{1}{f_a} \times \frac{1}{K[Q]} + \frac{1}{f_a} \qquad Eq. 2.$$

where ΔF is the change in fluorescence intensity through quenching. Accordingly plotted, $1/ f_a$ of a quenching experiment is given as the point of intersection with the y-axis.

Standard deviations were provided as error bars for all average values. The standard deviation of linear regressions was determined according to $SD = \frac{b}{R\sqrt{\frac{(n-2)}{1-R^2}}}$, with quenching efficiency b, R-value of the fit R and the number of experiments n. Welch's t-test was used to determine significance.

Relative quenching efficiencies were calculated by dividing values from experiments conducted under stress conditions with those conducted under control conditions.

Analysis of cellulose fibril spacing by confocal imaging

Roots of 7-d-old Arabidopsis seedlings were stained with 10 µg/mL S4B for 10 min and washed with PBS ~~(pH 7.2)~~ before imaging on a spinning-disk confocal system (Revolution WD, Andor, UK) equipped with a CSU-W1 spinning-disk head (Yokogawa, Japan) and an iXon Ultra 888 EMCCD (Andor, UK) ~~a spinning disc confocal microscope (Revolution WD, Andor, UK)~~. Using the HCX PL Apo 100~~×x~~ N.A. 1.49 objective, 561 nm laser, and a TR-F607/36 bandpass filter (Semrock BrightLine, USA)~~s allowing detection at 575 to 625 nm~~, z-stacks of the outer wall of epidermis cells were acquired. Image stacks were deconvolved using the Huygens software (Version 15.10, Scientific Volume Imaging, Netherlands) with the default settings using an automatically generated theoretical point spread function and the maximum likelihood estimator algorithm. Maximum projections were made from deconvolved image data in ImageJ. On these, the white level was adjusted so that the brightest cellulose fibrils of the outer wall are assigned the maximum value (= 65535 for the 16-bit images). The black level was left unchanged. Thereby, differences in signal intensity between samples were eliminated, ensuring that darker areas indicate reduced presence of cellulose. Rectangular ~~regions of interest~~ROIs were drawn on the in-focus areas of the outer epidermal wall and mean values determined using ImageJ's histogram function. At least 6 cells were measured for each sample.

Fluorescence lifetime measurement

Fluorescence lifetime imaging was performed on a Microtime 200 laser scanning confocal (PicoQuant, Germany). Bodipy FL was excited at 485 nm and fluorescence emission was detected at 500 to 525 nm by a photon-counting hybrid photomultiplier detector. Electrical signals were processed by a time-correlated single photon counting module (PicoQuant, HydraHarp 300). Analysis of FLIM images was performed using the SymPhoTime 64 software (PicoQuant, Germany), taking into account the instrument response function. Bodipy FL showed a single exponential decay. Time of photon arrival data is represented without fitting to a mathematical model. In these data, maximum peak height represents the fluorophore's lifetime under the given conditions.

Atomic force microscopy

Three-day-old dark-grown seedlings were bisected longitudinally and incubated in 2 M KOH at room temperature for 1 h and then in 1% Tween 20 for 30 min. After washing with ddH_2O until pH 7.0 was

reached, slices were placed between glass slides and a load of 5 g applied for 5 min. The innermost wall layer of primary cell walls was examined by scanning probe atomic force microscope (Bruker MultiMode V with NanoScope V Controller and SCANSYST-AIR probe). Contact AFM was performed in air at room temperature. Images of 2 μm^2 size with 512 x 512 pixel resolution were recorded using the software NanoScope Analysis (~~v1~~Version 1.10, (~~B~~Bruker, MA, USA). At least five areas per cell were scanned and at least six cells from three samples were analyzed.

Transmission electron microscopy

Whole, 7-day-old seedlings were vacuum infiltrated with 2.5% glutaraldehyde at 4°C for 6 h. After pre-fixing, the samples were gently rinsed with PBS solution (pH 6.8) for 4 times, 15 min each. Samples were fixed with 1% osmic acid at room temperature for 4 h followed by 3 times rinsing, 10 min each. The procedure of dehydration and infiltration with LR White resin was carried out as described by Verhertbruggen et al. (2017). After complete polymerization in capsules in the absence of oxygen, at 55°C, the root elongation zone of LR White embedded material were sectioned transversely with a diamond knife mounted on a Leica UC7 ultramicrotome to obtain ultra-thin sections of 70 nm of thickness. After transfer to copper grids, samples were stained with uranyl acetate for 20 min and gently rinsed with double-distilled water (ddH_2O). Then, samples were counterstained with lead citrate for 10 min, rinsed with ddH_2O and, after drying for 2 days, TEM images captured using a JEOL JEM-1230 under a voltage of 80 kV. Image files were analyzed by ImageJ to measure the thickness of the cell walls.

Yeast viability assay

Yeast cells of the strain BY4742 were cultured in yeast extract peptone dextrose medium at 30 °C in the presence of either CW (0.25 µg/mL), 2-DG (0.01 % (w/v)), SDS (2 µg/mL), Amphotericin B (0.5 µg/mL) or Voriconazole (0.25 µg/mL) or combinations of two of these substances. Every 2 h samples were taken, from which cell counts were determined using a hemocytometer. The data was analyzed using Microsoft Excel. Each assay was repeated three times. The synergistic effect was quantified as the average number of cells in culture after 24 h cultivation in the presence of two substances divided by the average number of cells of each treatment alone.

Field-emission scanning electron microscopy

Yeast cells were cultured as described above. For the different pretreatments 5 µg/mL CW, 0.04 mg/mL SDS or 0.02% (w/v) 2-DG were added 2 h before fixation. Cells were fixed in 4% glutaraldehyde at 4°C for 4 h followed by gradient dehydration in eight steps. Critical-point drying was performed using a Leica EM CPD300 (Leica Microsystems, Germany) in automatic mode. Dried samples were immobilized on double-sided carbon tape on a SEM sample stage and coated with Pt using a Quorum Q150T sputter coater (Quorum, UK) at 30 mA for 80 sec. Images were acquired using a FEI Nova Nano SEM-450 (FEI, USA) at a magnification of at least 10000 times with an in-lens detector at 5 or 10 kV. Images were analyzed with Image J (Schindelin et al. 2012).

Statistical analysis

Welch's two-sided t-test was used to assess significance of difference between data points. A difference was considered significant when $P < 0.05$. Data distribution was assumed to be normal but this was not formally tested. Pairwise Pearson Product Moment correlation was performed in SigmaPlot (Version 12.5, Systat Software, CA, USA). A correlation was considered significant when $P < 0.05$.

Supplemental material

Suppl. Fig. S1 complements Fig. 1 by providing further details on the properties of different quenchers. The correlation of quenching efficiency with spectral overlap of quencher absorption and dye emission (Suppl. Fig. S1) supports the hypothesis that Trypan Blue quenching is based on Förster Resonance Energy Transfer. Suppl. Fig. S2 provides an image from plasmolysis experiments performed on onion epidermis cells stained with FM4-64, indicating that the dye is associated with the plasma membrane and not the cell wall. Suppl. Fig. S3 illustrates the applicability of the quenching assay to plant leaves. Suppl. Fig. S4 provides quenchable fractions and quenching efficiencies in the various cell wall mutants used in this study, thereby complementing the information provided in Fig. 3 and Fig. 6. Suppl. Fig. S5 shows how the measurement regions to quantify fluorescence intensity were defined on different types of samples.

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Author contributions

Thomas Günther Pomorski~~GP~~ and ~~JL~~Johannes Liesche devised the quenching assay and control experiments. Xiaohui Liu~~XL~~ and Johannes Liesche devised the experimental plan for plant cell wall analysis. Johannes Liesche~~L~~ performed fluorescence lifetime microscopy and conducted the quenching experiments on vesicles and mammalian cells. Xiaohui Liu conducted quenching experiments on bacteria, yeast and plants, and measured cellulose fibril spacing and yeast viability. Xiaohui Liu and Jiazhou Li~~LJ~~ performed the electron microscopy. Shaolin Chen, ~~and~~ Heyu Zhao and~~,~~ Boyang Liu performed the atomic force microscopy. Johannes Liesche~~JL~~ wrote the manuscript with support of all authors.

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Abbreviations

2-DG – 2-Deoxyglucose

AFM – Atomic force microscopy

BHQ3 – Black Hole Quencher 3

CW – Calcofluor White

DCBN – 2, 6-dichlorobenzonitrile

ddH2O – double-distilled water

DOPC – 1,2-dioleoyl-sn-glycero-3-phosphocholine

FM4-64 – N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide

GUV – Giant unilamellar vesicle

HEK – Human embryonic kidney 293

MG – Malachite Green

MS – Murashige and Skoog

PB –Phosphate buffer

PBS – Phosphate buffered saline

PEG – Polyethylene glycol

ROI – Region of interest

S4B – Pontamine Fast Scarlet ~~S~~4B

SDS – Sodiumdodecylsulfate

TB – Trypan Blue

TBSS – Tyrodes balanced salt solution

TEM – Transmission electron microscopy

Tables

Table 1: Information on Arabidopsis cell wall mutants used in this study. HG – Homogalacturonan, RG – Rhamnogalacturonan.

Mutant name	**Gene**	**Description**	**Locus tag**	**Cell wall phenotype**	**Growth phenotype**	**References**
cesa3^{S211A}	CESA3	Cellulose synthase family protein	AT5g05170	↓cellulose	Slightly dwarfed	Chen et al. 2016
mur1	MUR1	GDP-D-mannose-4,6-dehydratase	At3g51160	↓ fucose, ↓RG II cross-linking	Slightly dwarfed	Reiter et al. 1997; O'Neil et al. 2001
mur10	CESA7	Cellulose synthase family protein	AT5G17420	↓fucose ↓xylose ↑arabinose ↑mannose	Slightly dwarfed	Reiter et al. 1997; Bosca et al. 2006
mur11	SAC9	sacI homology domain-containing protein	AT3G59770	↓fucose ↓xylose ↑arabinose	Dwarfed	Reiter et al. 1997; Austin et al. 2011
rol1	RHM1	UDP-L-Rhamnose synthase	At1g78570	↓RG II modified RG I	Normal	Diet et al. 2006
xxt1 xxt2	XXT1, XXT2	Xyloglucan Xylosyltransferase 1, 2	At4g02500	↓Xyloglucan	Dwarfed	Cavalier et al, 2008; Xiao et al. 2016

Figures captions

Figure 1. **Characteristics of the quenchers Black Hole Quencher 3 (BHQ3), Malachite Green (MG) and Trypan Blue (TB).** (**A)** Space filling model of hydrated quencher molecules in minimum energy configuration showing diameter and charges. **(B)** Fluorescence lifetime of Bodipy FL in PBS in the absence or presence of TB at the indicated concentrations showing a concentration-dependent reduction in lifetime. (**C)** Comparison of the absorption spectra of BHQ3, MG and TB in PBS with the emission spectrum of FM4-64 labeled *S. cerevisiae* cells. For details see Material and methods. Spectra were normalized to the respective maxima.

Figure 2. **Dependence of quenching of FM4-64 by Trypan blue (TB) on accessibility.** (**A)** FM4-64 labeled giant unilamellar vesicles (GUVs) imaged by fluorescence microscopy in the absence (control) or presence of TB at the indicated concentration, and (**B)** the corresponding intensity plot. (**C, D, E)** FM4-64 labeled human embryonic kidney (HEK) cells (**C**), *S. cerevisiae* cells (**D**), and *E. coli* cells (**E**) were imaged in the absence and presence of TB by fluorescence microscopy. (**F)** Stern-Volmer plots of FM4-64 fluorescence quenching by TB in GUVs, HEK cells, *S. cerevisiae* cells and *E. coli* cells. The slope of the regression line indicates quenching efficiency shown in panel (**G)**. (**H)** Corresponding modified Stern-Volmer in which the intersection of the linear regression line corresponds to the fraction of quenchable fluorophores shown in panel (**I)**. Dotted lines depict linear regression. All error bars indicate standard deviation of the mean ($n \geq 6$). Standard deviation of the

quenchable fraction (**I**) was extrapolated from standard deviations of measurements at high quencher concentrations (**H**). Asterisks in panels (**G)** and (**I)** indicate statistically significant ($P < 0.05$) difference to GUVs. Scale bars, 5 µm.

Figure 3. **Relationship of quenching efficiency and cell wall structure in epidermis cells of *Arabidopsis thaliana* wild-type plants and cell wall mutants.** (**A)** Wild-type, *cesa3*^{S211A} and *xxt1xxt2* plant root epidermis cells were labeled with FM4-64 and imaged by fluorescence microscopy in the absence (control) and presence of the indicated quenchers (10 µM). **(B)** Quenching efficiency of different quenchers (Malachite Green – MG, Trypan Blue – TB, Black Hole Quencher 3 - BHQ3) on wild-type ~~and,~~ mutant plants and wild-type plants treated with cell-wall modifying agents 2, 6-dichlorobenzonitrile (DCBN), isoxaben and polyethyleneglycol (PEG). Lines mark the quenching efficiency in wild-type plants. **(C)** Root epidermis cells stained with the cellulose-specific dye Pontamine Fast Scarlet ~~S~~4B ~~-~~imaged by fluorescence microscopy. Blue rectangles indicate the area corresponding to the overlaid histograms. Lower intensity mean value indicates larger spacing between cellulose fibrils. **(D)** Quenching efficiency plotted against mean intensity showing a significant correlation for all three quenchers. Both parameters were measured on all samples shown in (B), as well as other cell wall mutants listed in Table 1. Asterisks in (B) indicate significant difference ($P<0.05$) from wild-type. All error bars indicate standard deviation of the mean. Regression lines in (D) indicate significant correlation ($P<0.05$). Number of biological replicates $n \geq 4$. Scale bars, 20 µm (A), 10 µm (C).

Figure 4. **Atomic force microscopy images of the inner epidermal cell wall layer**. Spacing between wall polysaccharides appears larger in wild-type **(A)** than in the cesa3^{S211A} mutant **(B)** plants. Scale bars, 100 nm.

Figure 5. **Cell wall thickness of root epidermis cells of *Arabidopsis thaliana* wild-type plants and cell wall mutants**. **(A)** Cell wall thickness as determined by transmission electron microscopy plotted against Trypan Blue quenching efficiency showing no correlation between the two parameters. **(B-D)** Representative electron micrographs of epidermal cell walls from wild-type (B), *mur11* (C), and *rol1* (D). Error bars indicate standard deviation of the mean. Scale bars, 100 nm.

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Figure 6. **Correlation analysis of quenching efficiency, cell length and root length in *Arabidopsis thaliana* seedlings**. Trypan blue quenching efficiency was determined in the FM4-64 labeled roots of wild-type and cell wall mutant plants exposed to different levels of drought stress simulated by PEG treatment. In addition to quenching efficiency, cell length **(A)** and root length **(B)** were measured in the absence (0%) and presence of PEG at moderate (10%) and high (20%) concentrations. Changes relative to control conditions were quantified

for quenching efficiency **(C)**, cell length **(D)** and root length **(E)**. Quenching efficiency significantly correlated with cell length **(F)**, as did relative quenching efficiency with relative cell length **(G)**, and cell length with root length **(H)**. Regression lines indicate significant ($P<0.05$) correlation. All error bars indicate standard deviation of the mean. Asterisks indicate significant difference ($P<0.05$) compared to control conditions. Number of replicates n=5 (C), $n \geq 25$ (D), $n \geq 20$ (E). Scale bars in (A), 10 μm.

Figure. 7. **Effect of cell wall structure on antifungal drug efficiency in *S. cerevisiae***. **(A)** Relative FM4-64 quenching efficiency after treatment with cell wall-modifying factors compared to untreated cells (CW – Calcofluor White, 2-DG – 2-Deoxyglucose, EtOH – ethanol, SDS – Sodiumdodecylsulfate). **(B)** Cell counts over 24 h culture period in the absence (control) or presence of cell wall modifying chemicals and the antifungal drug Voriconazole (V). Combination of voriconazole and cell wall-modifying chemicals led to strong reduction in cell number. Similar results for this viability were observed with another antifungal drug Amphotericin B (A). **(C)** Synergism of cell wall-modifying chemicals and antifungal drugs plotted against quenching efficiency. **(D-G)** Field emission scanning electron micrographs of cells in the absence **(D)** or presence of the cell wall modifying chemicals CW (**E**), 2-DG (**F**) and SDS (**G**) at two magnifications. Only 2-DG-treated cells showed visible signs of compromised cell wall integrity (**F**). Error bars indicate standard deviation of the mean (n = 4). Asterisks indicate significant difference ($P<0.05$) compared to control conditions. Scale bars, 500 nm.

Figure. 8. **Illustration of four parameters potentially affecting extracellular quenching**: wall thickness, the barrier-function of polysaccharides close the plasma membrane, wall porosity and the binding capacity of quencher molecules to wall components. The experimental data indicate that wall porosity is the main parameter that determines quenching efficiency.

Supplemental figure captions

Suppl. Fig. S1. **Quencher characteristics**. (**A)** Stern-Volmer plots showing the quenching efficiency for the indicatedfluorescent dyes by Trypan Blue in water. (**B)** Comparison of absorption spectrum of Trypan Blue with the emission spectra of the same three fluorescent dyes. Correlation of quenching efficiency (**A**) with spectral overlap of quencher absorption and dye emission (**B**) supports the hypothesis that Trypan Blue quenching is based on Förster Resonance Energy Transfer. **C, D** Concentration-dependent absorbance of Black Hole Quencher 3 (BHQ3), Malachite Green (MG) and Trypan Blue (TB) at 488 nm (**C**) and 514 nm (**D**). It shows a low absorbance at the concentrations (up to 100 μM) that are used in the quenching assays.

Suppl. Figure S2. **Plasmolysis of onion epidermis cells stained with FM4-64.** A layer of onion epidermis was stained with 20 μM FM4-64 for 3 min, rinsed with PBS, covered with 30 % (w/v) sucrose on the slide and imaged after 5 min of incubation by phase contract and fluorescence microscopy. (**A)** Overview, (**B)** magnified area as indicated by black box in (**A)**. Scale bars, 10 μm (**A**), 1 μm (**B**).

Suppl. Figure S3. **Application of the quenching assay to maize leaves. (A)** Images of maize leaf epidermis cells labeled with FM4-64 in the absence (control) or presence of Trypan Blue at the indicated concentration. **(B)** Stern-Volmer plot in which the slope of the regression line indicates quenching efficiency. Error bars indicate standard deviation of the mean (n=4). Scale bar, 50 μm.

Suppl. Figure. S4**. Quenchable fraction and quenching efficiency in cell wall mutants**. (**A)** Modified Stern-Volmer plot indicating quenchable fraction in Arabidopsis wild-type and cell wall mutant plants. The fraction of FM4-64 fluorescence quenched by Trypan Blue can be calculated from the y-axis crossing point of regression lines according to equation 2. (**B)** Comparison of quenchable fractions indicates limited variation between wild-type and mutant plants. (**C-E)** Quenching efficiency (**C**), cell length (**D**) and root length (**E**) of *Arabidopsis thaliana* wild type and mutant plants grown under control conditions. Quenching efficiency (**C**) and cell length (**D**) were determined for epidermis cells of the elongation zone. All error bars indicate standard deviation of the mean. Asterisks indicate significant difference ($P<0.05$) from wild-type. Number of replicates N=4 (**A-C**), N>20 (**D**, **E**).

Suppl. Figure S5. **Quantification of peripheral membrane fluorescence.** Mean fluorescence was measured in a region of interest (yellow box) with constant size in each of the experimental systems. (**A)** Giant unilamellar vesicles, (**B)** *Saccharomyces cerevisiae*, (**C)** Cultured human embryonic kidney cells, (**D)** *Escherichia coli*, (**E)** Arabidopsis root cells. Scale bars, 5 μm.