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PERSPECTIVE

Best Practices and Pitfalls in Developing Nanomaterial Delivery Tools for Plants

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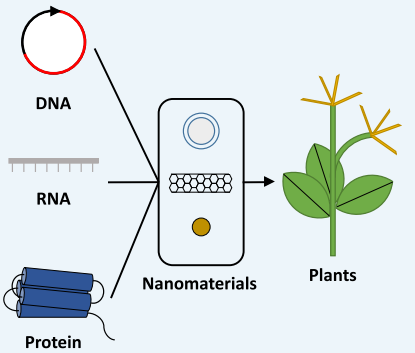
ABSTRACT: **Numerous reports of nanomaterial-assisted delivery of DNA, RNA, and protein to plants for biotechnology applications emerged over the past decade. While the field has experienced rapid growth, best practices for developing and validating nanomaterial delivery tools for plants have not yet been established. Best practices are well-established for clinical/animal cell delivery experiments, yet plants pose a distinct challenge requiring separate considerations due to their unique tissue structures and cellular morphology. In this Perspective, we provide recommendations and highlight pitfalls in developing nanomaterial tools for delivery of "Central Dogma" cargos to plants. Given the ongoing interest in the field, this discussion will aid in improving the rigor of this nascent field toward practical applications of nanomaterial delivery tools.**



KEYWORDS: *Plant biotechnology, nanomaterial delivery tools, drug delivery, biomolecule delivery, confocal microscopy*

FIELD HISTORY: FROM ANIMALS TOWARD PLANTS

The use of nanomaterials for drug delivery was first widely theorized in the mid-20th century.[1] Numerous clinical technologies have followed, with lipid nanoparticle-mediated delivery of mRNA for SARS-CoV-2 vaccines among the most notable recent successes.[2] Nanomaterial delivery approaches, initially optimized for animal cells in clinical applications, recently drew interest for applications in plant bioengineering with the potential to simplify plant genetic and post-transcriptional manipulation for applications in agriculture, bioenergy, and molecular farming. The first report of nanomaterial-assisted delivery of molecules to mature plants without biolistics appeared in 2009,[3] with numerous nanomaterial delivery tools developed since.[4] While nanomaterial-mediated biomolecule delivery for clinical applications in animal cells is relatively mature with established but continually refined best practices,[5−8] similar standards have yet to be established or broadly discussed in the context of plants. Established clinical practices provide a starting point for developing nanomaterial delivery tools for plants; however, animal and plant cells are distinct in tissue structure, genomic complexity, secondary metabolites, and cellular morphology and thus require separate considerations. Confocal microscopy experiments in plants, for example, require consideration of the plant cell wall, a highly exclusive cellulosic barrier, as well as autofluorescence from secondary metabolites and plastid organelles.[9]

To improve the rigor of the nascent but growing field of nanomaterial-mediated delivery of biomolecules to plants, best practices and pitfalls are discussed here based on common inconsistencies, omissions, or errors observed in the literature. We focus on nanomaterial delivery tools which carry "Central Dogma" cargoes, namely, DNA, RNA, and protein. First, we review experimental design considerations, focusing on the development of proper controls, repeatability, and use of multiple independent methods of experimental validation. Next, we highlight specific considerations for confocal microscopy and RT-qPCR due to the high frequency with which the field utilizes these techniques as well as the risk of misinterpretation of these techniques. Finally, we suggest more thorough integration and communication with the plant biology community to ensure that nanomaterial delivery tools are developed to address the most challenging plant biotechnology problems.

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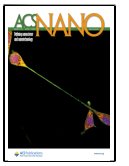

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CONSIDERATIONS FOR EXPERIMENTAL DESIGN

Many of the methodologies commonly used in the literature to evaluate nanomaterial delivery tools in plants are susceptible to type I errors, the false-positive assessment of delivery tool success. Careful experimental design can reduce type I errors. Here we provide recommendations for developing proper controls, choosing independent techniques for evaluating delivery success, and ensuring repeatability with biologically distinct plants based on omissions and errors we frequently observed in the literature.

The most common techniques for evaluating the effectiveness of a nanomaterial delivery tool include confocal microscopy, RT-qPCR, Western blotting, Northern blotting, genotyping, and phenotyping. Proper evaluation of a delivery tool should make exhaustive use of available assays at the DNA, RNA, and protein level. For example, a nanomaterial tool that delivers plasmid DNA that codes for a fluorescent protein should be evaluated by utilizing confocal microscopy to confirm that a functional protein is present, RT-qPCR to confirm that mRNA for the protein is present, and Western blotting to cross-confirm the presence of the protein. Confocal microscopy is fast but suffers from type I errors due to the potential to misinterpret plant autofluorescence for fluorescent protein signal. Similarly, RT-qPCR can suffer from type I errors due to amplification of any residual delivered plasmid. Western blotting, while time-consuming, suffers less from type I error. Combining the three techniques reduces the likelihood of falsely assessing a delivery tool as effective. Novel nanomaterial delivery tools might initially work with low efficiencies, outside the sensitivity of certain techniques, such as Western blotting. In these cases, we still encourage researchers to report "negative" results so the capabilities and limitations of the delivery tool are fully understood. Similar considerations apply to the delivery of other biomolecules such as dsRNA, siRNA, mRNA, and protein. Many publications developing nanomaterial delivery tools for plants rely on a single technique to verify delivery; regardless of the cargo delivered, multiple independent methods of assessing delivery success are necessary.

In addition to cross-method confirmation, experimental design also requires consideration of the proper controls. Certain cargoes may have a surprising capability to internalize to plant cells without a nanomaterial delivery tool. Recent examples include certain dsRNAs[10] and proteins.[11] Experiments should therefore include free cargo controls that attempt delivery without a nanomaterial tool. Additionally, controls with unloaded or mock-loaded nanomaterial delivery tools should be conducted. We note that nanomaterial delivery tools may damage or stress plant tissue, particularly if the tool contains a charged polymer.[12] Repeating confocal microscopy, RT-qPCR, or Western blot experiments with unloaded or mock-loaded nanomaterial delivery tools can control for any effects stress may have on evaluating the functionality of loaded tools, for example, stress-induced autofluorescence. We further note that loading a nanomaterial delivery tool with cargo often changes the tool's physical properties (size, ζ potential, etc.). Nanomaterial delivery tools loaded with nonfunctional cargoes such as scrambled/nontargeting siRNA or scrambled/nonfunctional pDNA serve as better controls than unloaded tools. Controls such as these are commonly absent in the published plant nanomaterial delivery tool literature.

Given the noise inherent in plant experimentation, multiple independent biological replicates are always necessary.[13] Many publications in the field rely on too few biological replicates or fail to report the nature of biological replicates (e.g., do not distinguish between biological or technical replicates). Frequently, publications report using three plants drawn from a single week of plant growth, which does not represent three biological replicates and fails to capture biological variability driven by unintentional variability in growth conditions. Plants grown distinctly in time (grown separately across different weeks, for example) respond differently to delivery tools and are important to include when probing biological variability. Other environmental factors such as day length and soil composition can influence biological variability and impact experimental interpretation;[14] we do not provide recommendations for specific conditions but instead emphasize that researchers should both control and report these parameters.

CONSIDERATIONS FOR PLANT CONFOCAL MICROSCOPY

Most publications reporting the development of a nanomaterial delivery tool for plants rely on confocal laser scanning microscopy (CLSM) to evaluate the fate of delivery tools and cargoes. Colocalization experiments with fluorophore-tagged cargoes are commonly used to establish whether a nanomaterial delivery tool is capable of internalizing into the cytosol of a plant cell. However, fluorophores may become dissociated from their cargoes or nanomaterial delivery tool and thus may not represent true biodistribution, as previously reported in mammalian systems.[6] Additionally, the physical limitations of CLSM require special consideration in plants. Due to the anatomy of walled plant cells and the physical resolution limits of traditional CLSM, colocalization analysis is merely suggestive of cargo and/or delivery tool internalization. Under ideal conditions, traditional CLSM is diffraction limited to a lateral (xy) resolution of 200−300 nm and an axial (z) resolution of 500−800 nm.[15] The plant cytosol in many cell types is compressed by the central vacuole into a small volume against the cell wall; given that the cell wall varies in thickness between 100 and 500 nm, traditional CLSM cannot reliably differentiate between cytosolic internalized cargo or cargo embedded/trapped in the cell wall. Super-resolution techniques have sufficient resolution to differentiate between these different cargo fates, but to date these techniques have not been used to confirm plant delivery. Separate from super-resolution techniques, researchers could consider methods which can provide unambiguous, quantitative localization data, such as split-protein[11,16] or Förster resonance energy transfer assays.[9] We encourage the field to avoid relying on colocalization to assay intracellular localization; at the very least, the limitations of traditional CLSM should be understood to prevent overinterpretation of results.

In addition to localization, CLSM is frequently used to verify functional delivery of cargoes such as plasmid DNA encoding fluorescent proteins. Researchers should consider that CLSM is susceptible to type I errors; fluorescent protein expression might be confused with plant autofluorescence, particularly at high laser powers or high gains. To avoid type I errors, we recommend that researchers consider the following. First, always utilize proper negative and positive controls, for example, nanomaterial delivery of a nonfunctional plasmid as a negative control and agrobacterium-mediated transfection as

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a positive control, as a direct comparison against nanomaterial delivery tools loaded with functional cargo. Importantly, researchers should use consistent laser powers and gains across negative control, positive control, and experimental treatment groups and also report these parameters.

We acknowledge that a positive control might not always be available, and thus, we additionally recommend consideration of the shape of objects viewed under CLSM, as fluorescent protein expression and autofluorescence exhibit different shapes. For example, cytosolic fluorescent protein expression typically appears as a puzzle-piece shape[17,18] with a fluorescent outline (cytosol), darker interior (vacuole), and swollen fluorescent nuclei[19] (Figure 1A,B). Nonspecific autofluorescence usually takes the form of irregular or punctate shapes that deviate from the prototypical puzzle-piece shape. In our experience, autofluorescence from tissue damage, debris, stomata, trichomes, and chloroplasts may all be misinterpreted as fluorescent protein expression (Figure 1C–E). Furthermore, use of nanomaterial delivery tools may induce plant stress or damage cells,[12] increasing the likelihood of observing autofluorescence. Time gating and spectral detection, common features on modern microscopes, can also reduce collection of autofluorescence signal;[20] see the work of Grossmann et al.[9] for further discussion on considerations for microscopy in plants. Regardless of the specific techniques or technologies used, in addition to utilizing proper negative and positive controls, careful inspection of the object shape can serve as a secondary check to reduce type I errors.

Due to the potential for misinterpretation, we again highlight the importance of utilizing multiple independent methods to validate a result. Even a carefully executed CLSM experiment for fluorescent protein expression, for example, should be cross-confirmed with a Western blot and RT-qPCR. As an alternative to fluorescent protein assays, we recommend that researchers consider time course luciferase assays instead. Plant phosphorescence presents less of a challenge relative to plant autofluorescence, reducing the chance of type I error. Some plant materials display phosphorescence briefly after excitation.[21] However, luciferase signal is stable over many minutes to hours while phosphorescence is short-lived; dark conditioning samples and conducting a time course to confirm that a signal is sustained can ensure that a true luciferase signal is observed. Ratiometric dual luciferase assays using an internal control luciferase can reduce the variability of time course experiments.[22] Finally, NanoLuciferase is significantly brighter than firefly or *Renilla* luciferase proteins;[23] we recommend that researchers consider NanoLuciferase for improved assay sensitivity.

CONSIDERATIONS FOR MRNA EXPRESSION QUANTIFICATION

Assays involving the quantification of mRNA are common for evaluating the effectiveness of a plant nanomaterial delivery tool. Typical mRNA assays include Northern blots and RT-qPCR. In general, we recommend RT-qPCR over Northern blots, as RT-qPCR provides higher-quality quantitative data. However, RT-qPCR assays are susceptible to type I errors, and thus, controls are critical to ensure proper interpretation of results. As an example, consider an experiment which attempts to quantify mRNA transcribed from a delivered plasmid DNA (pDNA) cargo utilizing RT-qPCR. After purification of RNA from the plant tissue, samples are typically treated with DNase prior to cDNA synthesis to remove genomic DNA and residual

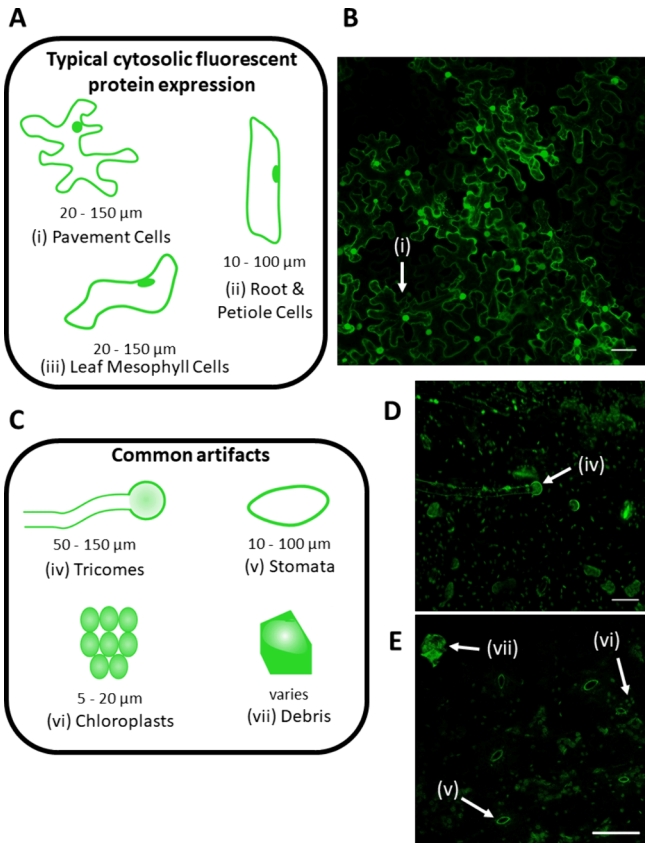

Figure 1. CLSM considerations in plants. (A) Representation of shapes typical of cytosolic fluorescent protein expression in different plant tissues viewed via CLSM, with approximate size scales of the objects listed. (B) Confocal maximum intensity projection of cytosolic expression of GFP in *Nicotiana benthamiana* leaf pavement cells induced by 3 days of infection with agrobacterium strain GV3101 carrying p35s:GFP:tNOS. The arrow indicates an example of a cell with the typical jigsaw puzzle shape and swollen nucleus. Laser power 4%, 488 nm excitation, 493–550 nm detection, gain 600. The scale bar represents 50 μm. See the Supporting Information (SI) for experimental details. (C) Representation of common artifacts which may be mistaken for fluorescent protein expression viewed via CLSM, with approximate scales of the objects listed. (D) Confocal maximum intensity projection of wild-type *Nicotiana benthamiana* leaf cells imaged at high laser power and gain displaying false fluorescent protein signals from trichomes, chloroplasts, and debris. The arrow indicates an example of a false fluorescent protein signal from a trichome. Laser power 100%, 488 nm excitation, 493–550 nm detection, gain 800. The scale bar represents 50 μm. See the SI for experimental details. (E) Confocal maximum intensity projection of wild-type *Arabidopsis thaliana* leaf cells imaged at high laser power and gain displaying false fluorescent protein signals from stomata, chloroplasts, and debris. Arrows indicate examples of false fluorescent protein expression from debris, chloroplasts, and stomata. Laser power 100%, 488 nm excitation, 493–550 nm detection, gain 650. The scale bar represents 50 μm. See the SI for experimental details.

pDNA. However, because most nanomaterial delivery tools protect nucleic acids from nuclease degradation,[24] residual pDNA may be present even after DNase treatment. Thus, a no reverse transcriptase control (NRTC), a sample that does not go through cDNA synthesis and thus contains no cDNA template to amplify, is critical to ensure that samples are not contaminated with DNA and to control for unintentional

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amplification of pDNA. If an NRTC returns significant RT-qPCR signal, samples were not sufficiently treated with DNase, which could lead to false-positive amplification of pDNA rather than amplification of cDNA originating from mRNA. Figure 2 illustrates how nanomaterial delivery tools may protect pDNA from DNase degradation, which subsequently may result in plasmid amplification in a RT-qPCR experiment and lead to the false conclusion that pDNA was successfully transcribed. To reiterate, many nanomaterial delivery tools are reported to protect cargoes from enzymatic degradation, and thus, false-positive amplification of pDNA with RT-qPCR is a generic problem independent of the specific nanomaterial delivery tool. We note that almost all papers reporting delivery of pDNA to plants and utilizing RT-qPCR quantification of mRNA do not include an NRTC.

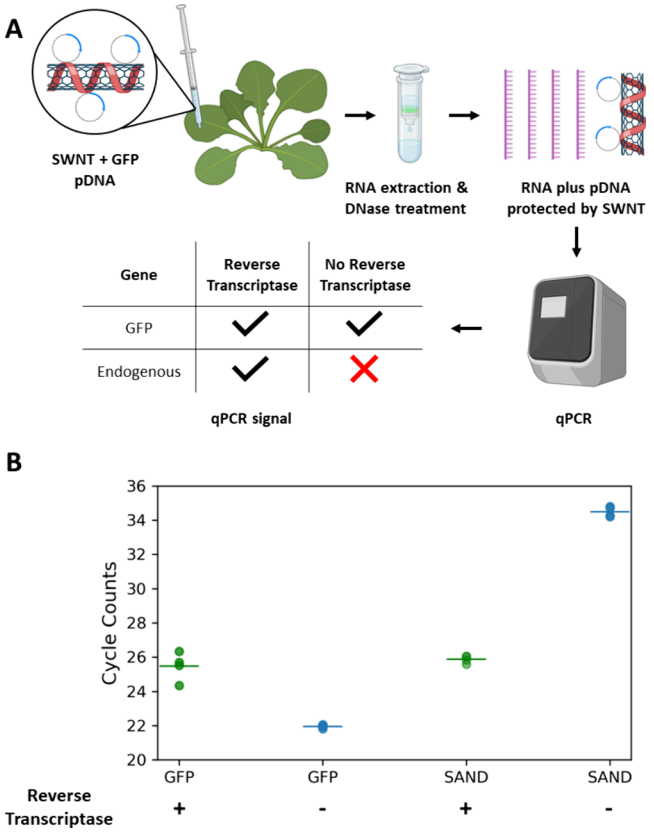

Figure 2. Nanomaterial delivery tools protect pDNA, potentially resulting in a false-positive RT-qPCR signal. (A) Schematic of experimental design. Single-walled carbon nanotubes (SWNTs) are functionalized with polyethylenimine (PEI) and loaded with plasmid DNA (pDNA) coding for GFP. PEI-SWNTs loaded with GFP pDNA are infiltrated into the leaves of wild-type *Arabidopsis thaliana*. Total mRNA is extracted from plant leaves, including a DNase treatment step to eliminate contaminating DNA. However, DNase treatment is ineffective against GFP pDNA due to the protective effect of the PEI-SWNT delivery tool; resultantly, pDNA contaminates the extracted mRNA. Samples are amplified via qPCR either with or without reverse transcriptase. In reactions with reverse transcriptase, both GFP gene expression and endogenous gene expression (SAND) are detected. In the control no reverse transcriptase control (NRTC) sample, GFP qPCR signal is detected, but SAND qPCR signal is not detected. The NRTC suggests that GFP pDNA contamination is present, interfering with quantification of cDNA originating from GFP mRNA. (B) Cycle count plot for qPCR experiments conducted for GFP (delivered via PEI-SWNT loaded with pDNA) and SAND (endogenous *Arabidopsis thaliana* gene). Samples that were reverse-transcribed and not reverse-transcribed are indicated with + and −, respectively; samples marked with − represent NRTCs. DNA contamination, likely from protected pDNA, can be clearly observed in the GFP qPCR sample lacking reverse transcriptase. Bars represent the average of four biological replicates. Each point represents an average of three technical replicates. See the SI for experimental details.

The use of an NRTC is standard practice in molecular biology, yet it is absent in many plant delivery publications. Other controls should also be run and reported per the well-established standards of RT-qPCR.[25] Primer efficiency should be determined and reported. The specificity of the RT-qPCR reaction should be tested and reported with melting curves and agarose gels. Well-established stable housekeeping genes should be used when evaluating fold change in mRNA transcripts. Finally, raw cycle counts should be reported. Improper use of RT-qPCR is common given the ease with which inexperienced users may conduct experiments with computationally designed primers, premade master mixes, and preprogrammed plate readers. Careful design, preparation, and execution is necessary for RT-qPCR assays.[25]

Experimental design can also simplify RT-qPCR experiments. For pDNA delivery experiments, the plasmid cargoes can be designed to contain introns. Designing RT-qPCR primers which span the exon−intron gap and thus amplify cDNA from mature mRNA but not pre-mRNA or the DNA template can reduce the chance of false-positive amplification of pDNA. As an aid to the field, we built three pDNA constructs containing introns that could be used in delivery experiments: two fluorescent proteins (GFP and mCherry) as well as NanoLuciferase. These plasmids are deposited on AddGene.

INTEGRATION WITH THE PLANT BIOLOGY COMMUNITY

In clinical applications, nanomaterial delivery tools serve as the basis for vaccines and therapeutics.[2] However, nanomaterial delivery tools have seen limited adoption by the plant biology community thus far. Most publications reporting a new nanomaterial delivery tool for plants highlight the limitations of agrobacteria and biolistic methods to motivate new tools. In light of the purported limitations of the current methods, why are new tools not widely adopted? We identify a few reasons. First, the plant delivery field is young, and new technologies are slow to be adopted; limited accessibility to nanomaterials and complex syntheses might also limit adoption. Second, lack of standards in the field and the relatively interdisciplinary nature of the work have resulted in missteps, improper use of techniques (such as RT-qPCR), and controversy[26] limiting confidence in the field. Finally, the lack of communication and integration with the broad plant biology community also limits nanomaterial delivery tool adoption. This disjunction results in misperceptions about the challenges that plant biologists face. For example, despite publications that report a new nanomaterial delivery tool frequently describing agrobacterium as a highly limited technique, plant biologists widely utilize and continue to improve this technique. This is highlighted by recent reports of improvements in agrobacterium efficiency in recalcitrant monocots using agrobacterium[27] or a combination of agrobacterium and viral delivery.[28] Separately, nanomaterial delivery tools are not typically tested in plant tissues or species that are of most interest to plant biologists, such as immature

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embryos and other germline tissues, but instead are typically tested in the somatic leaf cells of model plants. While initial tool development might reasonably take place in model species, we recommend that follow-up work should focus on more relevant tissues and species. Engagement and integration of nanotechnology scientists with the broad plant biology community will ensure that work on nanomaterial delivery tools attempts to solve the most challenging problems. We encourage researchers to actively seek out and work with potential end users throughout the nanomaterial delivery tool development process, from idea conception to *in planta* use.

SUMMARY OF RECOMMENDATIONS

Broad development of high-efficiency nanomaterial tools for delivering biomolecules to plants could improve plant biotechnology. Achieving this aim requires continued effort; given the recent growth of the field, we expect continued research, publications, and interest in the space. To aid the advancement of the field, we encourage researchers to consider the recommendations provided in this Perspective. The primary recommendations are briefly reiterated below:

1. Design experiments with multiple independent methods of validating delivery results at the DNA, RNA, and protein level
2. Design experiments with controls including free cargo, unloaded nanomaterial delivery tools, and nanomaterial delivery tools loaded with scrambled nonfunctional cargoes
3. Design experiments with multiple time-separated biological replicates and report the criteria used to define biological replicates
4. Consider the physical resolution limits of confocal laser scanning microscopy and avoid cytosolic colocalization experiments
5. Inspect object shape when utilizing confocal laser scanning microscopy and report relevant experimental parameters such as laser power and gain
6. Consider replacing fluorescent protein assays with time course luciferase assays
7. Rely on RT-qPCR for mRNA quantification and rigorously treat mRNA samples with DNase
8. Always perform and report RT-qPCR controls, including a no reverse transcriptase control
9. Design RT-qPCR primers to span exon−intron gaps to ensure that mature mRNA is quantified
10. Coordinate with the plant biology community when considering the development and application of new nanomaterial delivery tools
11. Consider testing nanomaterial delivery tools in non-model species and tissue types

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsnano.4c12116.

Plant growth conditions, confocal laser scanning microscopy methods, PEI-SWNT preparation, and RT-qPCR methods (PDF)

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Notes

The authors declare no competing financial interest.

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