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Review

Nanotechnology: A New Opportunity in Plant Sciences

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The agronomic application of nanotechnology in plants (phytonanotechnology) has the potential to alter conventional plant production systems, allowing for the controlled release of agrochemicals (e.g., fertilizers, pesticides, and herbicides) and target-specific delivery of biomolecules (e.g., nucleotides, proteins, and activators). An improved understanding of the interactions between nanoparticles (NPs) and plant responses, including their uptake, localization, and activity, could revolutionize crop production through increased disease resistance, nutrient utilization, and crop yield. Herewith, we review potential applications of phytonanotechnology and the key processes involved in the delivery of NPs to plants. To ensure both the safe use and social acceptance of phytonanotechnology, the adverse effects, including the risks associated with the transfer of NPs through the food chain, are discussed.

Trends

The field of nanotechnology has great potential within plant sciences and plant production systems.

The agronomic application of nanotechnology has thus far received comparatively little interest relative to the application within human systems.

We review the potential applications and future opportunities of nanotechnology in plant sciences, thereby assisting in bridging the divide between human and agricultural nanotechnology.

The application of nanotechnology in plant sciences will benefit from the development of improved analytical techniques that enable the *in situ* analysis of NPs *in planta* with a low detection limit and high lateral resolution.

Regardless of the benefits of nanotechnology for plant sciences, the principle of 'safety-by-design' must be heeded to address community concerns about the potential adverse effects of novel engineered nanoparticles (ENPs) on ecological systems.

Nanotechnology and Plants

Engineered NPs (ENPs, see Glossary) have unique physicochemical properties (e.g., small surface area, atypical surface structure, enhanced reactivity, etc.) that differ distinctively from those of their molecular and bulk counterparts. These properties typically result from the small size, chemical composition, surface structure, stability, shape, and agglomeration of the **nanoparticles** (NPs) [1]. Given these unique properties, ENPs are used increasingly in a range of consumer and commercial products, including semiconductors, microelectronics, catalysts, everyday domestic products (e.g., cosmetics and sunscreens), and for drug delivery.

Numerous applications in nanomedicine and nanopharmacology have developed ENPs as smart delivery systems. Specifically, ENPs can be loaded with a drug or other active substance (Figure 1A,B) for delivery to target-specific sites within a living organism. Considerable research efforts have been made to investigate the potential applications of ENPs within human systems, including for targeted drug delivery, cancer therapy, and treatment for loss-of-function genetic diseases [2]. Although the same principles can be applied to plant systems, the application of **nanotechnology** in plant sciences and plant production systems (**phytonanotechnology**) has received comparatively little interest thus far.

Phytonanotechnology could assist the development of 'smart' crops. Nanoscale materials can provide time-controlled, target-specific, programmed, self-regulated, and multifunctional capabilities [3]. For example, ENPs can deliver agrochemicals (e.g., fertilizers, pesticides, and herbicides) in an 'on-demand' manner, either for nutritional demand or protection against pests and pathogens. This provides an efficient way to avoid repeated applications of conventional agrochemicals and reduces adverse effects on plants and the environment. Additionally, the NP-mediated targeted delivery of nucleotides, proteins, and other phytoactive

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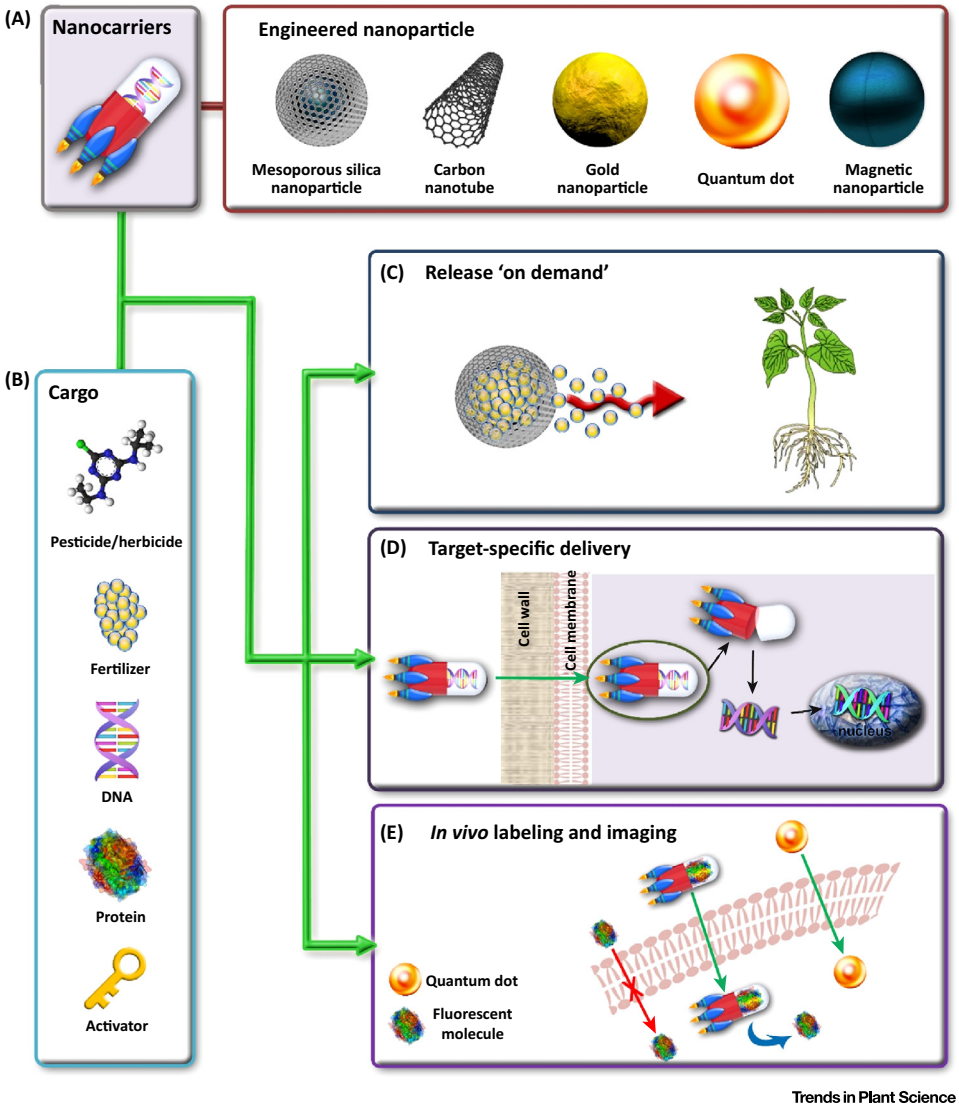

Figure 1. Schematic Representation of the Applications of Nanotechnology in Plant Sciences. (A) Engineering nanoparticles used as nanocarriers for delivery of various exogenous cargos (B), including agrochemicals and bioactive molecules. (C) Nanocarrier-mediated release of agrochemicals in a controlled manner. (D) Nanocarrier-mediated delivery of bioactive molecules into plant cells. (E) Nanocarrier-mediated intracellular fluorescent agents (e.g., quantum dots or fluorescent protein) delivery for intracellular labeling and imaging.

molecules has the potential for genetic modification and regulation of plant metabolism. However, despite all the benefits, the principle of safety-by-design must be heeded to address community concerns (e.g., use of ENPs in consumer products) about the potential adverse effects of novel ENPs on ecological systems [4].

Here, we review the applications of phytonanotechnology, and discuss the uptake, translocation, activity, and risks associated with the use of ENPs in this field. Given that the physiochemical properties of ENPs are key in understanding their interactions with plants, we also briefly review advanced analytical techniques used for their detection, quantification, and characterization both *in vivo* and *in vitro*.

Glossary

Carbon nanotubes (CNTs): allotropes of carbon that have a cylindrical nanostructure with diameters ranging from <1 nm to 50 nm. They are categorized as either single-walled nanotubes (SWNTs) or multi-walled nanotubes (MWNTs).
Engineered NPs (ENPs): nanomaterials that are intentionally produced and designed with specific properties related to their shape, size, surface properties, and chemistry.
Magnetic NPs: NPs that contain magnetic materials of elements such as Fe, Ni, Co, and their chemical compounds. This type of NP can be manipulated for targeted delivery using magnetic field gradients.
Mesoporous silica NPs (MSNs): NPs that comprise a honeycomb-like porous structure with tunable pore size and tunable outer particle diameter in the nanometer range. This type of NP has hundreds of empty channels that are capable of encapsulating or absorbing large amounts of agrochemicals or bioactive molecules.
Nano quantitative structure-activity relation (nano-QSAR): a model used to predict biological responses based upon the physicochemical properties of NPs.
Nanofertilizers: fertilizers contained within nanostructured formulations that can be delivered to targeted sites to allow release of active ingredients. This release can be in response to environmental triggers or plant demands.
Nanoparticles (NPs): materials that have external dimensions or internal surface structures with two or three dimensions between 1 and 100 nm.
Nanotechnology: refers to the science, engineering, and technology of controlling, building, and restricting materials and devices at the nanoscale.
Phytonanotechnology: the application of nanotechnology in the plant sciences and plant production systems.
Quantum dots (QDs): tiny particles or nanocrystals of a semiconductor material with diameters ranging from 2 to 10 nm. This type of NP can produce a distinctive fluorescence that can be used for subcellular labeling and imaging.
Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS): a type of time-resolved

Applications of Phytonanotechnology

Nanoparticles for the Controlled and Targeted Release of Agrochemicals

Nanotechnology enables target-specific delivery in a controlled manner, which can: (i) reduce applications of plant-protection products; (ii) decrease nutrient losses from fertilizers; and (iii) increase yields through optimized nutrient management. The ENPs can be designed as 'magic bullets' loaded with agrochemicals to deliver to specific tissues to release the agrochemicals in controlled manner (Figure 1A–D). It is possible to design this system to allow: (i) controlled release 'on demand' or 'on command'; (ii) target-specific delivery; (iii) reduced phytotoxicity; and (iv) use of optimized concentrations. For instance, it is possible to design NPs to protect agrochemicals from damage by external agents. **Mesoporous silica NPs** (MSNs) are designed to load pesticide (e.g., avermectin) into the inner core; this protects the pesticide from photodegradation, while also allowing for sustained release [5]. Similarly, MSNs have been functionalized to provide protection for plants against insects via physioabsorption into the cuticular lipids of insects, resulting in damage to the protective wax and subsequent death by dehydration [6]. NP-based herbicides have been used to control parasitic weeds at lower doses, thereby potentially avoiding adverse effects on the crops [7]. Furthermore, the release of active ingredients can be preprogramed or trigged by certain conditions within the parasitic weed or changes in external conditions.

The use of NP-based fertilizers (**nanofertilizers**) has been proposed to enhance nutrient-use efficiency (Figure 1C). Ideally, nutrients from nanofertilizers should be released in accordance with plant demands. This timely release also prevents the premature conversion of nutrients to chemical and/or gaseous forms that are unavailable to plants (e.g., volatilization of NH_3 from urea) [8]. The loading of urease enzymes or urease inhibitors into MSNs results in an ability to control the release of nitrogen from urea via hydrolysis [9]. More recently, apatite NPs have been used as a novel type of phosphorus (P) fertilizer with the sustained low release of P, as required by plants, which also decreases the risk of water eutrophication, a good compromise between agricultural benefits and environmental risks [10].

Nanoparticles as Delivery Vehicles of Bioactive Molecules

ENPs offer a new vector for the delivery of bioactive molecules (including nucleotides, proteins, and activators; Figure 1D). The use of MSNs to deliver DNA and its activator into isolated plant cells and intact leaves of tobacco is of particular interest [11]. A honeycomb-like MSN system with 3-nm pores was loaded with a gene (i.e., a plasmid containing a *GFP* gene) and its chemical inducer, with the ends of the MSNs capped with gold (Au) NPs. Once inside cells, an uncapping trigger was used to cleave the bonds attaching the Au NPs to the MSN, resulting in the release of the biomolecules, triggering gene expression. Proteins or enzymes can also be delivered by MSNs in plants, enabling a transient presence of the protein or enzyme that can be useful for biochemical analysis and genome modifications [12]. This method avoids DNA (transgene) integration into the genome and, thus, the transfer of the modified traits to future generations. Further to MSNs [12,13], single- or multi-walled **carbon nanotubes** (SWNTs or MWNTs) [14,15], Au NPs [16,17], magnetic virus-like NPs (VNPs) [18], carbon-coated **magnetic NPs** [19,20], **quantum dots** (QDs) [21], and starch NPs [22] have been with plants for this purpose. Indeed, NP-mediated genome editing has been used successfully to deliver plasmid containing a *GFP* gene [11] and the functional recombinase [12] into plant tissues, leading to successful genome editing. Compared with conventional delivery methodologies, this NP-mediated approach provides several benefits. First, it is easily operated and highly efficient. For example, the minimum amount of DNA required for detection of expression was 1000 times lower than that required for the conventional methods [11]. Second, it enables the transient DNA-free genome editing of plants in a controlled fashion (including gene silencing) and the generation of precisely modified 'nontransgenic' plants, which differs from conventional genetic engineering methods. Third, it is able to co-deliver more than one biomolecule simultaneously to the target,

ICP-MS technique that enables analyses of a single particle, particle size distribution, particle number, and compositions in both the external media and biological extracts.

Size exclusion limit (SEL): the size of the largest particle that can pass through a pore.

Synchrotron-based scanning transmission X-ray microscopy (STXM): a type of X-ray microscopy that allows mapping of elements at high lateral resolution within a specimen.

Synchrotron-based X-ray fluorescence microscopy (XFM): a mapping technique based upon the detection of the fluorescence emitted by relaxing atoms from an X-ray beam.

for example DNA and its activator [11], DNA and proteins [23], and different genes. Fourth, these ENPs can be easily tailored through surface functionalization with biological recognition molecules for specific, targeted delivery. Finally, unlike conventional methods, ENPs enable local translocation to individual cells, organs, or tissues.

ENPs also offer a new approach for intracellular labeling and imaging, both *in vitro* and *in vivo* (Figure 1E). NP-carried imaging agents (e.g., fluorescein isothiocyanate [14,15,24] and GFP [23]) are capable of circumventing biological barriers (e.g., cell walls and plasma membranes), resulting in the simultaneous and efficient localization of these agents at target sites. Using NP-carried imaging agents, we can also observe the movement of exterior genes along the expression of transferred genes by integrating these exterior genes on contrast or fluorescent ENPs. Significant progress has been made in biology with the application of fluorescent QD bioconjugates [25,26]. Notably, QDs can be excited by a single light source, providing distinct advantages over current fluorescent markers (e.g., organic dyes and fluorescent proteins), the latter having broad absorption and emission profiles and low photobleaching thresholds [25]. Different coating properties have been used for QDs to enable the *in vitro* imaging of solute uptake through fluid phase endocytosis by sycamore-cultured cells [21] and for *in vivo* imaging to trace the uptake and translocation of NPs by intact arabidopsis (*Arabidopsis thaliana*) [27,28] and the subsequent transfer in the food chain [28].

Uptake and Translocation of Nanoparticles in Plants

Evidence of Uptake and Translocation

It is important to understand whether intact NPs can be taken up by plants and transported to other plant tissues. Only a few studies have reported 'direct' uptake, translocation, and localization of ENPs in plants (Table 1). These studies utilized various insoluble ENPs, including MSNs, silica NPs (SNPs), CNTs, fullerene (C_{70}), QDs, Au-NPs, titanium dioxide NPs (TiO_2 NPs), iron (II,III) oxide (Fe_3O_4) NPs, and VNPs. These ENPs were delivered to intact plants, dissected plant organs, or protoplasts. Soluble ENPs, such as silver (Ag) NPs, copper oxide (CuO) NPs, zinc oxide (ZnO) NPs, and, to a lesser extent, cerium (IV) oxide (CeO_2) NPs and ytterbium (III)) oxide (YbO_3) NPs, have also been investigated, but in most cases it is unclear whether the NPs or their dissolution products (with subsequent reformation of NPs *in planta*) were taken up.

Researchers have shown that magnetic Fe_3O_4 NPs are directly taken up and translocated in pumpkin (*Cucurbita maxima*) with no toxic effects even at a NP concentration of 0.5 g/l [29]. Appling a magnetic field gradient, the magnetic NPs (<50 nm) could be positioned within the desired plant tissues [19], thereby suggesting that magnetic NPs could be used as a capping agent (e.g., gatekeeper) for porous NPs (e.g., MSNs) with uncapping by an external magnetic field at the target. Fullerene C_{70} has been reported to be translocated both upwards and downwards through the vascular system of rice (*Oryza sativa*) and can be transferred to the next generation through seeds [30]. The uptake and translocation of NPs has also been shown for MSNs [11–13,17,23] and CNTs [14,15,24].

Entry and Barriers for Nanoparticle Uptake and Transport in Plants

ENPs can enter plant tissues through either the root tissues or the aboveground organs and tissues (e.g., cuticles, trichomes, stomata, stigma, and hydathodes), including through wounds and root junctions (Figure 2A). For uptake and translocation, ENPs must traverse a series of chemical and physiological barriers, which control the **size exclusion limits** (SELs, Figure 2B). For the apoplastic transport pathway, movement is restricted by the SEL of cell walls (5–20 nm) [31–33]. The Casparian strip, with a SEL of <1 nm [34], provides a barrier to movement into the vascular system. From the cell wall, ENPs can be internalized into the cells through endocytosis [21,35]. Subsequent symplastic transport then depends upon the SEL of the plasmodesmata, typically 3–50 nm in diameter [32,36], with particles up to this size able to enter [33,37]. Although

Table 1. Studies that Demonstrated the Direct Uptake of NPs by Plants[a]

NP	Size	Coating	Plant	Remarks	Refs
MSNs	Sphere 20 nm	Fluorescein isothiocyanate, aminopropyl triethoxysilane	*Arabidopsis thaliana*	MSN translocation through entire leaf	[13]
Silica NPs	Sphere 14–200 nm	Bare	*A. thaliana*	Size-dependent uptake by roots; no toxicity even at doses up to 1000 mg l^{-1}	[64]
CNTs	Tube D × L: ~4 nm × ~1 μm	Fluorescein isothiocyanate	*Catharanthus roseus*	Short CNTs penetrated various subcellular membranes and targeted specific cellular substructures	[15,24]
	Tube L: <500 nm	Fluorescein isothiocyanate	Bright Yellow cells	SWCNTs traversed cell walls and delivered fluorescein and DNA into cell organelles	[14]
Fullerene (C_{70})	Sphere 1.2 nm	Natural organic matter	*Oryza sativa*	C_{70} taken up by plants and accumulated C_{70} transferred to progeny through seeds	[30]
QDs	Sphere 18–53 nm	Poly(acrylic acid-ethylene glycol), polyethylenimine, poly (maleic anhydride-alt-1-octadecene)-poly (ethylene glycol)	*A. thaliana*	QDs with different coatings taken up by plants, with amount of translocation being coating dependent	[28]
Au-NPs	Sphere 6–10 nm	Thiolated poly-(ethylene glycol), thioalkylated oligo (ethylene glycol)	*O. sativa*	Surface charge-dependent accumulation of Au NPs detected in shoots	[71]
	Sphere 40 nm	Citrate	*Solanum lycopersicum*	Translocation of Au NPs from roots into shoots detected, with major size of intercellular Au NPs being 40 nm	[93]
	Sphere 15–50 nm	Bare	*Populus deltoids* x *nigra*	Au NPs observed in cytoplasm and various organelles of root and leaf cells	[37]
TiO_2 NPs	Sphere 2.8 nm	Alizarin red S	*A. thaliana*	Ultrasmall TiO_2 easily penetrated cell walls and accumulated in specific subcellular sites	[39]
	Sphere 14–655 nm	Bare	*Triticum aestivum*	Size-dependent translocation observed, with only NPs <36 nm translocated into leaves	[44]
Fe_3O_4 NPs	Sphere 20 nm	Bare	*Cucurbita maxima*	Uptake and translocation of magnetic NPs detected throughout whole plant	[29]
	Sphere 5–200 nm	Carbon	*Cucurbita pepo*	Penetration and translocation of magnetic NPs in whole living plants, with a maximum size of 50 nm observed inside	[19,20]
VNPs	18.6 nm	Carboxyl-terminated PEGylated phospholipids	*Nicotiana benthamiana*	VNPs penetrated plant tissues, with long-distance transfer through vasculature	[18]

[a]Uptake was termed 'direct' if insoluble ENPs were directly determined or visualized within the cytoplasm, vasculature, or after long distance translocation.

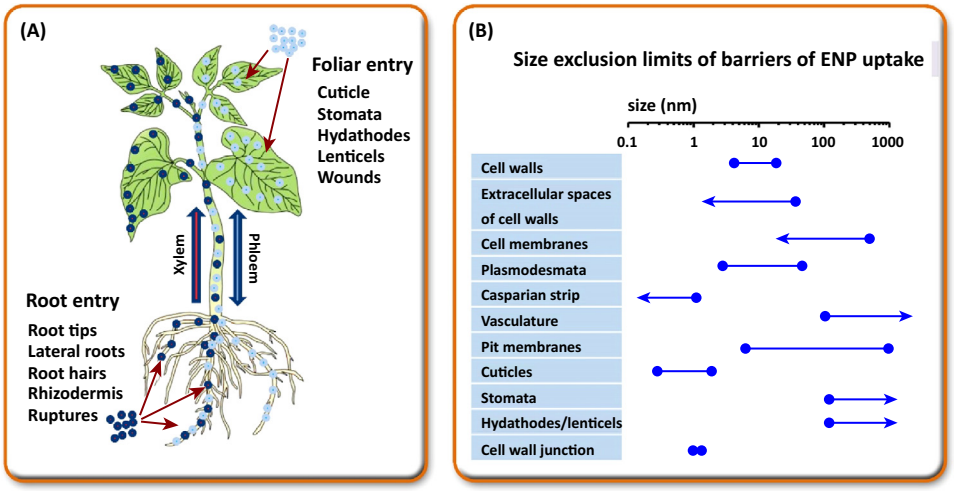

Figure 2. Entry Mode and Size Exclusion Limits of a Series of Barriers for the Uptake and Transport of Nanoparticles in Plants. Abbreviation: ENP, engineered nanoparticle.

ENPs have been detected both in the apoplast and symplast, it remains unclear which pathway is more important.

Although the uptake and translocation of ENPs depends upon the SELs, it has been reported that ENPs up to 36–50 nm have been internalized and translocated (Table 1), this being larger than the SELs of cell walls, plasmodesmata, and the Casparian strip (Figure 2B). One possible explanation is that SELs appear to be dynamic and influenced by calcium, silicon, proteins, virus, environmental stresses, as well as by ENPs themselves [33,38]. Indeed, ENPs such as Ag NPs, TiO_2 NPs, and ZnO NPs, have been reported to induce the formation of new and larger pores in cell walls [39–42] and cuticles [43], and cause structural changes (e.g., ruptures and disruption of microfilaments) [44,45], which in turn facilitate the internalization of larger NPs. ENPs can also enter the vascular system at the root tip meristem, where the Casparian strip has not yet fully formed, or the sites of lateral root formation, where the Casparian strip is broken [46].

Nanotoxicology in Plants

Beneficial and Toxic Effects

The increasing use of ENPs led to a public debate about their potential adverse effects in ecosystems. Over the past decade, studies have shown contradictory results regarding the effects of ENPs in plants [32,33,47,48] (Figure 3A). One cause of these apparent discrepancies is that different toxicity endpoints have been used, ranging from easily scored parameters (e.g., seed germination and seedling growth), to cytotoxicity [e.g., enzyme activities, pollen development, photosynthetic system, reactive oxygen species (ROS), etc.] and genotoxicity (e.g., nucleic acid integrity and expression). Furthermore, it is apparent that plant species differ in their response to ENPs. For example, while it has been shown that CNTs facilitated germination by penetrating the seed coat, which then increased water uptake in seeds of tomato (*Solanum lycopersicum*) [8] and rice [3], they had deleterious effects on root elongation in tomato and lettuce (*Lactuca sativa*) [49]. Similarly, TiO_2 NPs have been reported to enhance photosynthesis capacity [50] and nitrogen metabolism [51], but they also caused antioxidant stress [52].

Several methodological problems commonly encountered in plant nanotoxicology studies should also be recognized [53]. First, some studies use unrealistically high concentrations of ENPs (e.g., up to 10 g/l). Second, many studies use ENPs in their pristine form rather than

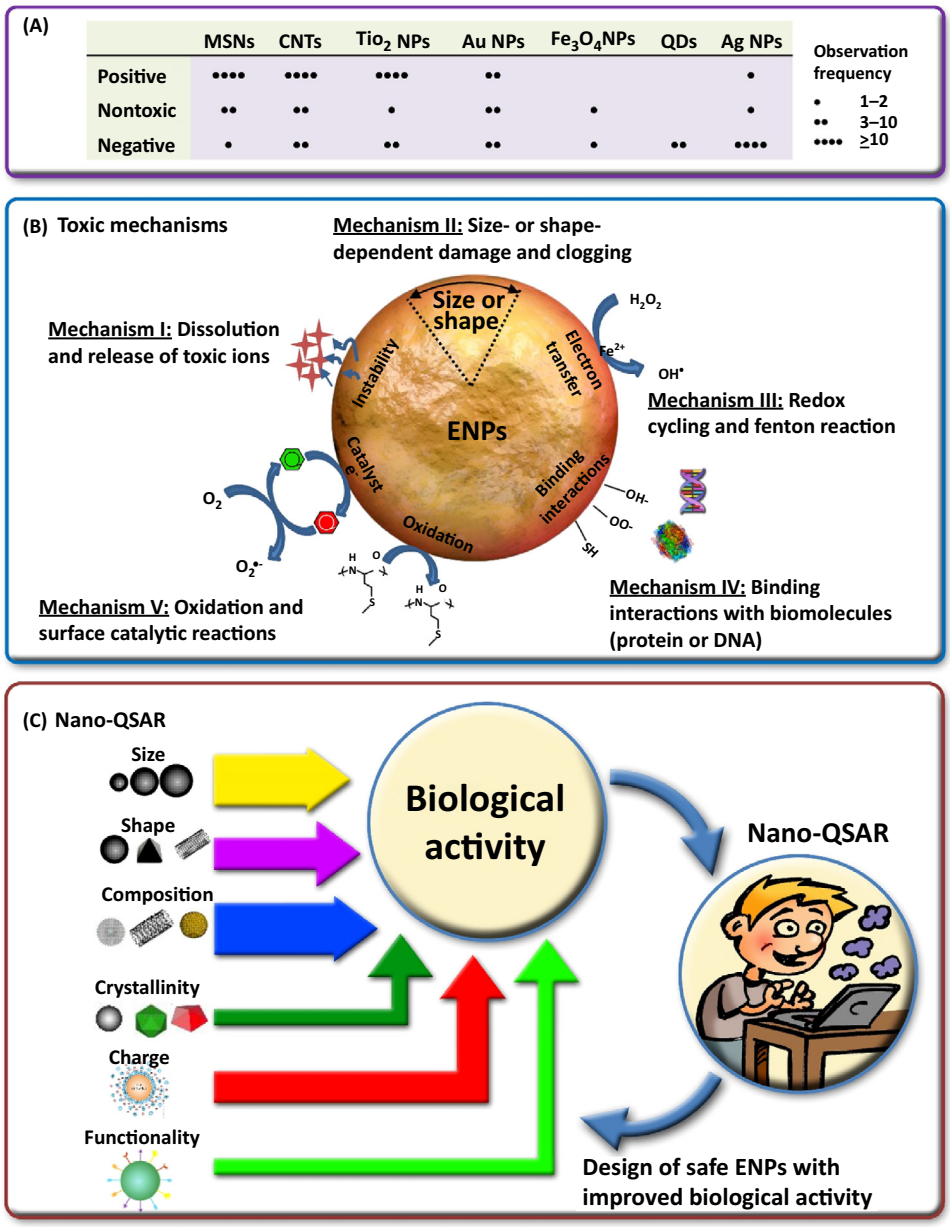

Figure 3. Nanotoxicology in Plants. (A) Observed effects of engineered nanoparticles (ENPs) on plants. (B) Several proposed mechanisms by which ENPs cause phytotoxicity. (C) Conceptual framework of a nano quantitative structure–activity relation (nano-QSAR), including key parameters of ENPs that have important impacts on plant–NP interactions. For definitions of abbreviations, please see the main text.

realistic forms of NPs to which plants would in fact be exposed. Finally, some studies have largely ignored the requirement for appropriate control treatments (e.g., the use of an ionic Zn^{2+} treatment to compare against the effects of ZnO NPs).

Trophic Transfer of Nanoparticles in the Food Chain

Given that plants are the basis of terrestrial food chains, some studies have investigated the terrestrial trophic transfer of Au NPs [54,55], CeO_2 NPs [56], and lanthanum oxide (La_2O_3) NPs [57]. For example, in a simulated terrestrial food chain, Au NPs were transferred from tobacco

(*Nicotiana tabacum*) and tomato (*Lycopersicon esulentum*) to a primary consumer, tobacco hornworm (*Manduca sexta*) [54]. Similarly, the trophic transfer of CeO_2 NPs and La_2O_3 NPs was also noted at higher-level trophic organisms fed with leaves of plants grown in NP-amended soils [56,57]. Although these studies did not find biomagnification of ENPs, they clearly demonstrated the trophic transfer of ENPs. Therefore, human exposure to ENPs via food dietary uptake or food chain contamination needs to be considered when phytonanotechnologies are developed.

Phytotoxicity and Influencing Factors

ENPs can cause toxicity via: (i) the dissolution and release of toxic ions, such as Ag^+, Zn^{2+}, and Cu^{2+}; (ii) size- or shape-dependent mechanical damage and clogging [58]; (iii) the production of excess ROS through redox cycling (e.g., redox instability of the CeO_2 NP surface) and the Fe^{2+}-mediated Fenton reaction [59,60]; (iv) binding interactions that release surface free energy, leading to surface reconstruction of biomolecular structures [59,61,62]; and (v) oxidation of biomolecules through catalytic reactions [60] (Figure 3B).

The nature of the interactions between plants and NPs depends upon the intrinsic properties of ENPs, including their size, chemical composition, shape and angle of curvature, crystal structure, surface roughness, and hydrophobicity or hydrophilicity [1,61,63]. From a toxicological perspective, particle size and surface area are critical: a decrease in particle size increases the surface area and the proportion of the atoms or molecules of the particle on the surface layer [1]. These size-dependent features influence the interfacial reactivity and the ability to traverse physiological barriers. Several studies have shown that the uptake and phytotoxicity of ENPs is dependent upon particle size, with smaller particles generally accumulating to higher levels and being more toxic compared with their bulk particles [44,64,65]. However, it is not yet clear whether this variation in toxicity results from changes in the size-dependent specific surface area. For instance, for a given mass, 12.5-nm SiO_2 NPs were more toxic to *Pseudokirchneriella subcapitata* than 27.0 nm NPs, but once normalized by the surface area, no significant difference existed [66]. It has also been reported that crystal structure and shape influence NP toxicity and uptake. Anatase TiO_2 NPs are more biologically active than rutile TiO_2; anatase NPs induce cell necrosis and membrane leakage, while rutile NPs initiate apoptosis through the formation of ROS [67]. ZnO nanopyramids showed the strongest inhibition of a typical enzyme β-galactosidase compared with ZnO nanoplates and spheres [68].

Not only do the intrinsic properties of ENPs influence their interactions with plants, but their extrinsic properties are also of importance, including surface charge (zeta potential) and coating, stability characteristics (e.g., dissolution), particle aggregation, and valence of the surface layer. For instance, the uptake by, and toxicity of, soluble NPs can result either directly from the NPs themselves or from their dissolution and release of soluble ions. Indeed, some studies have demonstrated direct nano-specific effects for Ag NPs and CuO NPs [40], while no nano-specific effects on plants were observed when ZnO NPs were applied to soils [69]. It has been shown that positively charged surfaces are more likely to induce endocytosis across cell membranes [38,70], whereas particles with negatively charged surfaces are more likely to be translocated through vascular tissues [71,72]. Therefore, the behavior of ENPs can be altered by modifying the surface coating, such as coating MSNs with triethylene glycol to facilitate penetration into plant cells [11]. Moreover, ENP coatings are an effective means of reducing the dissolution and release of toxic ions [73]. These extrinsic properties of ENPs are also influenced by the characteristics of the suspending media [63], including the ionic strength, pH, and the presence of organic molecules or surfactants.

The use of nanomaterials in agriculture should be dictated by a safety-by-design principle and should be guided by plant physiology, NP functionalization, and nanomedicine-inspired nanodelivery systems to efficiently supply nutrients, pesticides, and bioactive molecules to crops with minimizing adverse effects on plants and environment.

Nanoquantitative Structure–Activity Relations

Probing the various interactions between NP properties and biological systems enables the development of **nano quantitative structure-activity relations** (nano-QSARs) (Figure 3C). This method has been applied to predict the cytotoxicity of various metal oxides using *in vitro* cell-based assays [74–76] and the adsorption of various molecules onto NPs [77]. A enthalpy descriptor (ΔH_{Me} +) [74] or a biological surface adsorption index (log k_i) [77] was proposed as a comprehensive nanodescriptor (which quantifies the chemical stability, surface activity, molecular interactions of the NPs at the nanobiological interface, etc.) to predict cytotoxicity. Moreover, some studies used computational chemistry to simulate the translocation of NPs, including nanotubes with different shapes across a phospholipid biolayer [78,79]. These studies are of particular importance in providing guidance for the design of functionally desirable and safe NPs that are environmentally benign.

Detection and Characterization of Nanoparticles

Research into phytonanotechnology will benefit from the development of improved analytical methodologies. Current techniques (Table 2) can be divided into those that: (i) detect intrinsic or extrinsic NP properties in suspending media or following acid/enzyme-mediated digestion media of plant tissues; and (ii) permit the *in situ* analysis of NPs *in planta*. The advantages and limitations of these fit-for-purpose techniques have been reviewed previously [80–82].

The recent development of transmission electron microscopy (TEM) coupled with electron transparent windows enables imaging, with high lateral resolution (e.g., 4 nm), of a single particle in living cells in liquid [83]. This could be used to trace the dynamics of individual NPs in a living cell or plant tissues. For example, total internal reflection fluorescence microscopy with high spatial resolution has been used to observe the endocytosis process of La(III) particles in plant cells [84] (Figure 4A). Dynamic light scattering (DLS) and ultraviolet-visible spectroscopy (UV-Vis) are not able to characterize NPs in a biological media due to interference from nontargeted particles, but they provide an easy way to characterize particle size distribution (associated with the agglomeration behavior) [85] and band gap energy (associated with electron transfer) [76], both of which have a large impact on oxidative stress and subsequent cytotoxicity.

Synchrotron-based **X-ray fluorescence microscopy** (XFM) provides a unique capability for examining the *in situ* localization (e.g., 2D and 3D distribution) of elements in biological specimens (reviewed in [80,82,86]). Currently, this technique has limited resolving power (submicrometer), which makes analysis of individual NPs challenging. With the development of improved X-ray optics, nanometer-scale resolution will soon be available, enabling the detection of single NPs. Synchrotron-based **scanning transmission X-ray microscopy** (STXM) at a lateral resolution of 60 nm was used to examine the 3D distribution of Ag NPs inside a human monocyte [87] (Figure 4B). Synchrotron-based X-ray absorption spectroscopy (XAS) (including μ-XANES) can be used for the *in situ* assessment of chemical speciation (i.e., transformation) within cells and plant tissues [45,87] (Figure 4C).

Nano secondary ion mass spectrometry (Nano-SIMS) uses an energetic primary ion beam to remove particles from the top few atomic layers of a sample surface to achieve elemental distribution with a low detection limit (mg/kg range) and high lateral resolution (down to 50 nm). It is capable of measuring most elements in the periodic table. Nano-SIMS has been used for the analyses of NPs in animal cells or aquatic organisms [88–90] and should also be applicable to plant studies.

Although inductively coupled plasma mass spectrometry (ICP-MS) is not laterally resolved, it offers an exceptional detection limit (ng/l range) for a range of elements. Hyphenation of field flow

Table 2. Analytical Techniques and the NP Parameters that They Are Capable of Detecting

Technique	Concentration	Composition/ Speciation	Size or Distribution	Shape	Surface Chemistry	Distribution	*In vitro*	*In vivo*	Detection Limit	Lateral Resolution[a]
Scanning/transmission electron microscopy (SEM/TEM)	×	×	✓	✓	✓	✓	✓	✓	mg/kg	nm
Atomic force microscopy (AFM)	×	×	✓	✓	✓	✓	✓	✓	mg/kg	nm
Dynamic light scattering (DLS)	×	×	✓	✓	×	×	✓	×	mg/l	n.r.
Ultraviolet-visible spectroscopy (UV-Vis)	✓	✓	×	×	×	×	✓	×	mg/l	n.r.
Inductively coupled plasma mass spectrometry (ICP-MS)	✓	✓	×	×	×	×	✓	×	ng/l	n.r.
Single-particle ICP-MS (SP-ICP-MS)	✓	×	✓	×	×	×	✓	×	ng/l	n.r.
Flow field flow fractionation (FFF)-ICP-MS	✓	✓	×	✓	×	×	✓	×	ng/l	n.r.
Laser ablation ICP-MS (LA-ICP-MS)	✓	✓	×	×	×	✓	✓	×	ng/l	µm
X-ray fluorescence microscopy (µ-XRF)	×	×	×	×	×	✓	✓	✓	mg/kg	nm–µm depth
X-ray absorption spectroscopy (XAS)	×	✓	×	×	×	×	✓	✓	mg/kg	µm depth.
Transmission X-ray microscopy (TXM)	×	✓	×	×	×	✓	✓	✓	mg/kg	10 nm
Nano secondary ion mass spectrometry (nano-SIMS)	×	×	×	×	×	✓	✓	✓	mg/kg	50 nm
Hyperspectral microscopy	×	×	×	×	×	✓	✓	✓	µg/kg	nm

[a]n.r., no lateral resolution ability.

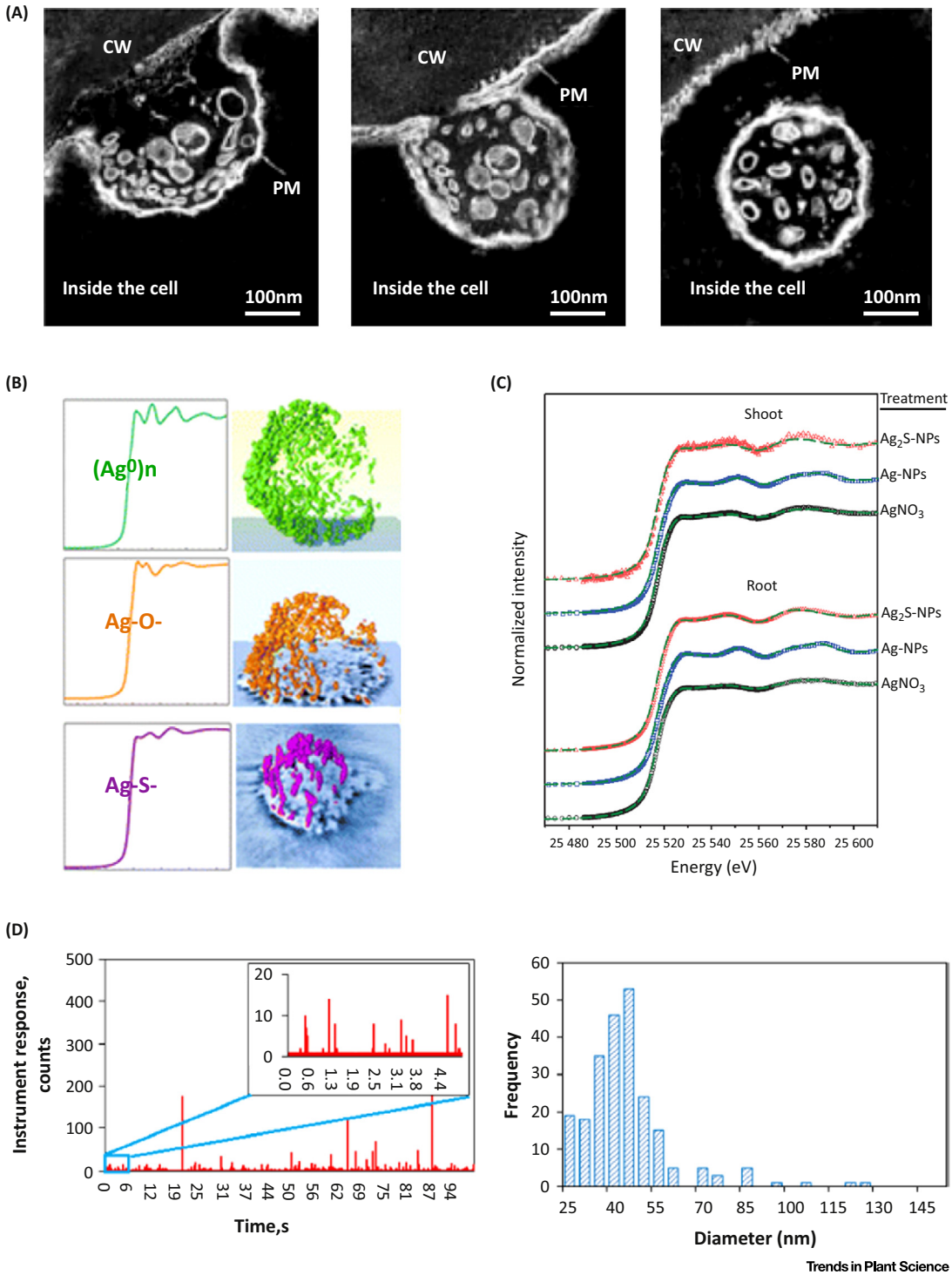

Figure 4. Detection and Characterization of Nanoparticles (NPs). (A) Real-time endocytosis observed through a total internal reflection fluorescence microscopy, demonstrating an internalized uptake of NPs in plant cells by endocytosis [84]. (B) Synchrotron-based scanning transmission X-ray microscopy (STXM) examining 3D distribution and transformation of silver (Ag) NPs inside a human monocyte, revealing the chemical origin of Ag NP cytotoxicity [87]. (C) Synchrotron-based X-ray absorption spectroscopy (XAS) examining the chemical speciation and transformation of silver sulfide (Ag_2S) NPs and Ag NPs, demonstrating a direct upward translocation of Ag_2S NPs in plant tissues [45]. (D) Single-particle ICP-MS examining the size distribution of gold (Au) NPs in the enzymatically digested leaves of tomato, indicating an uptake of intact particles without altering the properties of the Au NP [93]. Reproduced, with permission, from [45,84,87,93].

fractionation and ICP-MS (FFF-ICP-MS) has been used to separate and characterize natural colloids and NPs in environmental systems and food samples [91,92], while time-resolved ICP-MS has enabled analyses of single particles (**SP-ICP-MS**). SP-ICP-MS has been recently developed for the simultaneous determination of Au NP size, size distribution, particle concentration, and dissolved Au concentration in tomato tissues [93] (Figure 4D).

Concluding Remarks and Future Perspectives

Although phytonanotechnology is in its infancy, it has the potential to generate: (i) new tools for smart delivery of agrochemicals; (ii) new ways to deliver particular bioactive molecules to manipulate plant breeding and genetic transformation; and (iii) new approaches for intracellular labeling and imaging.

Unlike animal cells, NPs in plants must traverse cell walls before entering the cytoplasm. The interactions between NPs and cell walls are still poorly understood. Future studies should focus on the generation of novel NPs that can either enlarge or induce pores in cell walls [11,38] to facilitate the transfer of NPs across cell walls and other barriers (see Outstanding Questions). The surface of NPs can be functionalized with a tag [24], magnetic field [19], or biological recognition molecules [94] to allow for specific, targeted delivery. This could provide new ways of manipulating gene expression, either transiently or permanently at a single cell or tissue level. Additionally, NPs could be engineered so that the opening or closing of nanopores can be controlled to release cargo in response to changes of external conditions. This could be achieved through the use of pH-sensitive nanovalves [95] or ultrasound-responsive gatekeepers of the pores [96]. This could make NPs 'smarter' for the controlled release of cargo upon reaching certain targets where the required conditions are met or applied (e.g., the neutral pH environment of the cytosol and nucleus or acidic pH of the vacuole). Finally, it is possible to design NPs to carry nucleotides or activators that can trigger plant defences in response to biotic or abiotic stress. These advances in phytonanotechnology will generate new opportunities in the plant sciences and plant production systems.

However, despite these exciting opportunities, to ensure both the safe use and social acceptance of phytonanotechnology, studies need to investigate the toxicity and trophic transfer of NPs under environmentally realistic and relevant conditions. In this context, the development of nano-QSARs to elucidate the relations between the characteristics of NPs and subsequent plant responses is particularly important. This will support the establishment of blueprints for safe-by-design NPs. For instance, NPs could be tailored to adhere to specific plant surfaces, transverse barriers at the cellular level, and translocate to targets before decomposing once no longer required.

Outstanding Questions

Are the general principles regarding the use of ENPs within human systems also applicable to plant systems? If so, how can we design 'smart' crops?

How can we design ENPs to deliver agrochemicals (for example fertilizers, pesticides, and herbicides) to meet the nutritional demands of the crop or provide protection against pests and pathogens in a controlled manner?

Can ENPs be used as a new vector to deliver bioactive molecules, including nucleotides, proteins, and activators, into plant cells for genome modification and the regulation of plant metabolism?

How can we tailor ENPs to enable target-specific delivery in a controlled manner?

Is it possible to deliver proteins and enzymes using ENPs, thereby allowing the transient presence of these proteins and enzymes? Could this be a useful method for biochemical analyses and genome modifications?

Can ENPs be directly taken up by plants? If so, how do ENPs traverse the complex series of chemical and physiological barriers in plants? Can we tailor ENPs to circumvent or interact with these barriers to facilitate the internalization of these ENPs?

Does the release of ENPs into the environment represent a threat to soil ecosystem health and food security? If so, to allow the future design of functionally desirable and safe NPs, is it possible to establish the relations between the characteristics of NPs and the corresponding plant responses?

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