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Engineered silver nanoparticles are sensed at the plasma membrane and dramatically modify physiology of *Arabidopsis thaliana* plants

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Summary

Silver nanoparticles (Ag NPs) are the world's most important nanomaterial and nanotoxicant. The aim of this study was to determine early stages of interactions between Ag nanoparticles and plant cells and investigate their physiological roles. We have shown that addition of Ag NPs to cultivation medium, at levels above 300 mg L^{-1}, inhibited *Arabidopsis thaliana* root elongation and leaf expansion. This also resulted in decreased photosynthetic efficiency and extreme accumulation of Ag in tissues. Acute application of Ag NPs induced transient elevation of $[Ca^{2+}]_{cyt}$, and accumulation of ROS (partially generated by NADPH oxidase). Whole-cell patch-clamp measurements on root cell protoplasts demonstrated that Ag NPs slightly inhibited plasma membrane K^+ efflux and Ca^{2+} influx currents or caused membrane breakdown. However, in excised outside-out patches, Ag NPs activated Gd^{3+}-sensitive Ca^{2+} influx channels with unitary conductance of approximately 56 pS. Bulk particles did not modify the plasma membrane currents. Tests with electron paramagnetic resonance spectroscopy showed that Ag NPs were not able to catalyse hydroxyl radical generation but they directly oxidised the major plant antioxidant, L-ascorbic acid. Overall, the presented data

sheds the light on mechanisms of the impact of nanosilver on plant cells and show that these include induction of classical stress signalling reactions (mediated by $[Ca^{2+}]_{cyt}$ and ROS) and a specific effect on the plasma membrane conductance and the reduced ascorbate.

Introduction

Silver nanoparticles (Ag NPs) have gained particular attention from industrialists due to their relatively low cost of production and tremendously enhanced physical/chemical characteristics (Nowack *et al.,* 2011). Since the 19th century, the unique antimicrobial and fungicidal properties have been encouraging very wide utilization of Ag NPs in medical products, fabrics, antiseptics, food containers, cosmetics, paints, and even plush toys (Morones *et al.,* 2005; Kim *et al.,* 2007, 2009; Rai *et al.,* 2009; Nowack *et al.,* 2011). Nowadays, nearly 25% of all nanotechnology consumer products include Ag NPs (according to Inventory of Nanotechnology Consumer Products). Between 320 to 480 tons (different estimates) of Ag NPs are industrially produced and consumed every year (Nowack *et al.,* 2011). The dramatic increase in industrial use of Ag NPs has raised considerable concern about their potential release and effects on flora and ecosystems, as well as the possibility of it entering human food chain through plants (Monica & Cremonini 2009; Gottschalk & Nowack 2011).

Indeed, Ag NPs from NP-containing goods are released to the environment in large amounts, where they accumulate in the soil or water reservoirs and affect biota (Benn & Westerhoff 2008; Roh *et al*., 2009; Kaegi *et al.*, 2010; Gottschalk & Nowack 2011; Quadros and Linsey 2011). The part of metallic Ag NPs that are released from NP-containing products is converted in the soil and sewage sludge to less toxic substances, such as Ag-sulphide NPs (Choi & Hu 2008; Kim *et al.*, 2010; Luther & Rickard 2005). Nevertheless, some studies clearly indicate that a significant portion of Ag NPs can reach the soil in their metallic "nano-form" (Geranio *et al.*, 2009; Nowack *et al.*, 2011). Modelling conducted by Gottschalk *et al.*, has demonstrated that the soil concentration of Ag NPs in agricultural land (estimates for USA, 2012) exponentially increases, mainly due to the treatment with Ag-NP-polluted sludge, with the average predicted concentration of Ag NPs in the soil of approximately 8 mg kg^{-1} (Gottschalk *et al.*, 2009). This is an average number while "hotspots" contain much more nanosilver. Concentrations of Ag NPs in surface water and sewage treatment have also been increasing significantly in recent years according to several reports (Blaser *et al.*, 2008; Gottschalk *et al.*, 2010; Fabrega *et al.*, 2011; Gottschalk & Nowack 2011).

Silver is believed to be not very toxic for living organisms (Ratte, 1999). This metal does not have any established function in plants or animals and has been acknowledged as a trace element (Ratte, 1999). The ability of Ag NPs to release Ag^+ at a slow rate probably underlies its commercial usage as

an antiseptic agent. On the other hand, this results in the long-term effects on living systems. Ag NPs affect the growth of bacteria, fungi and algae as well as reproduction of viruses (Liu & Hurt 2009). High levels of Ag NPs also appear to be toxic for animals and humans (Gaul & Staud 1935; Braydich-Stolle *et al.*, 2005; Hussain *et al.*, 2005; Rahman *et al.*, 2009; AshaRani *et al.*, 2009; Ji *et al.*, 2007). Higher plants can also be affected by Ag NPs. For example, Stampoulis *et al.*, (2009) have demonstrated that Ag NPs (> 100 mg L^{-1}) inhibit seed germination, growth and "transpiration volume" of zucchini plants. Gubbins *et al.*, (2011) have found that the growth of the aquatic plant *Lemna minor* is very sensitive to Ag NPs. Lee *et al.*, (2012) have demonstrated that root growth of *Phaseolus radiatus* and *Sorghum bicolor* is inhibited by Ag NPs. Similar results were observed by Yin *et al.*, (2011) for growth of *Lolium multiflorum* and Geisler-Lee *et al.*, (2013) for *Arabidopsis thaliana* seedlings. Geisler-Lee *et al.*, (2013) have shown that Ag NPs accumulate in root tissues. Nair and Chung (2014, 2015) showed increase in lipid peroxidation and ROS production in plant tissues as well as activation of genes related to oxidative stress. Qian et al. (2013) demonstrated that chronic exposure to Ag NPs modifies thylakoid membrane and transcription of antioxidant and aquaporin genes. Nevertheless, effects of Ag NPs on plant cell Ca^{2+} and redox signalling as well as other intracellular phenomena have not been examined to date. Thus, an identification of the primary reactions induced by Ag NPs in plant cells is currently a key step in this research. These reactions, such as an elevation in cytosolic free Ca^{2+}, generation of reactive oxygen species (ROS) and modification of ion channel activities, have recently been studied in a number of species and have been shown to play a pivotal role in encoding developmental and stress signals (Demidchik, 2015). Whether they can be involved in sensing 'nano-signals' is unknown. Another important issue that has not yet been properly addressed is how nanosilver affect free radical production, photosynthetic efficiency and plant cell antioxidants.

In this work, we have aimed to establish the pattern of regulatory and stress reactions of manufactured Ag NPs in model plant *Arabidopsis thaliana*, at the organismal level (root and leaf growth) and at the cellular level (Ca^{2+} signalling, ROS generation, plasma membrane conductances, photosynthetic efficiency). We also examined the Ag accumulation in higher plants cultivated on Ag NP-containing media, and the potential action of Ag NPs on extracellular L-ascorbic acid.

Results

Effect on root elongation and leaf expansion

The elongation of the main root has been measured in the presence of NPs, bulk material and supernate (supernatant) (Fig. 1). The addition of 300, 500, 1000, 1500, 3000 and 5000 mg L^{-1} Ag NPs to the media reduced root length by 41.5 ± 2.1%, 50.8 ± 5.7%, 59.1 ± 5%, 70.4 ± 1.4%, 87.5 ± 6.1% and 91.2 ± 6.2%, respectively, relative to the control (± SD; n = 15; Fig. 1). No significant difference

for NPs and bulk was found for 300-1000 mg L^{-1}. However at concentrations higher than 1500 mg L^{-1}, NPs caused more pronounced inhibition than bulk (Fig. 1E; P<0.01; ANOVA test). Surprisingly, low concentrations of 'supernate' stimulated elongation (300-1000 mg l^{-1}; Fig. 1 A-C; P<0.01; ANOVA test). The higher concentrations of supernate (1500-5000 mg L^{-1}) did not cause this effect (this effect was not studied in details).

Fig. 1E shows the dose dependence of root growth changes in response to Ag NPs and bulk. These values were calculated for a six day period, starting from the fourth day from the beginning of cultivation (from day four to ten). The half-maximal effective concentration (EC_{50}) was estimated as 515 ± 12 mg L^{-1} and 785 ± 10 mg L^{-1} for Ag NPs and Ag bulk respectively (Concentration-Response Curve Analysis Tool, SigmaPlot). Surprisingly, even 5000 mg L^{-1} Ag NPs did not induce death of seeds (they still germinated and showed some growth).

Addition of 300 to 1500 mg L^{-1} Ag NPs triggered reduction in the growth rate of *A. thaliana* leaves (Fig. 2A-C). The average leaf area of the control plants (cultivated without NPs in the medium) was 152.9 ± 3.9 mm^2 (± SE; n = 10), after thirteen days of cultivation. Treatment with 300, 500, 1500 and 3000 mg L^{-1} Ag NPs caused a reduction in the average leaf area to 111.2 ± 5.4, 99.9 ± 4.6, 82.2 ± 5.5 and 74.2 ± 9.6 mm^2, respectively (± SE; n = 10; Fig. 2A-C). Plants treated with the bulk material were not significantly different to the control plants. The difference between the average leaf area measured for the NPs and bulk was particularly pronounced for longer treatment times (13 days). For example, for 3000 mg L^{-1} Ag, it was 51.8 ± 2.1% (± SE; n = 10; P<0.01, ANOVA test) (Fig. 2C).

Change of chlorophyll fluorescence

Changes in the fluorescence parameter F_v/F_m in response to Ag NPs (300 to 3000 mg L^{-1}) and the corresponding bulk material are shown in Fig. 2D (measured 4 days after germination in NP or bulk-containing media). The mean F_v/F_m ratio for the control plants was found to be 0.68 ± 0.01 (± SE; n = 10). Treatment with 300, 500, 1500 and 3000 mg L^{-1} Ag NPs, resulted in mean F_v/F_m values of 0.67 ± 0.01, 0.47 ± 0.05, 0.42 ± 0.03 and 0.29 ± 0.04 (± SE; n = 8-9; Fig. 2D). Statistical analysis indicates that there was significant difference in F_v/F_m at 500 mg L^{-1} NPs and the higher NP concentrations (P<0.01, ANOVA test). Bulk induced a less pronounced decrease of the F_v/F_m parameter.

Accumulation of Ag in plants exposed to Ag nanoparticles

Negligibly low level of Ag was detected in control plants that were grown in the absence of Ag. Dramatic Ag accumulation was found when plants were cultivated on Ag NP- and bulk-containing media. In the case of NPs, a significantly high amount of Ag was found to be translocated into the

plant as compared to the bulk (Fig. 3). For the lowest NP concentration, 100 mg L^{-1}, the total concentration of Ag metal was 0.31 ± 0.04 g kg^{-1}, while, at 1500 mg L^{-1}, it increased to 1.35 ± 0.14 g kg^{-1} (± SD; n = 6). Accumulation of Ag from supernate- and $AgNO_3$-containing media was significantly lower (Fig. 3; also see insert).

Elevation of cytosolic free Ca^{2+}

The application of Ag NPs at concentrations from 300 to 3000 mg L^{-1} resulted in a significant transient elevation of $[Ca^{2+}]_{cyt}$ as compared to their bulk counterparts (Fig. 4). The basal $[Ca^{2+}]_{cyt}$ level was 76.6 ± 6.2 nM (± SE; n = 5). Peak values were calculated by subtracting this level (tested in each individual trial) from the obtained maximal (peak) increase. Addition of buffer solution without NPs or bulk caused a mean peak of 18.9 ± 2.7 nM (± SE; n = 5). The minimal concentration of Ag NPs inducing a statistically significant increase of $[Ca^{2+}]_{cyt}$ was 100 mg L^{-1} (ANOVA test, $P<0.01$). The same concentration of bulk did not induce a significant increase in $[Ca^{2+}]_{cyt}$. 1500 and 3000 mg L^{-1} Ag NPs caused peaks of 238.7 ± 25.5, and 301.1 ± 24.7 nM (± SE; n = 5), respectively (Fig. 4A,B). The same Ag bulk levels yielded negligible effects with mean peaks of 13.8 ± 5.7 and 6.7 ± 14 nM (± SE; n = 5).
The relationship between the mean peak $[Ca^{2+}]_{cyt}$ increase and the concentration of applied NPs and bulk is demonstrated in Fig. 4B. Half-maximal $[Ca^{2+}]_{cyt}$ elevation was observed at 350-400 mg L^{-1} Ag NPs. The radical scavengers (thiourea and dimethyl sulfoxide) (Halliwell & Gutteridge 1999; Demidchik *et al.,* 2003, 2010; Demidchik 2012) and, the blocker of plasma membrane Ca^{2+} influx channels, Gd^{3+} (Demidchik *et al.,* 2002), were used to define the pharmacological characteristics of Ag NP induced $[Ca^{2+}]_{cyt}$ elevation. The results showed that Gd^{3+} and thiourea, which is considered by some authors as a hydroxyl radical-specific scavenger (Liszkay *et al.,* 2004), are very effective inhibitors of cytosolic calcium burst induced by 500 mg l^{-1} Ag NPs (Fig. 4C). At the same time, dimethyl sulfoxide did not decrease NP induced $[Ca^{2+}]_{cyt}$ elevation. Application of supernate (30-3000 mg L^{-1}) resulted in very small $[Ca^{2+}]_{cyt}$ elevation (Fig. 4B). Ag^{+}-binding agent, Cys (0.3 mM; added together with Ag NPs) decreased NP-induced $[Ca^{2+}]_{cyt}$ elevation by only 20-25% (Fig. 4B). This suggests that the effect on cytosolic Ca^{2+} was not only related to the leakage of silver ions from NPs to solution.

Generation of reactive oxygen species in roots exposed to Ag NPs

The generation of H_2O_2 was measured in intact *Arabidopsis thaliana* roots exposed for 30 min to 300-3000 mg L^{-1} Ag NPs and bulk material (Fig. 5). No significant difference was observed upon application of 300 mg L^{-1} Ag NPs as compared to the control (Fig. 5A and Fig. 5B). However higher

concentrations of Ag NPs produced a significant elevation in Ampliflu Red™ fluorescence (ANOVA test; $P<0.01$). An application of Ag bulk produced a much weaker signal (Fig. 5A and Fig. 5B). ROS scavengers inhibited NP-induced fluorescence signal (Fig. 5C). Plants lacking functional NADPH oxidase RBOHC (*rhd2*) (Foreman *et al*., 2003) demonstrated significantly lower signal than wild type.

NP-induced changes in plasma membrane conductances

Addition of 1000 mg L^{-1} Ag NPs to an extracellular solution containing 20 mM $CaCl_2$ (pipette solution: 50 mM K^+, 45 mM gluconate$^-$ and 5 mM Cl^-), in the whole-cell mode, resulted in rapid irreversible increase of plasma membrane conductance leading to breakdown in 57% of protoplasts (in 230 out of 405 tested protoplasts; Group 1). Other protoplasts demonstrated decrease of whole-cell inward and outward currents (Fig. 6A-C; Group 2). The Group 2 was studied in details. Removal of intracellular K^+ and addition of K^+ channel blocker TEA^+ (pipette solution: 10 mM TEA^+, 10 mM Cl^-) abolished outward currents showing that they were mediated by K^+ efflux (Fig. 6B). NPs did not inhibit outward currents in this case. This indicates that NPs inhibited K^+ efflux channels previously characterised in Arabidopsis root cells (Demidchik *et al*., 2003, 2010). Removal of extracellular Ca^{2+} resulted in three-fold decrease in inwardly-directed current and disappearance of NP-sensitive inwardly-directed component of this current (Fig. 6C). This shows that the effect of Ag NPs on the inward current was due to inhibition of Ca^{2+} influx channels, which also were also previously characterised in this tissue (Demidchik *et al*., 2002). Exogenously applied Ag^+ (1 µM $AgNO_3$) and Gd^{3+} (1 µM $GdCl_3$) induced inhibitory effects on outwardly- and inwardly-directed currents, which were similar to Ag NPs (Fig. 6A). Ag bulk was not effective (Fig. 6A).

In tests with excised outside-out patches (Fig. 6D-G), K^+-containing pipette solutions were not used because they destabilised patches causing loss of seal. Replacement of K^+ by Na^+ in the pipette solution improved the stability of measurements. This allowed to detect the unique single channel-like conductance activated by Ag NPs (Fig. 6D). An exogenous application of Ag NPs to excised outside-out patches activated single channel-like currents in approximately 50% of protoplasts (in 37 out of 72 patches) (Fig. 6D). Ag bulk was not capable of inducing these effects although often caused seal instability (NP_{open} = 0; n = 35). NP-activated currents operated as short-term opening events with unitary conductance of 56±9 pS (SE; n = 6-7). Note that constitutive NP-insensitive cation channels appeared in some patches however they had ten times smaller unitary conductance (5-6 pS) which corresponds to well-characterised Ca^{2+}-permeable nonselective cation channels (Demidchik *et al.,* 2002).

The opening times of NP-induced unitary conductaces varied from 50 ms to few seconds. The open state probability (NP_{open}) did not change over time or at different holding potentials; but its value increased with concentration of NPs. Analysis of I-V curve showed that permeability ratio P_{Ca}:P_{Na} was 1.41:1 (Fig. 6F; calculated using Goldman-Hodgkin-Katz equation).

NP-induced single channel-like conductances were insensitive to K^+ channel blocker TEA^+ (10 mM), Ca^{2+} channel blocker verapamil (50 μM), anion channel blocker anthracene-9-carboxylic acid (100 μM) and hydroxyl radical scavenger thiourea (1 mM) but they were inhibited by 0.1 mM Gd^{3+} (blocker of nonselective cation channels and mechanosensitive channels). Decrease of [Ca^{2+}] in the bathing solution from 20 mM to 0.01 mM abolished NP-induced currents, showing that they were mediated by Ca^{2+}-permeable ion channels. Moreover, previous studies showed that Cl^- efflux current (component of inwardly directed conductance) is very low in the conditions which were used here (Diatloff *et al*., 2004).

Generation of free radicals and oxidation of L-ascorbic acid

To examine the capacity of inducing hydroxyl radical generation by NPs, 5,5-dimethyl-pyrroline N-oxide (DMPO) was applied with Fenton-like mixture with Ag NPs or bulk used instead of the transition metal (40 mM DMPO, 1 mM L-ascorbic acid, 1 mM H_2O_2, 30-15000 mg L^{-1} Ag NPs or bulk). In contrast to the standard mixture (containing 1 mM $CuCl_2$ instead Ag NPs), the NP-containing mixture was ineffective in the producing characteristic four-peak spectrum of hydroxyl radical DMPO adducts (Fig. 7; n = 6-7). However, Ag NPs caused the appearance of an ascorbyl radical-specific signal which showed the classical two-peak shape spectrum (Fig. 7A; n = 6-7).

An addition of natural plant radical scavenger mannitol (1 mM) or key antioxidant glutathione (1 mM) resulted in the disappearance of the ascorbyl radical signal (Fig. 7B and Fig. 7C), suggesting that these substances are capable of protecting ascorbate from Ag-NP mediated oxidation. Interestingly, Ag NPs did not induce generation of mannitol or glutathione radicals. Thiourea, caused an effect, which was similar to that of glutathione while bulk or supernatant did not induce ascorbyl radical signal (Fig. 7C; n = 4-7).

Application of Ag NPs (3000 and 15000 mg L^{-1}) to intact roots resulted in characteristic ascorbyl radical peaks, which were sensitive to a number of free radical scavengers (Fig. 8). High concentrations of H_2O_2, which are known to cause the oxidation of L-ascorbic acid, also induced the formation of the ascorbyl radicals. Addition of bulk particles or supernate did not result in the formation of ascorbyl radicals in intact roots (n = 5).

Discussion

Results of this study demonstrated that growth of both root and leaf was sensitive to Ag NP concentrations above 300 mg L^{-1} (Figs. 1 and 2). The inhibitory effect tended to saturate at 3000 mg L^{-1}. The results of root growth tests are comparable with the findings of other studies, which tested some concentrations of Ag NPs and other metal-containing NPs (Lee *et al.,* 2008, 2010, 2012; Monica and Cremonini 2009, Stampoulis *et al.,* 2009; Gubbins *et al.,* 2011; Yin *et al.,* 2011; Geisler-Lee *et al.,* 2013). Overall, Ag NPs appear to be more toxic agents for root elongation than Fe_3O_4, SiO_2, TiO_2 and Al_2O_3 NPs, but less toxic than ZnO and Cu NPs.

The effect of NPs on leaf expansion has never been tested before. An inhibition of leaf expansion by Ag NPs found here is indicative of the complex phytotoxicity (affecting all organs). Leaf expansion was slightly less sensitive to Ag NPs as compared to root elongation and its inhibition correlated with decrease of photosynthetic efficiency (F_v/F_m parameter) (Fig. 1 and Fig. 2). This can probably be explained by the fact that root cells are directly exposed to NPs while leaves are mainly affected by the changes of root physiology and those NPs that are transported by roots to the xylem. The latter has recently been confirmed for Ag NPs (Geisler-Lee *et al.,* 2013) and CuO NPs (Wang *et al.,* 2012). The effect of bulk particles on root and leaf growth as well as on photosynthetic efficiency was less pronounced as compared to NPs (Figs. 1 and 2). Moreover, supernatant did not inhibit root growth (Fig. 1). Thus, the observed effects were related to the unique properties of nanosilver.

The minimal threshold concentration of Ag NPs, which induced statistically significant changes in growth characteristics of *A. thaliana* plants, was 300 mg L^{-1} or 0.03% (weight per volume). Interestingly, *A. thaliana*, as well as many other higher plant species, can tolerate as much as 3% ethanol or methanol, 1% chloroform or dimethyl sulfoxide, 0.3% NaCl, 0.1-0.01% of most organic xenobiotics, 0.001-0.005% heavy metal ions (Demidchik & Tester 2002; Fujishige *et al.,* 2004; Schröder & Collins 2011). This places Ag NPs at a position of moderate toxicity, actually, similar to most xenobiotics. Thus, Ag NPs are not extremely toxic for higher plants, although they can cause significant damage to wild flora and crops, under severe pollution (above 0.03%). Significantly higher toxicity of Ag NPs was demonstrated in algae (Navarro *et al*., 2008).

It seems unlikely that soil pollution by Ag NPs reaches 300 mg L^{-1} (Gottschalk *et al.,* 2009). However, the level of Ag NPs in soils shows exponential increase. Surprisingly high levels of Ag NPs (1.3 mg L^{-1}) were detected in the sewage water (Benn and Westerhoff, 2008). Commercial products containing Ag NPs can release up to 4.6 mg of Ag NPs per kg of the product, after washing-up with tap water during 1 h (Benn *et al.,* 2010). Kaegi *et al.,* (2010) have reported that one runoff from Ag-NP-

containing roof paints contains approximately 150 μg L^{-1} Ag NPs. All these "discharges" can potentially accumulate in the soil and result in very high levels of nanosilver.

The specific effect of Ag NPs on growth and photosynthesis can be at least partially explained by greater uptake of silver by plants from NP-containing media as compared to bulk-containing media (Fig. 3). Data on Ag accumulation in plants (Fig. 3) have demonstrated that Ag content can reach a dramatic level that is probably toxic for man and animals (up to 1 g per kg of dry weight). Very similar accumulation of metals in living tissues exposed to a range of metal NPs has been reported for a number of non-photosynthesising organisms as well as some higher plants (Scown *et al.,* 2010; Montes *et al.,* 2012). Nevertheless this has not yet been shown for Ag NPs and higher plants. High levels of Ag accumulation suggest an existence of very efficient pathway for nanoparticle transport into the plant cell.

This study provides the evidence that Ag NPs are capable of inducing the signalling reactions in root cells (Figs. 4-6), suggesting that Ag NPs can be sensed at the plasma membrane. Ag NPs caused Ca^{2+} elevation, which is, by amplitude and shape, similar to Ca^{2+} signals produced in response to heavy metals, salinity, pathogen elicitors or some hormones (Demidchik & Maathuis 2010). Ca^{2+} elevation was not induced by supernatant up to 1500 mg L^{-1} Ag NPs (Fig. 4B), and only slightly decreased in the presence of Cys (Ag^{+} chelator). These facts strongly suggest that metallic nano-form was required for induction of Ca^{2+} signal. Increase of resorufin fluorescence, demonstrating ROS generation, was also typical for a number of stresses (Demidchik 2012).

The patch-clamp analyses provided the mechanistic explanation for Ca^{2+} influx and ROS generation (Fig. 6). Ag NPs caused a little inhibition of K^{+} efflux and Ca^{2+} influx conductances, which was probably mediated by Ag^{+}. This can be considered as a classical effect of heavy metal treatment on ion channels (Kiss and Osipenko, 1994). However Ag NPs also induced currents, which were not previously measured in plant membranes. NP-induced single-channel conductances allowed Ca^{2+} entry (which usually catalyses $[Ca^{2+}]_{cyt.}$ elevation) and showed sensitivity to Gd^{3+}, a blocker of nonselective and mechanosensitive cation channels in *A. thaliana* (Demidchik *et al.*, 2002; Haswell *et al.*, 2008). Hypothetically, the NP-induced breakdown of the plasma membrane in Group 1 protoplasts can be a result of activation of these channels in whole-cell mode.

Observed single channels showed much higher unitary conductance (56 pS) than constitutive (5-6 pS) and ROS-activated (14-15 pS) Ca^{2+}-permeable channels in Arabidopsis root plasma membrane (Demidchik *et al.*, 2002, 2007). They resembled those that were recorded for *A. thaliana* mechanosensitive channels (Haswell *et al.*, 2008). Nevertheless NP-activated channels were selective to cations (Haswell *et al.*, 2008) while mechanosensitive channels are preferably permeable to anions (Maksaev and Haswell, 2012). The activation of mechanosensitive channels by metallic NPs

was previously shown in animal cells (Hughes *et al.,* 2005). NP-induced conductances could also be related to formation of pores in the plasma membrane (Klein *et al*., 2008; de Planque *et al*., 2011). For example, an application of 12 nm diameter CdSe quantum dots (Klein *et al*., 2008) or silica nanospheres (Planque *et al*., 2011) caused formation of temporary pores in the lipid bilayer that allowed ion movement.

Calcium entry through the plasma membrane ion channels may lie upstream of ROS generation because NADPH oxidase, an enzyme producing ROS, is stimulated by cytosolic Ca^{2+} (Demidchik & Maathuis, 2007). Hypothetically, in intact root, $^{\bullet}O_2^-/H_2O_2$ produced by NADPH oxidase can be converted to $^{\bullet}OH$ by cell wall ascorbate and transition metals and activate ROS-sensitive Ca^{2+}-permeable cation channels (Demidchik, 2015). Supporting this, $^{\bullet}OH$ scavenger thiourea inhibited Ag NP-induced Ca^{2+} influx in roots (Fig. 4C) while it did not change currents in protoplasts (Fig. 6G). Moreover, *rhd2* plants (lacking NADPH oxidase RBOHC) showed significantly smaller generation of ROS in response to Ag NPs as compared to the wild type (Fig. 5).

Ag NP-catalysed formation of ascorbyl radicals (Figs. 7 and 8) was not previously shown. The ability to catalyse oxidation of ascorbate and other organic substances has a great potential for a number of biotechnological applications. We found that reduced glutathione protected ascorbate from Ag NP-induced oxidation (Fig. 7 and Fig. 8). This finding might be useful for both plant stress biologists and medical toxicologists, as providing a route for lowering Ag NP-induced toxicity.

It was recently shown that, under very alkaline pHs, Ag NPs react with H_2O_2, forming Ag^+ and $^{\bullet}O_2^-$, and further with $^{\bullet}O_2^-$ to give "negatively charged Ag NPs" and O_2 (He *et al.,* 2011; Jones *et al.,* 2011). Thus Ag NPs can potentially behave as a transition metal ions (such as copper or iron) and catalyse Haber-Weiss cycle. However, EPR spectroscopy data presented here have ruled out Haber-Weiss-like reactions (Fig. 7). Thus, Ag NPs, which are known to promote redox imbalance and oxidative stress in a number of organisms (Kim *et al.,* 2009; Fabrega & Luoma 2011), probably make this mainly through affecting lipid bilayer and cell ascorbate pool (not via production of hydroxyl radicals).

In conclusion, it has been established that Ag NPs induce complex physiological modifications in the model plant *Arabidopsis thaliana*. At the organismal levels, they inhibit growth and photosynthetic efficiency, and cause dramatic increase of Ag content in plants (raising food safety issues). At the cellular level, Ag NPs trigger Ca^{2+} and ROS signalling through formation of Ca^{2+}-permeable pores and oxidation of apoplastic L-ascorbic acid.

Experimental procedures

Plant material

Wild type (WT) *Arabidopsis thaliana (A. thaliana)*, ecotype Wassilewskija (WS), was used to study the impact of Ag NPs on growth, accumulation of Ag, production of ROS and chlorophyll fluorescence. Transgenic *A. thaliana* (Columbia ecotype) were used to measure changes in $[Ca^{2+}]_{cyt}$ by aequorin luminometry. RBOHC knockout mutants (*rhd2*) (Foreman *et al*., 2003) were from NASC (arabidopsis.info). For all experiments, except chlorophyll fluorescence tests, the plants were grown vertically on a surface of sterilised gel (in Petri dishes), which contained full-strength Murashige and Skoog (MS) medium, 1% sucrose and 0.35% Phytagel (Sigma, UK) (Murashige & Skoog, 1962). To test the chlorophyll fluorescence, plants were cultivated in 12-well plates (one plant per well) containing the same medium composition (13 days; sterile conditions). Fresh hot medium was poured into the Petri dishes or 12-well plates immediately after preparation (Phytogel solidified in few minutes), which avoided nanoparticle precipitation. Constant temperature and irradiance conditions were maintained in growth cabinets (19±1°C, 16-h daylight, halogen lamps, 200 µmol m^{-2} s^{-1} irradiance).

Nanoparticle samples and plant growth medium preparation

Ag NPs and the corresponding bulk material (d > 250 µm) were purchased from American Elements and Sigma respectively, and used after confirming their physical parameters. These tests have been carried out with Transmission Electron Microscopy (TEM) using a Philips CM200 HR-TEM. Ag NPs were dispersed in to ethanol and sonicated for 30 minutes. A drop of the stable suspension (approximately 10 µL) was deposited on to a 300 mesh copper grid with an amorphous carbon film. The mean individual spherule diameter determined by TEM analysis was 41±1.5 nm (n = 50) that corresponded to the certificate supplied by manufacturer.

For the root elongation assay, atomic absorption spectroscopy and chlorophyll fluorescence imaging, plants were grown in media containing different concentrations of NPs, bulk particles or metal ions (supernatant produced by centrifugation of the Ag-NP-containing solutions; 5000 g). The dispersion of NPs and the respective bulk particles in the plant growth medium was carried out by sonicating the nanoparticle suspension for 30 min in the solution containing only MS medium (modified technique from Lee *et al.,* 2008). Phytagel and sucrose were added to this NP-containing MS, mixed thoroughly, rapidly autoclaved and used for plant cultivation.

Measurement of changes in root growth

The growth response of fifteen roots exposed to different concentrations of Ag NPs, "bulk Ag" particles and "Ag supernatant" was continuously recorded over a period of six days, starting from the fourth day of cultivation (root elongation rates from days 4 to 10 were analysed). The location of the root tip was measured and marked (by ultrafine permanent marker) at the base of the transparent plastic Petri dish and then scanned at the last day of the experiment (this allowed to avoid plant growth disturbance). Concentration-response curves were fitted and analysed using SigmaPlot 10.0 software (Hill Equation, Systat Software Inc., USA).

Luminometric measurements of cytosolic free Ca^{2+}

Standard aequorin chemiluminometry techniques were used to measure changes in $[Ca^{2+}]_{cyt}$. (Knight *et al.,* 1996, 1997; Demidchik *et al.,* 2003). The experiments were carried out on 7-12 day-old seedlings of *Arabidopsis* plants constitutively expressing apoequorin, under control of 35S CaMV promoter (Polisensky & Braam 1996; Knight *et al.,* 1996, 1997). Recording of the Ca^{2+} activity was carried out continuously for 15-30 min with a Berthold Lumat LB 9501 single-tube luminometer controlled by a Berthold PC interface and WinTerm software (Berthold, Germany) or Turner BioSystems Luminometer Model TD-20/20 (Turner BioSystems; USA; controlled by Spreadsheet Interface Software). The remaining aequorin was discharged for 5 min using a solution containing 2 M $CaCl_2$, 20% ethanol and 0.01% Triton X-100. The amount of free Ca^{2+} entering the cells was calculated using the calibration equation derived empirically: pCa = 0.332588 (- log *k*) + 5.5593, where *k* = (luminescence counts s^{-1}) / (total luminescence counts remaining) (Knight *et al.,* 1996, 1997). L-Cysteine (Cys) solution was freshly prepared and kept on ice as described elsewhere (Navarro *et al*., 2008). Concentration-response curves were fitted and analysed using SigmaPlot 10.0 software (Systat Software Inc., USA).

ROS imaging with 10-acetyl-3,7-dihydroxyphenoxazine

10-acetyl-3,7-dihydroxyphenoxazine (Ampliflu Red™ Kit including horse radish peroxidise; Sigma) was used to test the occurrence of an oxidative burst in *Arabidopsis thaliana* roots (adapted from Laohavisit *et al.,* 2009). Seedlings were incubated in the presence of varying concentrations of NPs, bulk and 0.2 mM Ampliflu Red™ for 30 min in the dark (other salines: 0.3 mM $CaCl_2$, pH 6 adjusted by 2 mM Tris/4 mM Mes). Images were obtained using an Olympus BX-41 fluorescence microscope with Olympus analySIS Imaging System and software (resorufin fluorescence was measured with

standard Olympus 560/590 nm excitation/emission filters). The images were analysed using Image J software (National Institute of Health, USA).

Chlorophyll fluorescence and leaf area measurements

Chlorophyll a fluorescence imaging techniques were applied to assess whether Ag NPs affected photosynthetic efficiency and leaf growth (Barbagallo *et al.,* 2003; Baker 2008). The ratio of F_v/F_m was examined, which is a measure of the maximum quantum efficiency of PSII and is an important parameter in detection of stress levels in plants (Lawson *et al.,* 2002). Healthy plants have a conserved F_v/F_m value of approximately 0.8-0.85, whilst any decrease in Fv/Fm is indicative of damage or stress to the plant (Lawson *et al.,* 2002; Barbagallo *et al.,* 2003; Baker 2008). Images of chlorophyll fluorescence were obtained using a FluorImager Chlorophyll Fluorescence Imaging System (Technologica Ltd., Colchester, UK) provided with a progressive scan CCD camera. Prior to measurements, plants were dark-adapted for 20 min, after which an image of Fo was captured at <1 µmol m^{-2} s^{-1} PPFD followed by an image of Fm, obtained during a 800 ms saturating pulse of ca 6000 µmol m^{-2} s^{-1}. To estimate leaf area and expansion, pixel numbers were determined from the Fm images capture on day 4 and 13 of the experiment.

Patch-clamp measurements

Conventional patch-clamp and protoplast isolation techniques were used (Demidchik & Tester, 2002; Demidchik *et al*., 2010). The standard bathing solution contained (mM): 20 $CaCl_2$, 2 Tris, adjusted to pH 5.8 with Mes and to 290-300 mosM with sorbitol. A freshly-prepared mixture of this solution with Ag NPs or bulk particles was applied in whole-cell or excised patches (outside-out configuration; single-channel recordings). The standard pipette solution contained (mM): 50 K^+ (or Na^+ for recording in excised patches), 45 gluconate$^-$ and 5 Cl^- (100 nM Ca^{2+} was adjusted with 1 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; pH 7.2 with 2 mM Tris/Mes). To examine the sensitivity of whole-cell outward current to TEA^+, 10 mM TEA^+ and 10 mM Cl^- were used instead of 50 mM K^+/Na^+, 45 gluconate$^-$ and 5 Cl^- in the pipette solution.

Liquid junction potentials were calculated (corrections were made by amplifier if it exceeded ±5 mV). Voltage was held at -90 mV, then square 3-min long depolarising or hyperpolarising voltage pulses were applied. Conductances were measured and analysed using Axon Instruments Axopatch 200B/ Digidata 1320/Axon Laboratory software (Demidchik and Tester, 2002; Demidchik *et al*., 2010). Single-channel statistical analyses were carried out using Clampfit 10.4.1.10 (Molecular

Devices LLC). Concentration-response curves were fitted and analysed using SigmaPlot 10.0 software (Hill Equation, Systat Software Inc., USA).

Detection of radicals by electron paramagnetic resonance spectroscopy

The EPR spectroscopy has been used to examine the NP-induced generation of radicals *in vitro* in the buffer solution imitating plant extracellular fluid (pH 6 adjusted by Tris/Mes, 0.1 mM $CaCl_2$, liquid phase, 20 ^{o}C) (Halliwell & Gutteridge 1999; Liszkay *et al.,* 2004; Demidchik *et al.,* 2010). To investigate potential Fenton-like activity of Ag NPs, the hydroxyl radical specific spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) has been applied at the concentration of 40 mM (Demidchik *et al.,* 2010). Reagents have been mixed with DMPO for 60 s before recording EPR spectrum in oxygen free conditions. ESR Grade Water (Noxygen) was used and DMPO was purified using ultrafine activated charcoal powder (Sigma) to remove residues of transition metals. A 400-µL Bruker EPR cuvette was filled with the reaction mixture through a disposable syringe. The EPR spectra were recorded on a Bruker EMX (X-band) spectrometer equipped with ER 4103TM Cylindrical Mode resonator and analysed using WinEPR (Bruker). The lineshape of DMPO adduct spectra was consistent with trapping the $^{\bullet}$OH (Yamazaki *et al.,* 1990; Ikeda *et al.,* 2002).

Ascorbyl radical spectra were detected using same hardware and software but without the spin-trap (Buettner & Jurkiewicz 1993). In cell-free conditions, L-ascorbic acid was directly mixed with buffer solution containing NPs (pH 6; Tris/Mes). To measure ascorbyl radical generation in intact roots, fifty intact one-week-old seedlings were removed from the medium and carefully washed several times in a buffer solution containing 0.1 mM KCl, 0.1 mM $CaCl_2$ (pH 7.0; Tris/Mes). Their 2.5 cm root-tip parts were immersed during 15 min in the buffer solution containing NPs or other treatments. After treatment, the eluate from the vial was directly collected in a Bruker AquaX system (multiple-bore design of closely spaced capillaries allowing much higher sensitivity as compared to capillary as well as to flat-cell) for liquid samples measurements.

Atomic absorption spectrometry

Conventional atomic absorption spectrometry techniques were used to determine Ag concentration in plants (Radojevic & Bashkin 1999). *A. thaliana* WS plants were cultivated vertically with NPs in medium during five weeks as described above. They were washed several times with deionised water to remove the medium, dried out and crushed into a fine powder. 0.1 g of this powder was dissolved in 5 mL of primary grade nitric acid (70%) overnight at room temperature (in digestion

vessels). Digestion then followed in a Teckam PTC-2 digestion block at 140^{o}C for 8 h. Standard Graphite Furnace Atomic Absorption Spectrometry techniques (UNICAM 939, Cambridge, UK) were used. 1-2 µL of digested plant samples were diluted up to 2 mL with deionised water and loaded into 1.5 mL auto sampler cups (AA cup, Technicon). The UNICAM 959 spectrometer was calibrated with Ag standards (Sigma).

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Legends to Figures

Fig. 1. Changes in *Arabidopsis thaliana* root elongation induced by Ag NPs (NPs). 300 (A), 500 (B), 1000 (C) and 1500 (D) mg L^{-1} Ag NPs, Ag bulk, supernatant of Ag NPs (supernate), respectively, were added to the growth media. Supernatant was obtained by centrifugation of NPs. (E) Mean root length reduction (%) induced by different concentrations of NPs and bulk particles (n = 15; ± SD). Insert: plants with typical reaction to 3000 mg L^{-1} Ag NPs and Ag bulk (8-day-old seedlings). "***": P<0.001; "n.s.": not significant (P>0.05) (comparison to control).

Fig. 2. Changes in *Arabidopsis thaliana* leaf area and chlorophyll fluorescence parameter, F_v/F_m, triggered by Ag nanoparticles (NPs). 300 (A) and 3000 (B) mg L^{-1} Ag NPs and Ag bulk particles were added to the growth medium. (C) mean leaf area (± SE; n = 10) measure in plants exposed to different levels of Ag NPs at day 4 and 13 of cultivation. (D) mean±SE chlorophyll fluorescence parameter, F_v/F_m (n = 10). Measurements were carried out at fourth day after germination in NP- or bulk-containing media. "*" and "***": P<0.05 and P<0.001, respectively (comparison of NPs and bulk or control).

Fig. 3. Accumulation of Ag in *Arabidopsis thaliana* plants cultivated in medium containing Ag NPs or Ag^+ ($AgNO_3$; insert). Data are mean ± SD (n = 6). Analysis was carried on whole 5-week old plants. "**" and "***": P<0.01 and P<0.001, respectively (compared to NPs).

Fig. 4. Changes in *Arabidopsis thaliana* root $[Ca^{2+}]_{cyt}$ in response to Ag NPs. (A) Transient elevation of $[Ca^{2+}]_{cyt}$ in response to 300, 500, 1500 and 3000 mg L^{-1} Ag NPs or bulk, respectively. Arrow indicates the time when NPs or bulk were applied to roots. Curve obtained in the presence of 1 mM thiourea is indicated as "NPs + thiourea". (B) Dependence of mean peak increase in $[Ca^{2+}]_{cyt}$ (basal level was subtracted; ± SE; n = 5-6) on the applied concentrations of NPs, bulk or NP supernatant. (C) Mean peak $[Ca^{2+}]_{cyt}$ obtained upon application of 500 mg L^{-1} Ag NPs in the control (Ag NPs) and in the presence thiourea (1 mM), dimethyl sulfoxide (DMSO; 0.1 %) and Gd^{3+} (0.3 mM) (basal level was subtracted; ± SE; n = 5). The bath solution contained 2 mM Ca^{2+} (pH 6). "*" and "***": P<0.05 and P<0.001, respectively; "n.s." not significant (P>0.05) (panel B: compared to 3000 mg L^{-1} Ag NPs; panel C: compared to 500 mg L^{-1} Ag NPs).

Fig. 5. Generation of reactive oxygen species in response to Ag NPs. (A) Resurfin fluorescence (571/585 nm; Ampliflu Red™, Sigma; indicative to H_2O_2 production) and bright field images measured in *Arabidopsis thaliana* roots treated with Ag NPs and bulk (30-min exposure). Control buffer solution contained 0.1 mM Ca^{2+}, pH 6 by Tris/MES. (B) Mean fluorescence intensities of resurfin (n = 3; ± SE) obtained after 30 min treatment with 300-3000 mg L^{-1} Ag NPs and bulk ('0' – control without NPs). (C) Effects of 1 mM thiourea on the resurfin fluorescence (n = 5; SE), which was induced by 500 mg L^{-1} Ag NPs, and resurfin fluorescence in *rhd2* plants (lacking functional RBOHC) treated by 500 mg L^{-1} Ag NPs (n = 5; ± SE). Control solution: 0.3 mM $CaCl_2$, pH 6. Images were obtained using Olympus BX-41 fluorescence microscope. "*" and "**": P<0.05 and P<0.01, respectively; "n.s.": not significant (P>0.05) (comparison of NPs and bulk).

Fig. 6. Changes in ion currents induced by Ag NPs in the plasma membrane of root cell protoplasts. (A) Whole-cell currents obtained in control conditions and after addition of 1000 mg L^{-1} Ag NPs to the bathing solution. Mean (±SE) I-V curves obtained in the following bathing solutions: control, 1000 mg L^{-1} Ag NPs, 1000 mg L^{-1} Ag bulk, 1 µM Gd^{3+} and 1 µM Ag^{+}. (B) Mean I-V curves measured when 50 mM K^{+} was replaced by 10 mM TEA^{+} in the pipette solution (1000 mg L^{-1} Ag NPs were added to the bathing solution). (C) Mean I-V curves measured at 0.1 mM Ca^{2+} in the bathing solution instead 20 mM Ca^{2+}, which was normally used (1000 mg L^{-1} Ag NPs were added to the bathing solution). (D) Typical NP-induced Gd^{3+}-sensitive Ca^{2+} influx currents (holding potential = -80 mV) in excised outside-out plasma membrane patches (500 or 1500 mg L^{-1} Ag NPs were added to the bathing solution). (E) Mean (±SE) open probability values (NP_{open}; vertical axis) of Ag NP-activated currents measured after exogenous addition (1-2 min) of different concentrations of Ag NPs (horizontal axis). (F) Mean (±SE) Ag NP-induced currents measured after exogenous application (1-2 min) of 500 mg L^{-1} Ag NPs at different holding potentials (horizontal axis). Mean unitary conductance was determined as 56±5.5 pS (±SE; n = 6-7). (G) Mean (±SE) open probability values (NP_{open}; horizontal axis) of Ag NP-activated conductances measured after addition (1-2 min) of 500 mg L^{-1} Ag NPs in the presence of 10 mM TEACl, 0.1 mM $GdCl_3$, low bath Ca^{2+} (0.1 mM $CaCl_2$), 0.1 mM anthracene-9-carboxylic acid (A-9-CA), 50 µM verapamil and 1 mM thiourea. Pipette solutions (mM): 50 K^{+}, 45 $gluconate^{-}$ and 5 Cl^{-} (A and C); 10 TEA^{+}, 10 Cl^{-} (B); 50 Na^{+}, 45 $gluconate^{-}$ and 5 Cl^{-} (D-G). In all pipette solutions, 100 nM Ca^{2+} was adjusted with 1 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; pH 7.2 with 2 mM Tris/Mes; 290-300 mosM (adjusted by ultrapure sorbitol). Bathing solution (mM): 20 (0.1 in C) $CaCl_2$, 2 Tris, pH 5.8 (adjusted by Mes), 290-300 mosM (adjusted by sorbitol). Single channel activity (NP_{open}) was evaluated using current recorded at all open states (O_{1-n}).

Fig. 7. The effect of Ag NPs on generation of hydroxyl and ascorbyl radicals in cell-free conditions studied with Electron Paramagnetic Resonance Spectroscopy. (A) Examination of possible Fenton-like activities of Ag NPs. Top spectrum represents control generation of hydroxyl radical adducts of spin trap DMPO (40 mM) in the presence of 1 mM $CuCl_2$, 1 mM L-ascorbic acid (AA) and 1 mM H_2O_2. Bottom spectrum represents the lack of DMPO-hydroxyl radical adducts when 1 mM Cu^{2+} was replaced by 3000 mg L^{-1} Ag NPs. The analysis of the two-peak spectrum (observed in the bottom spectrum), which appeared after addition of Ag NPs, is shown in the panel B. (B) Typical EPR spectra of ascorbyl radical induced by addition of 3000 and 15000 mg L^{-1} Ag NPs to the solution containing 1 mM L-ascorbic acid. Second spectrum is control without AA (in the presence of Ag NPs). (C) Mean EPR signal intensities (ascorbyl radical spectra; $\pm$ SD; n = 7) obtained in different conditions (concentrations of Ag NPs, bulk and supernatant are given in mg L^{-1}; AA, D-mannitol, reduced glutathione, thiourea: 1 mM). "*" and "**": $P<0.05$ and $P<0.01$, respectively (compared to AA, ascorbic acid).

Fig. 8. The effect of Ag NPs on generation of ascorbyl radicals in intact roots studied with Electron Paramagnetic Resonance Spectroscopy. (A) Typical EPR spectra of ascorbyl radical induced by addition of 10 mM H_2O_2, 3000 mg L^{-1} Ag NPs and 15000 mg L^{-1} Ag NPs to the bathing solution (pH 7 adjusted by Tris/Mes, 0.1 mM $CaCl_2$). 15000 mg L-1 Ag NPs were also added together with 1 mM D-mannitol and 1 mM reduced glutathione. (B) Mean intensities of ascorbyl radical spectra (n = 4-6; $\pm$ SD) obtained in different conditions (concentrations of Ag NPs are given in mg L^{-1}; AA, D-mannitol and reduced glutathione: 1 mM). "**": $P<0.01$ (compared to 10 mM H_2O_2).