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Engineered Nanomaterial Exposure Affects  
Organelle Genetic Material Replication in  
*Arabidopsis thaliana*

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Organelle Genetic Material Replication in

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**Engineered nanomaterial exposure controls organelle  
genetic material replication in Arabidopsis thaliana**

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3 1 **Engineered nanomaterial exposure controls organelle genetic material replication**  
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34 15 **Abstract**  
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38 17 Although a robust literature has been investigating biological effects of engineered nanomaterial  
39 exposure (ENMs) at cellular, tissue and organism levels, wide differences in experimental design  
40 18 confound a systemic analysis. Mitochondria and chloroplast are not only the cellular energy sources  
41 but also have important regulatory and developmental roles in cell function. CeO<sub>2</sub>, FeOx ENMs, ZnS,  
42 CdS QDs and relative metal salts were utilized *in vitro* at different concentrations and times of  
43 19 exposures. Analysis of physiological and molecular response of *A. thaliana* chloroplast and  
44 mitochondrion demonstrates that ENMs modify functionality and organelle genome replication.  
45 20 Exposure to nanoscale CeO<sub>2</sub> and FeOx induced significant increase in biomass, whereas ZnS QDs  
46 and CdS QDs yielded neutral or negative effects on growth. Differential effects between ENMs and  
47 21 their corresponding metal salts highlight nanoscale-specific response pathways. Data from the  
48 22 different Fe forms suggest that the extent to which an ENM and its metal salt differ is a direct function  
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3 28 of ENM dissolution rate, toxicity of the metal ion released, and eventual biotransformation processes  
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5 29 occurring within the plant. With regard to specific effects on ptDNA and mtDNA, CdS QD exposure  
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7 30 triggered potential variations at sub-stoichiometric level in the two organellar genomes, while  
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9  
10 31 nanoscale FeOx and ZnS QDs caused an increase in ptDNA and mtDNA copy number. Nanoparticle  
11  
12 32 CeO<sub>2</sub> exposure did not affect ptDNA and mtDNA stoichiometry. These findings suggest that  
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14 33 modification in stoichiometry as potential morpho-functional adaptive response to ENMs exposure,  
15  
16 34 triggered by modifications of bioenergetic redox balance which leads to reduce the photosynthesis or  
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18 35 cellular respiration rate.  
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22  
23 37 **Keywords:** nanomaterials, copy number variation, mitochondria, chloroplasts, *Arabidopsis thaliana*.  
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31 40 In the last decade, a wide range of engineered nanomaterials (ENMs) of different types have  
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33 41 seen increasing use in industrial applications, consumer and medical products, and agriculture;  
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35 42 application of these materials is projected to continue increasing.<sup>1</sup> Concerns over the environmental  
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37 43 fate and effects of these materials have fostered studies to predict environmental concentrations in  
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39 44 air, water, and soil, as well as efforts to determine threshold concentrations for eco-toxicological  
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41 45 effects on terrestrial or aquatic biota.<sup>2</sup> Therefore, it is important to develop exposure biomarkers to  
42  
43 46 the different types of nanoparticles that are potentially accumulating in the environment.<sup>3</sup> These  
44  
45 47 assessments can be complicated by the nature of ENM use and release. For example, the occurrence  
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47 48 of ENMs in agriculture can be through intentional means to achieve benefits after uptake by plants  
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49 49 roots and foliage,<sup>4,5</sup> but also may occur incidentally through aerial deposition or presence in land  
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51 50 applied biosolids.  
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54  
55 51 A robust literature has developed in recent years evaluating plant interactions with ENMs,  
56  
57 52 from both an application and implication perspective,<sup>6</sup> and the involvement of both the chloroplast  
58  
59 53 and mitochondrion as key targets of ENMs response has become clear.<sup>7,8,9</sup> Plastids and mitochondria  
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3 54 are thought to have originated from independent endosymbiotic events,<sup>10</sup> wherein cyanobacteria-like  
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5 55 and  $\alpha$ -proteobacteria-like organisms became fully integrated with their host eukaryotic cells.  
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7 56 Although mitochondria were likely derived from a single endosymbiotic event, plastids are thought  
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9  
10 57 to have evolved through multiple endosymbiotic events.<sup>11</sup> Although many genes moved from the  
11  
12 58 organellar DNA to the “host” nucleus over evolutionary time, both plastids and mitochondria retain  
13  
14 59 their own DNA that is unique in terms of architecture, size and content.<sup>12,13</sup> As the mitochondria and  
15  
16 60 chloroplasts involve highly dynamic oxidation-reduction (RedOx) processes to support their  
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18 61 bioenergetic activity, the organelle genomes may be particularly sensitive to mutational damage  
19  
20 62 caused by excessive production of reactive oxygen species (ROS).<sup>10</sup> In addition, this organelle DNA  
21  
22 63 lacks the protective protein scaffold found in nuclear DNA, and the overall higher replication rate  
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24 64 make these genomes highly susceptible to mutation caused by a number of effectors (e.g. mutagens,  
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26 65 stressors, environmental conditions).<sup>10,14</sup> Due to their structure of prokaryotic origin (a collection of  
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28 66 linear, highly branched and occasionally circular molecules), limited size, and high copy number per  
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30 67 cell, the organellar genomes are subject to a physiological balance between fission/fusion processes  
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32 68 and selective pressure dynamics that lead to a certain degree of heteroplasmic variance.<sup>10,15</sup> These  
33  
34 69 recombinational phenomena are well known in the mitochondria of different organisms, from simple  
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36 70 eukaryotes (e.g. *Saccharomyces cerevisiae*) to plants and humans.<sup>10,12-14</sup> In plastids, these  
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38 71 mechanisms are recognizable, although their significance and their modes of action are still unclear.<sup>16</sup>  
39  
40 72 Indeed, selective effectors like abiotic stress may cause organellar DNA damage and/or copy number  
41  
42 73 variation, as reported for plastid DNA in *Arabidopsis thaliana* under salt stress.<sup>17</sup> In general, both the  
43  
44 74 whole number of plastids and mitochondria and their full functionality constitute an indicator of plant  
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46 75 health and as such, disturbance of these levels may hold a value as a biomarker of stress exposure.<sup>8</sup>  
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53 76 A number of ENMs present significant risk of exposure to plant materials. For example,  
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55 77 nanoscale CeO<sub>2</sub> has been investigated as a novel nano-fertilizer, with effects varying as a function of  
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57 78 both plant species and dose.<sup>18,19</sup> These applications have led to the frequent detection of CeO<sub>2</sub> ENMs  
58  
59 79 in the environment, with agricultural soil being the predominant sink.<sup>20</sup> The impact of nanoscale  
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3 80 Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>3</sub>O<sub>4</sub> on plants has been investigated by a number of groups, with variable results reported  
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5 81 and overall impact often depending on the EMN form utilized: reduced chlorophyll production and  
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7 82 biomass or improved root elongation.<sup>21-23</sup> For example, Kokina et al. (2017)<sup>24</sup> reported that Fe<sub>3</sub>O<sub>4</sub>  
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10 83 NPs did have beneficial effects on photosynthesis and growth. CdS QDs have exhibited toxicity to a  
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12 84 number of species.<sup>8,25,26</sup>

14 85 The present study assessed the potential genetic effects of a range of engineered nanomaterials  
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17 86 at different doses and exposure times. Importantly, measured endpoints included structural integrity  
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19 87 and abundance of the organelle genetic information, both mitochondrial DNA (mtDNA) and plastid  
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21 88 DNA (ptDNA). Two sets of experiments were conducted: the first focused on evaluating the  
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23  
24 89 responses of wild type *Arabidopsis thaliana* to CdS QDs, as well as corresponding metal salt, at  
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26 90 different treatment times and concentrations. The second compared plant response to CdS QDs with  
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28 91 the response to CeO<sub>2</sub>, Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub> ENMs and ZnS QDs, as well as their corresponding metal salt  
29  
30 92 forms. By evaluating the correlation between the physiological parameters associated with  
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33 93 mitochondrial and chloroplast function with the organellar DNA copy number, we aimed to uncover  
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35 94 the significance of organelle involvement in ENMs response and to demonstrate the potential utility  
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37 95 of mtDNA and ptDNA copy number modulation as biomarker of exposure.

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## 44 98 **Results and Discussion**

### 46 99 *Replication of ptDNA and mtDNA during CdS QDs and CdSO<sub>4</sub> exposure*

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49 100 As shown in Figure 1 and Table S1, treatment of plants with CdS QDs and CdSO<sub>4</sub> resulted in  
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51 101 different effects on ptDNA and mtDNA copy number and integrity, depending both on the exposure  
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53 102 time and concentration. All data were normalized to the untreated control (T0 NT). In addition, some  
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56 103 changes in ptDNA gene copy number are also evident in the controls T10 NT and T20 NT (*YCF1*  
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58 104 and *PSBD*) (Figure 1c). Considering the T10 Cd(II) and T20 Cd(II) treatments with CdSO<sub>4</sub>, it appears  
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60 105 that the copy numbers were quite similar across all markers considered. The genes that showed a

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3 106 significant copy number increase were *YCF1* and *PSBA*, while results obtained for *ORF31* (*PETL*)  
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5 107 and *PSBF* (very close on the ptDNA map) were more similar to the untreated controls (T10 NT, T20  
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8 108 NT) (Figure 1c, Table S1). For the treatments (T10 QD 40, T10 QD 80, T20 QD 40 and T20 QD 80),  
9  
10 109 the ptDNA gene copy number increased as observed for the CdSO<sub>4</sub> exposure. Similarity between the  
11  
12 110 effect of nanoscale and ionic Cd treatments increased with the time of exposure. This can be explained  
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14  
15 111 by the time-dependent increase in ionic Cd after long exposure to CdS QDs, and specifically to the  
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17 112 intracellular modification or biotransformation of CdS QD within the plant, as recently demonstrated  
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19 113 in *Arabidopsis thaliana* by Marmiroli *et al.* (2020).<sup>27</sup> When considering each single target gene more  
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22 114 closely, it is evident that the increase in ptDNA copy number was not generalized to the whole  
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24 115 organellar genome. For example, *PSAC* and *YCF1*, located in the short single copy region (SSC), as  
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26 116 well as *PSBA* and *PSBD*, located in long single copy region (LSC), all showed a consistent copy  
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29 117 number across all the treatments. Conversely, *ORF31* and *PSBF* showed a general similarity to the  
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31 118 untreated control in nearly all conditions, with the exception of T10 QD 40 (Figure 1c). This  
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33 119 phenomenon suggests a potential sub-stoichiometric shifting of the ptDNA, according to the  
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35 120 definition of Woloszynska & Trojanowski (2009),<sup>15</sup> has been previously observed during plant  
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38 121 development or stress response.<sup>10,17,28</sup> This can be considered a dynamic process by which the  
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40 122 organellar DNA copy number (or DNA fragments) may undergo changes over the course of a single  
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42 123 generation. Sub-stoichiometric shift was proposed as result of recombination increased frequency or  
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45 124 selective replication of DNA fragments, which may also occur by selective transmission of DNA  
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47 125 molecules during organelle division.<sup>15</sup> Interestingly, several ptDNA markers, including *ORF31*, are  
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49 126 co-expressed with the gene *ZATI2*, which encodes for a transcription factor involved in abiotic stress  
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52 127 response, and plays a key role in ROS signaling pathway.<sup>29</sup> Treatment with CdS QDs at 150-250 mg  
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54 128 L<sup>-1</sup> produced effects more similar to the untreated controls with regard to ptDNA gene copy numbers.  
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56 129 Since the growth inhibition concentration was previously estimated at 130 mg L<sup>-1</sup>,<sup>30</sup> this response is  
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58 130 suggestive of complete inhibition of the organelle functions, which results in the maintenance of the  
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60 131 ptDNA copy number during this high level of stress or during plant senescence.<sup>10</sup>

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3 132 The mtDNA analyses (Figure 1d, Table S1) had some similarity to those of ptDNA for the  
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5 133 CdSO<sub>4</sub> treatments (T10 Cd(II), T20 Cd(II) treatments). Specifically, mtDNA showed that for *COX1*  
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7 and *COB*, the gene copy number was increased in several treatments (T10 QD 80, T20 Cd(II) 50, T20  
8 134 Cd(II) 100), whereas *CCB206*, *CCB256* and *CCB382* remained largely unchanged. These findings  
9  
10 135 may suggest a potential mtDNA reorganization after exposure. It remains uncertain whether the  
11  
12 136 observed effects on mtDNA were due to replication or recombination (or both), and if they derived  
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14 137 directly from the Cd<sup>2+</sup> ion, considering its known genotoxic effects on nucleic acids,<sup>31</sup> or from the  
15  
16 138 stress-induced production of ROS related to CdS QDs interaction with the inner mitochondrial  
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18 139 structures.<sup>8,26,32</sup> Similar to what observed in ptDNA, the amplification of several mtDNA markers has  
19  
20 140 been associated with response to abiotic stress conditions, such as cold or drought.<sup>33</sup> Interestingly, by  
21  
22 141 comparing gene copy number and gene expression of the target genes (Figure S1 and Table S2), it is  
23  
24 142 possible to observe a correlation between stoichiometric modification of the organellar DNA and the  
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26 143 relative expression of the same mitochondrial genes.  
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#### 32 33 145 34 35 146 *Effect on organellar DNA during ENMs exposure*

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37 147 Analyses of gene copy number for ptDNA highlights some important features. The heatmaps  
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39 148 (Figure 1e, Table S1) show that for FeCl<sub>3</sub>, ZnSO<sub>4</sub>, ZnS QDs, Fe<sub>3</sub>O<sub>4</sub> ENMs treatments, the copy  
40  
41 149 number of the six target genes was uniformly increased, whereas a negligible effect was observed  
42  
43 150 upon exposure to Fe<sub>2</sub>O<sub>3</sub> NPs, CeO<sub>2</sub> NPs and CeCl<sub>3</sub> as compared with the untreated control. In all  
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45 151 cases, it is clear that the amplification phenomenon may involve all markers along the entire ptDNA  
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47 152 sequence, whereas for CdS QDs or CdSO<sub>4</sub> treatments, the amplification was specific only to certain  
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49 153 markers of ptDNA (Figure 1c, Table S1). A chord diagram was constructed (Figure 2a) to clarify the  
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51 154 relationships between the different amplification phenomena that occurred during treatment, in terms  
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53 155 of differences and commonalities. For example, Fe- and Zn-based treatments produced a significant  
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55 156 overlap, with all gene targets that increase in copy number ranging between 1 to 3-fold. For Cd-based  
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57 157 treatments, there were differential trends in amplification of the targets: increases of *YCF1* or *PSAC*,

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3 158 *PSBA*; decreases of *ORF31*, *PSBF*), whereas for Ce-based materials, there were no significant  
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5 159 changes as compared to the control.  
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7  
8 160 The presence of a specific metal in the treatment, as nanoscale or ionic form, determined the  
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10 161 differences and similarities in the responses. For example, in the Ce-based treatments (both as ionic  
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12 162 or nanoscale), the copy number was similar to the untreated controls, whereas for the Zn-based  
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14 163 treatments, there was an increase in ptDNA copy number. Treatment with different Fe-based  
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16 164 compounds showed an increase in ptDNA gene copy number with the order: Fe<sub>2</sub>O<sub>3</sub> ENMs < Fe<sub>3</sub>O<sub>4</sub>  
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18 165 ENMs < FeCl<sub>3</sub>. A potential explanation may be related to the differential stability of the three forms  
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20 166 of Fe (Tables 1, 2). While the FeCl<sub>3</sub> salt has a theoretical value of dissolution (in ddH<sub>2</sub>O) of 100%,  
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22 167 the Fe<sub>2</sub>O<sub>3</sub> and Fe<sub>3</sub>O<sub>4</sub> ENMs showed a much lower percentage of dissolution (Table 1), suggesting a  
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24 168 correlation between ptDNA amplification and with ROS generation by Fe ion presence,<sup>17,34,35</sup> and  
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26 169 with the modulation of the cellular redox state.<sup>10,28,36</sup> ZnSO<sub>4</sub> and ZnS QDs treatments caused a  
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28 170 significant amplification in ptDNA markers; this effect could be partially attributed to released Zn  
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30 171 ions, but this does not explain the entirety of the effects with ZnS QDs, whose dissolution is limited  
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32 172 in ddH<sub>2</sub>O (Table 1). This may suggest, as for CdS QDs, that in planta biotransformation processes  
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34 173 were responsible for the generation of active Zn or Cd forms within the tissues that in turn promoted  
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36 174 ptDNA amplification.<sup>27</sup>  
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42 175 A similar trend in target gene amplification was observed for mtDNA (Figures 1f, 2b; Table  
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44 176 S1). Specifically, FeCl<sub>3</sub>, ZnSO<sub>4</sub>, ZnS QDs, and Fe<sub>3</sub>O<sub>4</sub> ENMs treatments caused a significant increase  
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46 177 in copy number, with values in the range of 1 to 3-fold for all the target genes analyzed. Conversely,  
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48 178 exposure to Fe<sub>2</sub>O<sub>3</sub> ENMs, CeO<sub>2</sub> ENMs and CeCl<sub>3</sub> decreased or had no effect on copy number.  
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50 179 Treatment with CdSO<sub>4</sub> and CdS QDs induced a different response, causing a 2-fold decrease in copy  
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52 180 number for the target genes *CCB382* and *CCB206*. Comparison between the amplification profiles of  
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54 181 ptDNA and mtDNA suggests that for these treatments, a general uniform response was evident for  
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56 182 both organelles with regard to DNA replication.  
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### 184 *Effects of ENMs treatments on plant physiology*

185 The results of the physiological analyses from plants exposed to the ENMs and the  
186 corresponding metal salts are reported in Figure 3 and Tables S3-S5. Treatment with Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub>  
187 and CeO<sub>2</sub> ENMs doubled the total biomass, as compared to the untreated control (Figures 3, S2; Table  
188 S3). Conversely, a decrease in biomass was evident after exposure to the four metal salts (CeCl<sub>3</sub>,  
189 FeCl<sub>3</sub>, ZnSO<sub>4</sub> and CdSO<sub>4</sub>) and to CdS QDs (Figure 3). ZnS QDs treatment had no effect on biomass,  
190 with results equivalent to the untreated control. Normalizing the biomass parameter of the treated  
191 samples to the control value allowed to distinguish three effects on biomass: positive, neutral and  
192 negative (Figure S2). With regard to photosynthetic efficiency (Table S4), Fe<sub>3</sub>O<sub>4</sub> NPs exposure  
193 caused a marked increase in chlorophyll *a* and *b* accumulation, respectively;  $1.852 \pm 0.636$  and  $0.767$   
194  $\pm 0.197$ , as compared with the control values of  $0.989 \pm 0.367$  and  $0.535 \pm 0.271$ , respectively. Plants  
195 treated with CdSO<sub>4</sub> showed a significant decrease in chlorophyll *a*, and exposure to CeCl<sub>3</sub>, ZnSO<sub>4</sub>,  
196 ZnS QDs, CdSO<sub>4</sub>, CdS QDs significantly decreased chlorophyll *b* production. No significant  
197 differences were observed in carotenoid levels of the treatments, because this class of pigments is  
198 rapidly deployed to quench the initial ROS burst likely induced by exposure.<sup>26</sup> For CdS QDs, the  
199 results were consistent with previous studies of exposure at different concentrations and exposure  
200 times,<sup>26</sup> where the biomass, and photosynthetic pigments were significantly decreased. Analyzing the  
201 results from the plant cell respiration (TTC assay, Table S5), exposure to Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub> and CeO<sub>2</sub>  
202 ENMs treatments significantly increased respiration, suggesting adequate mitochondrial functionality  
203 and aligning with the positive effects on plant biomass. Conversely CdSO<sub>4</sub> and CdS QDs treatments  
204 showed a decrease in respiration, suggesting an inhibition of mitochondrial function. These findings  
205 are also consistent with the observed biomass reductions for these treatments.<sup>8,26</sup>

206 The effects of nanoscale Fe<sub>2</sub>O<sub>3</sub>, Fe<sub>3</sub>O<sub>4</sub> and CeO<sub>2</sub> treatments differed from those obtained with  
207 corresponding ionic forms. Concerning CeO<sub>2</sub> NPs and CeCl<sub>3</sub>, the results obtained fit with reports for  
208 *Arabidopsis thaliana*<sup>37</sup> and other crops species.<sup>3,9,18,38</sup> Nanoscale CeO<sub>2</sub> stimulates vegetative growth;  
209 the effect being related to the antioxidant properties of co-existing Ce<sup>4+</sup> and Ce<sup>3+</sup> valence forms.<sup>39,40</sup>

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3 210 For the iron oxide-based ENMs ( $\text{Fe}_2\text{O}_3$ ,  $\text{Fe}_3\text{O}_4$ ), the results are also in keeping with the current  
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5 211 literature, where positive or neutral effects on the vegetative growth of *Arabidopsis thaliana* and  
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7 212 *Triticum aestivum* L.<sup>24,41</sup> have been reported, but also with negative effects on biomass during  $\text{FeCl}_3$   
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9  
10 213 exposure.<sup>42</sup> With regard to CdS QDs and  $\text{CdSO}_4$ , the negative effects on biomass and other  
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12 214 physiological parameters were also consistent with previous studies<sup>26,30</sup> as with a strong decrease in  
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14 215 photosynthetic activity and cellular respiration as a consequence of disrupted organellar functions  
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16  
17 216 with increasing exposure time and CdS QDs concentration.<sup>8</sup> It has been demonstrated that CdS QDs  
18  
19 217 and  $\text{CdSO}_4$  trigger different regulatory responses at the level of mRNA and proteins.<sup>25,27,30,37,43</sup>  
20  
21 218 Concerning the effects observed for ZnS QDs, the literature related to this pristine type of QD is  
22  
23  
24 219 minimal. However, information related to ZnO NPs effects on plant species is abundant,<sup>9</sup> and mostly  
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26 220 in agreement with the results reported in Ruotolo *et al.* (2018):<sup>37</sup> decreased chlorophyll production  
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28 221 and induction of secondary metabolite biosynthesis.

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### 32 33 223 *Analysis of physiological and molecular components of ENM and ionic response*

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35 224 A Principal Component Analysis (PCA) based model was constructed for both the  
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37 225 mitochondria and chloroplasts to provide a mechanistic interpretation of each organelle involvement  
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39  
40 226 in response to ionic and nanoscale metal exposure and to explain the positive, neutral and negative  
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42 227 effects on ptDNA and mtDNA replication and that plant physiological effects (Figure 4). The  
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44 228 observed effects depend mainly on i) the elemental composition of the ENM, ii) whether the form  
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47 229 was nanoscale or ionic, iii) the physico-chemical stability of the ENM in water, as compared with  
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49 230 salt taken as reference. The parameters considered for this analysis were: biomass index (Figure S2),  
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51 231 ptDNA and mtDNA copy number variation (Figure 1), photosynthetic activity (Table S4) and cellular  
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53 232 respiration (Table S5). Analyzing the two major components based on the total variance revealed that  
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56 233 for the chloroplast and mitochondrion, a similar correlation was observed, not only demonstrating  
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58 234 how the effects were common to both organelles but also that the overall organellar function was  
59  
60 235 deterministic of the overall plant phenotypic response under stress conditions.<sup>14,44</sup> For the chloroplast,

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3 236 the first two components represent 51.6% and 34.7% of the total variance (Figure 4a), whereas for  
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5 237 mitochondrion, the first two components represent the 63.2% and 33.5% of the total variance (Figure  
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7  
8 238 4b). In both cases, the first component was determined by the type and form of the compound used  
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10 239 (nanoscale or ionic form), as shown by the different effects between nanoscale FeOx or CeOx-based  
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12 240 treatments and their associated ionic forms in terms of biomass index. Conversely, for CdS QD and  
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14 241 ZnS QD treatments, the response was similar to that obtained with the respective ionic forms. In the  
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17 242 case of ZnS QDs, it should be considered that the release of Zn<sup>2+</sup> ions is higher than Cd<sup>2+</sup> from CdS  
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19 243 QDs, at least when considering dissolution in water (Tables 1, 2). However, ZnS QDs dissolution  
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21  
22 244 does not completely explain the observed results, suggesting that biotransformation phenomena may  
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24 245 occur within the plant tissues and organs after ZnS QDs uptake. Indeed, for CdS QDs Marmioli *et*  
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26 246 *al.* (2020)<sup>27</sup> recently demonstrated that particle biotransformation can occur within the plant cell,  
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29 247 resulting in a chemical form that is not consistent with ionic Cd. This difference between ionic and  
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31 248 nanoscale form has been also demonstrated through unique genetic responses to CdS QDs and Cd<sup>2+</sup>  
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33 249 exposure,<sup>8,30</sup> and by a nanoscale specific proteomic response.<sup>43</sup>

35 250 The second component (Figure 4a) is related to the ptDNA and mtDNA copy number,  
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37  
38 251 separating the treatments that triggered a copy number increase (Fe<sub>3</sub>O<sub>4</sub> NPs, FeCl<sub>3</sub>, ZnS QDs, ZnSO<sub>4</sub>)  
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40 252 from those that had no effect (CeCl<sub>3</sub>, CeO<sub>2</sub> NPs, Fe<sub>2</sub>O<sub>3</sub> NPs) or those that had a non-uniform impact  
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42 253 on ptDNA and mtDNA (CdS QDs, CdSO<sub>4</sub>). With the Ce-based treatments, the effects observed on  
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44  
45 254 the biomass were different (Figure 3; Table S3), whereas the effects on ptDNA and mtDNA copy  
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47 255 number were uniformly non-significant (Figure 1). These results are consistent with the fact that CeO<sub>2</sub>  
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49 256 ENMs did not interfere with the functionality of either organelle, but did promote a ROS scavenging  
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52 257 activity within the plant cell.<sup>39,40</sup> Conversely, CeCl<sub>3</sub> decreased the biomass production, as observed  
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54 258 also in other plant species.<sup>3</sup> Interestingly, the Fe-based treatments decreased mitochondrial and  
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56 259 chloroplast function (Figure 3, Tables S4, S5), but not the biomass index (Figure S2), and these  
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58 260 materials caused an increase in ptDNA and mtDNA copy number. As noted above, this phenomenon  
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60 261 is likely a function of the increase in Fe<sup>2+/3+</sup> concentration within the plant cell from FeCl<sub>3</sub> and,

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3 262 partially, from FeOx (Table 1). Ionic Fe can increase ROS production through Fenton reactions that  
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5 263 may trigger a stoichiometric increase in ptDNA and mtDNA in order to balance the physiological  
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8 264 need of organelle function.<sup>10,14,28,36,45</sup>

9  
10 265 In analyzing the physiological effects of exposure, it is clear that ptDNA and mtDNA copy  
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12 266 number increased when biomass and photosynthesis activity or cellular respiration decreased. This  
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15 267 may be an evidence of an interplay between the morpho-functional integration of the organelles and  
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17 268 their replication/recombination machinery.<sup>10,46,47</sup> Upon exposure to an external stress, mtDNA and  
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19 269 ptDNA were able to alleviate and accommodate the functional damage by modulating the copy  
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22 270 number of some of their essential genes (Figure 1, Table S1). Evidence of this relationship can also  
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24 271 be found at the gene expression level (Figure S1, Table S2), suggesting a role in response and  
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26 272 adaptation to a broad range of different stressors.<sup>29,33</sup> Another consideration is that the oxidative stress  
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29 273 generated by some ENMs or equivalent salts could also be an effector of the response at the systemic  
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31 274 level, including functional modifications in the regulation of chloroplast and mitochondrial DNA  
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33 275 replication.<sup>7,8</sup> This mechanism may be peculiar for organellar genomes and for their ability to adjust  
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35 276 gene expression levels during stress conditions.

## 37 38 277 39 40 278 **Conclusions**

41  
42 279 The primary finding of significance in this study is that organellar DNA molecules may  
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45 280 change drastically in copy number during exposure to select metals in either nanoscale or ionic form.  
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47 281 These nucleic acid effects may or may not be correlated with overt physiological changes in the plants.  
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49 282 Importantly, the different physiological effects between ENMs and their corresponding metal salts  
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51 283 highlight nanoscale-specific response pathways. These size-specific changes may rest upon  
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54 284 differential metal availability from the salt or nanoscale forms, from the inherent metal toxicity  
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56 285 (Cd>Zn>Fe>Ce), and from biotransformation processes that impact the material in the plant.<sup>27</sup> CdS  
57  
58 286 QDs and CdSO<sub>4</sub> show the greatest potential to modify both ptDNA and mtDNA copy number,  
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60 287 whereas CeO<sub>2</sub> NPs does not affect organellar DNAs. The results suggest that the effect on organellar

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288 DNA replication is a plant adaptive response to the stress imposed by metal exposure and to the  
289 subsequent ROS production at organellar level.<sup>10-14</sup> A similar phenomenon was described for  
290 organellar DNAs in developing plants.<sup>12,48</sup> With recent experimental evidence, the conventional idea  
291 of organellar DNAs as exclusively closed loops have been updated with the idea that organellar DNA  
292 contains both linear and circular molecules.<sup>12</sup> During stress conditions, organellar proteins involved  
293 in DNA repair/replication, persist at similar expression levels by increasing the copy number of  
294 specific genes, as occurs for organellar ribosomes components.<sup>49</sup> This type of regulation appears  
295 increasingly relevant when considering all the cases of DNA abandonment where selected copies of  
296 organellar DNA (exclusively) are degraded because of damage accumulated during replication under  
297 conditions of stress.<sup>50</sup> Molecular integrity can change drastically during exposure to some metals in  
298 ionic and nanoscale forms as a consequence of inhibition of respiration and photosynthesis, as well  
299 as from activation of photorespiration and ROS production.<sup>10,14</sup> Disturbance at the organelle level  
300 produces a retrograde hormone signaling, likely modulated by ROS.<sup>51</sup> This may impact nuclear gene  
301 expression related to growth and development, as well as abiotic stress response. Abandonment of  
302 larger (unrepaired) organellar DNA molecules in this condition and replication of smaller circular  
303 DNAs in the organelles effectively block lesion transmission and modulate the availability of some  
304 organellar genes to the existing treatment stress (Figure 5). Interestingly, the same nanoscale or ionic  
305 responses were observed both for ptDNA and mtDNA. An explanation can be coordination to  
306 produce dual localized protein complexes involved in DNA replication/repair that are targeted to both  
307 chloroplast and mitochondria.<sup>48,52</sup> In this way, mitochondria and plastids respond similarly to stimuli  
308 such as metal stress, which change the redox state of the cell. When the need for protein involved in  
309 repair and replication is increased, the production of additional DNA able to restore organellar  
310 functionality is also increased.<sup>10,12,14</sup>

311 This study highlights the important relationship between ptDNA and mtDNA replication and  
312 organelle function, and serves as further evidence of the significant role of chloroplast and  
313 mitochondria in mediating plant response and adaptation to stress induced by exposure to metals in

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3 314 nanoscale or salt forms. As such, changes in the copy number of ptDNA and mtDNA may be  
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5 315 considered a putative biomarker of metal exposure (in both nanoscale or ionic form) in higher  
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8 316 eukaryotes. This tool could be used when assessing potential exposure to ENMs, as well as for the  
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10 317 monitoring of ENM presence and transfer through different environmental compartments.

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## 13 14 15 319 **Experimental section**

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### 18 19 321 *ENMs characterization*

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22 322 CdS quantum dots (QDs), ZnS QDs, Fe<sub>2</sub>O<sub>3</sub> NPs, and Fe<sub>3</sub>O<sub>4</sub> NPs were synthesized by IMEM-  
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24 323 CNR (Parma, Italy). CeO<sub>2</sub> NPs were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany).  
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26 324 All ENMs were characterized by X-ray diffraction (XRD) and transmission electron microscopy  
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28 325 (TEM, Talos F200S G2, SEM FEG Thermo Fisher Scientific, Waltham, MA, USA). Additional  
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31 326 information on ENM synthesis is reported in the Supporting Information (SI). The average particle  
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33 327 size (*dh*) and zeta ( $\zeta$ ) potential of the ENMs (100 mg L<sup>-1</sup>) were determined in ddH<sub>2</sub>O on a Zetasizer  
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35 328 Nano Series ZS90 (Malvern Instruments, Malvern, UK). ENM dissolution was investigated by  
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38 329 ultracentrifugation ENMs standards (100 mg L<sup>-1</sup>) prepared in ddH<sub>2</sub>O after 20 days at 30000 rpm, 10  
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40 330 min, 20°C (Optima Max-XP Ultracentrifuge, Beckman-Coulter Inc., Brea, CA, USA). A 1 ml aliquot  
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42 331 of the resulting solutions was digested in 4 mL of 1M HNO<sub>3</sub> for 20 min at 200°C using a VELP DK20  
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45 332 digester (VELP Scientifica, Usmate, Italy). The digests were analyzed by flame atomic absorption  
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47 333 spectroscopy (FA-AAS) (AA240FS, Agilent Technologies, Santa Clara, CA, USA). ENM  
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49 334 characterization and dissolution data are reported in Table 1, Figure 6 and Figures S3-S7.

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### 52 53 54 336 *Plant treatments*

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56 337 Wild type *Arabidopsis thaliana* (L.) Heynh, ecotype Landsberg erecta (Ler-0) was grown on  
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58 338 a Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) containing  
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339 1% w/v sucrose, 0.8% w/v agar, at 24°C. Plants were grown under 30% relative humidity with a 16

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3 340 h photoperiod (light intensity  $120 \mu\text{M m}^{-2} \text{s}^{-1}$  photosynthetic photon flux). After 10d of growth on  
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5 341 non-treated MS medium, the seedlings were transferred to MS medium amended with the individual  
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8 342 ENMs at a range of concentrations for different exposure periods as reported in Table 2. The ENMs  
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10 343 were probe sonicated by a Fisher Scientific Model 505 Sonic Dismembrator (Fisher Scientific,  
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12 344 Waltham, MA) at 40% amplitude for 60s to maximize dispersion before addition to the MS medium.  
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15 345 The respective salts were included as controls. All the treatments were conducted with ten biological  
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17 346 replicates.

#### 18 19 347 20 21 22 348 *Real Time qPCR on mtDNA and ptDNA*

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24 349 DNA extraction was performed from the whole plant tissues using the 2x CTAB protocol.<sup>53</sup>  
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26 350 The copy number for mtDNA and ptDNA selected markers (Figure 1; Table S6) was determined by  
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28 351 Real Time quantitative PCR (qPCR) using Applied Biosystems Power SYBR Green Master Mix  
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31 352 chemistry (Applied Biosystems, Foster City, CA, USA) in an optical 96-well plate with the Applied  
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33 353 Biosystems ABI PRISM 7900HT Sequence Detection System. Specific primers for each selected  
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35 354 target were designed (Table S6) using the Primer3 software (primer3.ut.ee). Target genes were chosen  
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38 355 on the basis of their position on the mtDNA and ptDNA biomolecules, so as to effectively characterize  
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40 356 the organellar DNA sequences (Figure 1a, b).<sup>10</sup> Amplification followed the thermal profile: 95°C for  
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42 357 10", 95°C for 15" and 60°C for 60" (for 40 cycles). Synthesized primers (Sigma-Aldrich, St. Louis,  
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45 358 MO, USA) were assessed by qPCR in four serial dilutions (1, 1:10, 1:100, 1:1000). Amplicons were  
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47 359 confirmed by size and sequencing, performed by BMR Genomics service (Padova, ITA). Alignments  
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49 360 were performed by BLAST tools (blast.ncbi.nlm.nih.gov/Blast.cgi). The relative quantity of DNA  
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52 361 was estimated through the  $\Delta\Delta\text{Ct}$  method, using *rrn16* (encoding rRNA16) as reference gene. The  
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54 362 relative quantity of each DNA sample was determined by normalization using the reference gene  
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56 363 number of copies, and the arithmetic mean was calculated for three independent repeated reactions.

#### 57 58 364 59 60 365 *Physiological Parameters*

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3 366 Several physiological parameters were analyzed after plant harvest: fresh biomass,  
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5 367 photosynthetic activity, and cellular respiration were determined according to the protocols below.  
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8 368 The biomass index was calculated as the ratio between the fresh weight biomass values of each  
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10 369 different treatments versus the untreated control. Photosynthetic pigment content was evaluated  
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12 370 according to Ni *et al.*, (2009)<sup>54</sup> in order to assess photosynthetic activity. Briefly, portions of leaves  
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15 371 (200 mg) were suspended in 800  $\mu$ L 95% acetone. After incubation for 10 min on ice, the samples  
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17 372 were centrifuged at 3000g and 4°C for 10 min, and chlorophyll *a*, chlorophyll *b*, and total carotenoids  
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19 373 were determined by spectrophotometric analysis (Varian Cary 50, Agilent Technologies, Santa Clara,  
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22 374 CA, USA) at 662 nm, 647 nm, 480 nm, respectively. The TTC (2,3,5-triphenyltetrazolium chloride)  
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24 375 reduction assay was used as a quantitative method to assess the plant cell viability through respiration  
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26 376 activity.<sup>55</sup> A 200 mg aliquot of fresh tissue was added to 3 mL of TTC buffer (TTC 0.18 M, 78%  
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29 377 Na<sub>2</sub>HPO<sub>4</sub> 0.05 M, 22% KH<sub>2</sub>PO<sub>4</sub> 0.05 M). Samples were incubated at 30°C for 15h. Formazan, which  
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31 378 results from TTC reduction, was extracted in 10 ml of 95% ethanol for 10 min at 80°C and was  
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33 379 quantified spectrophotometrically at 530 nm (Varian Cary 50, Agilent Technologies, USA).

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### 37 381 *Statistics and bioinformatic analysis*

38 382 Physiological parameters were evaluated by a one-way ANOVA with a pairwise Tukey's  
39  
40 383 multiple comparison test (IBM SPSS v. 26.0). A two-tail Student *t* test was also applied to qPCR  
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42 384 results. Chord diagram multiple comparisons were constructed through the Circos table viewer tool  
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45 385 (<http://mkweb.bcgsc.ca/tableviewer/>). The R software (<https://www.r-project.org/>) was used for gene  
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47 386 clustering and principal component analysis (PCA) of different treatments.  
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### 52 388 **Author contributions**

53  
54 389 NM, MM, with contribution of JCW and LP supervised the project and designed all the  
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56 390 experiments. LP, JM and RR performed the experiments with the contribution of MM. MV and AZ  
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59 391 provided the FeOx ENMs, CdS QDs and ZnS QDs. All authors contributed and approved the final  
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17  
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26 402 **Notes**  
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28 403 The authors declare no competing financial interest.  
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35 406 **Supporting Information (SI)**  
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37 407 Supporting information (SI) contain methods related to ENMs synthesis and characterization,  
38  
39 408 sequence alignments and total RNA extraction with relative comparison to DNA amplification.  
40  
41 409 Figure S1: schematic representation of the dissolution protocol. Figure S2: Biomass Index  
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43 410 representation. Figures S3-S6: ENMs characterization by XRD; Figure S7: CdS QDs treatments  
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45 411 comparison (gene copy number vs gene expression); Tables S1-S2: data from heatmaps (included in  
46  
47 412 Figures 1, S1); Tables S3-S5: physiological parameters measurements and statistics; Table S6:  
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49 413 primers sequences utilized in qPCR analyses.  
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8 574 **Figure captions**

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12 576 **Figure 1.** Target gene copy number variations on ptDNA and mtDNA. Schematic representation of  
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14 *A. thaliana* ptDNA and mtDNA and relative position of the target genes taken into account (a, b).  
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17 578 LSC: long single copy region; SSC: short single copy region; IRA, IRB: inverted repeated regions.  
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19 579 Target genes were chosen so as to cover the entire ptDNA and mtDNA. Details related to the target  
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21 genes are reported in the Supporting Information (SI). Distances were calculated on the basis of the  
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24 581 NCBI reference sequences: chloroplast (AP000423.1), mitochondrion (NC\_037304.1).  
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26 582 Heatmaps representing the chloroplast (c) and mitochondrial (d) relative target gene abundance  
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28 583 during treatment with CdS QDs or CdSO<sub>4</sub>, at different times of exposure and concentrations (Table  
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30 2). Data, normalized on the untreated control (T0 NT), showed a non-uniform target gene abundance  
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33 585 across the entire ptDNA and mtDNA structures.  
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35 586 Relative target gene abundance of chloroplast (e) and mitochondrial (f) target genes taken into  
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37 account during treatments with different ENMs and relative metal salts (Table 2). Data, normalized  
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40 588 on the control untreated, highlighted the different ranges of similarity in the response (measured as  
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42 589 relative target gene abundance) between the treatments with ENMs and relative metal ion  
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44 counterparts. Numerical data are reported in Table S1.  
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49 592 **Figure 2.** Chord diagram graphical comparison between the relative gene abundance under treatment  
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51 593 with ENM or relative metal salts in the chloroplast (a) and mitochondrion (b). Ribbons show the  
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53 overlap between over- and under-abundant genes in term of copy number, normalized on the  
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56 595 untreated control in the different treatments performed.  
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60 597 **Figure 3.** Heatmaps representing the physiological parameters measured: fresh biomass (mg);

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3 598 photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and carotenoids absorbance) and cellular  
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5 599 respiration (formazan absorbance). Data are reported in shades from lilac (higher values) to light blue  
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8 600 (lower values). Data and statistics are reported in Tables S3-S5.  
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12 602 **Figure 4.** Principal Component Analysis (PCA) related to the effects on the chloroplast (a) and  
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14 603 mitochondrion (b). For the chloroplast, parameters taken into account are the relative target gene copy  
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16 604 number (ptDNA response), photosynthetic activity and biomass; for mitochondrion, the relative  
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18 605 target gene copy number (mtDNA response), cellular respiration and biomass. The first component,  
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20 606 representing 51.59% and 63.15% of the total variance for chloroplast and mitochondrion, respectively,  
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22 607 is related to differential effects between the ENMs and their metal ion counterparts, as supported by  
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24 608 biomass indices and DNA analyses.  
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30 611 **Figure 5.** Representation of mechanisms involved in the organellar DNA replication in response to  
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32 612 oxidative stress due to ENMs exposure, with particular regard to CdS QDs. Molecular integrity can  
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34 613 change drastically as a consequence of inhibition of respiration and photosynthesis, as well as from  
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36 614 activation of photorespiration and ROS production. Abandonment of unrepaired organellar DNA  
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38 615 molecules and replication of smaller DNA fragments have the role of blocking lesion transmission  
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40 616 and modulating the availability of several gene functions.  
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45 619 **Figure 6.** ENMs characterization by transmission electron microscopy (TEM): (a) CeO<sub>2</sub> NPs, (b)  
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47 620 Fe<sub>2</sub>O<sub>3</sub> NPs, (c) Fe<sub>3</sub>O<sub>4</sub> NPs, (d) ZnS QDs, (e) CdS QDs. Additional characterization data are reported  
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49 621 in Table 1 and in the Supporting Information (SI).  
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## Tables

**Table 1.** ENMs characterization related to aggregate average particle size ( $dh$ ), zeta-potential ( $\zeta$ ), and dissolution after 20 days (in ddH<sub>2</sub>O).

ENM	size (nm)	purity (%)	$\zeta$ Z-potential (mV)	$(dh)$ hydrodynamic		
				range (nm)	%metal	%dissolution
CeO <sub>2</sub> NPs	<25	99.99	+ 42.5	243.9	81.3	1
Fe <sub>2</sub> O <sub>3</sub> NPs	<15	99.9	+ 3.8	978	69.8	16
Fe <sub>3</sub> O <sub>4</sub> NPs	<10	99.9	+ 44.2	271.6	72.4	13
ZnS QDs	<5	99.99	+ 61.6	1190	63.2	3
CdS QDs	<5	99.99	+ 15.8	178.7	78	2

27 630 Concentration utilized for dissolution experiments was 100 mg L<sup>-1</sup> for all the ENMs standards. Additional information  
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29 631 related to ENM characterization has been reported in the Supporting Information (SI).

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**Table 2.** ENMs treatments exposure time and concentrations used in experiment 1(a) and experiment  
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40 636 2(b).

### (a)

name	exposure time	treatment	concentration	metal content	metal in solution*
T0 NT	0 days	untreated	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>
T10 NT	10 days	untreated	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>
T10 QD 40	10 days	CdS QDs	40 mg L <sup>-1</sup>	31.2 mg L <sup>-1</sup>	0.62 mg L <sup>-1</sup>
T10 QD 80	10 days	CdS QDs	80 mg L <sup>-1</sup>	62.4 mg L <sup>-1</sup>	1.24 mg L <sup>-1</sup>
T10 QD 150	10 days	CdS QDs	150 mg L <sup>-1</sup>	117 mg L <sup>-1</sup>	2.34 mg L <sup>-1</sup>
T10 QD 250	10 days	CdS QDs	250 mg L <sup>-1</sup>	195 mg L <sup>-1</sup>	3.90 mg L <sup>-1</sup>
T10 Cd(II) 50	10 days	CdSO <sub>4</sub> ·7H <sub>2</sub> O	38.4 mg L <sup>-1</sup>	12.8 mg L <sup>-1</sup>	12.8 mg L <sup>-1</sup>
T10 Cd(II) 100	10 days	CdSO <sub>4</sub> ·7H <sub>2</sub> O	76.9 mg L <sup>-1</sup>	25.6 mg L <sup>-1</sup>	25.6 mg L <sup>-1</sup>
T20 NT	20 days	untreated	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>
T20 QD 40	20 days	CdS QDs	40 mg L <sup>-1</sup>	31.2 mg L <sup>-1</sup>	0.62 mg L <sup>-1</sup>
T20 QD 80	20 days	CdS QDs	80 mg L <sup>-1</sup>	62.4 mg L <sup>-1</sup>	1.24 mg L <sup>-1</sup>
T20 QD 150	20 days	CdS QDs	150 mg L <sup>-1</sup>	117 mg L <sup>-1</sup>	2.34 mg L <sup>-1</sup>
T20 QD 250	20 days	CdS QDs	250 mg L <sup>-1</sup>	195 mg L <sup>-1</sup>	3.90 mg L <sup>-1</sup>

T20 Cd(II) 50	20 days	CdSO <sub>4</sub> ·7H <sub>2</sub> O	38.4 mg L <sup>-1</sup>	12.8 mg L <sup>-1</sup>	12.8 mg L <sup>-1</sup>
T20 Cd(II) 100	20 days	CdSO <sub>4</sub> ·7H <sub>2</sub> O	76.9 mg L <sup>-1</sup>	25.6 mg L <sup>-1</sup>	25.6 mg L <sup>-1</sup>

**(b)**

<b>name</b>	<b>exposure time</b>	<b>treatment</b>	<b>concentration</b>	<b>metal content</b>	<b>metal in solution*</b>
untreated	20 days	untreated	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>	0 mg L <sup>-1</sup>
CeO <sub>2</sub> NPs	20 days	CeO <sub>2</sub> NPs	500 mg L <sup>-1</sup>	407 mg L <sup>-1</sup>	4.07 mg L <sup>-1</sup>
CeCl <sub>3</sub>	20 days	CeCl <sub>3</sub>	175 mg L <sup>-1</sup>	99.3 mg L <sup>-1</sup>	99.3 mg L <sup>-1</sup>
Fe <sub>2</sub> O <sub>3</sub> NPs	20 days	Fe <sub>2</sub> O <sub>3</sub> NPs	500 mg L <sup>-1</sup>	349.9 mg L <sup>-1</sup>	55.98 mg L <sup>-1</sup>
Fe <sub>3</sub> O <sub>4</sub> NPs	20 days	Fe <sub>3</sub> O <sub>4</sub> NPs	500 mg L <sup>-1</sup>	361.4 mg L <sup>-1</sup>	46.98 mg L <sup>-1</sup>
FeCl <sub>3</sub>	20 days	FeCl <sub>3</sub>	75 mg L <sup>-1</sup>	25.4 mg L <sup>-1</sup>	25.4 mg L <sup>-1</sup>
ZnS QDs	20 days	ZnS QDs	500 mg L <sup>-1</sup>	334 mg L <sup>-1</sup>	6.64 mg L <sup>-1</sup>
ZnSO <sub>4</sub>	20 days	ZnSO <sub>4</sub>	175 mg L <sup>-1</sup>	70.8 mg L <sup>-1</sup>	70.8 mg L <sup>-1</sup>
CdS QDs	20 days	CdS QDs	80 mg L <sup>-1</sup>	62.4 mg L <sup>-1</sup>	1.24 mg L <sup>-1</sup>
CdSO <sub>4</sub>	20 days	CdSO <sub>4</sub> ·7H <sub>2</sub> O	76.9 mg L <sup>-1</sup>	25.6 mg L <sup>-1</sup>	25.6 mg L <sup>-1</sup>

After 10 days growth on unamended MS medium, plants were transferred on to MS medium amended with the desired amount of ENMs or metal salts, (\*) expected metal released in ddH<sub>2</sub>O calculated from ENMs dissolution studies (Table 1).

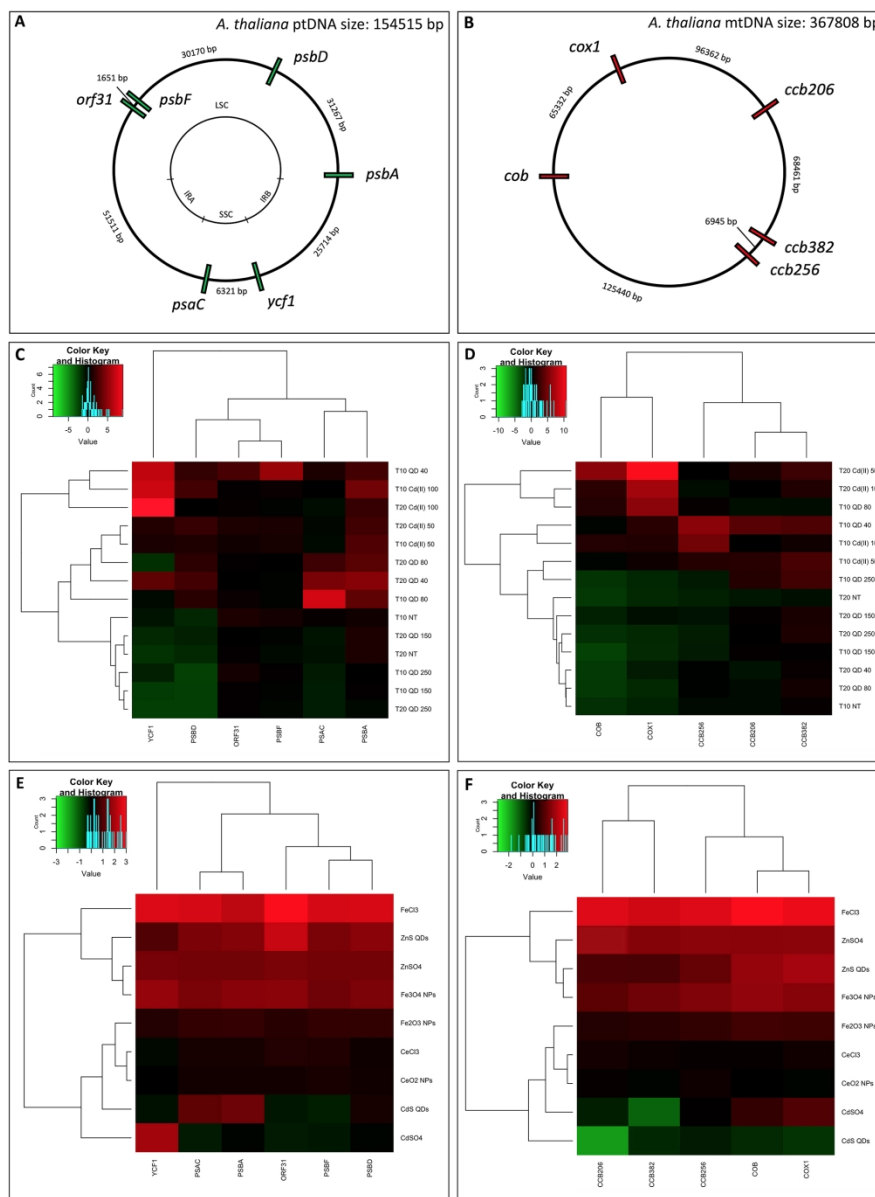


Figure 1. Target gene copy number variations on ptDNA and mtDNA. Schematic representation of *A. thaliana* ptDNA and mtDNA and relative position of the target genes taken into account (a, b). LSC: long single copy region; SSC: short single copy region; IRA, IRB: inverted repeated regions. Target genes were chosen so as to cover the entire ptDNA and mtDNA. Details related to the target genes are reported in the Supporting Information (SI). Distances were calculated on the basis of the NCBI reference sequences: chloroplast (AP000423.1), mitochondrion (NC\_037304.1).

Heatmaps representing the chloroplast (c) and mitochondrial (d) relative target gene abundance during treatment with CdS QDs or CdSO<sub>4</sub>, at different times of exposure and concentrations (Table 2). Data, normalized on the untreated control (T0 NT), showed a non-uniform target gene abundance across the entire ptDNA and mtDNA structures.

Relative target gene abundance of chloroplast (e) and mitochondrial (f) target genes taken into account during treatments with different ENMs and relative metal salts (Table 2). Data, normalized on the control untreated, highlighted the different ranges of similarity in the response (measured as relative target gene abundance) between the treatments with ENMs and relative metal ion counterparts. Numerical data are

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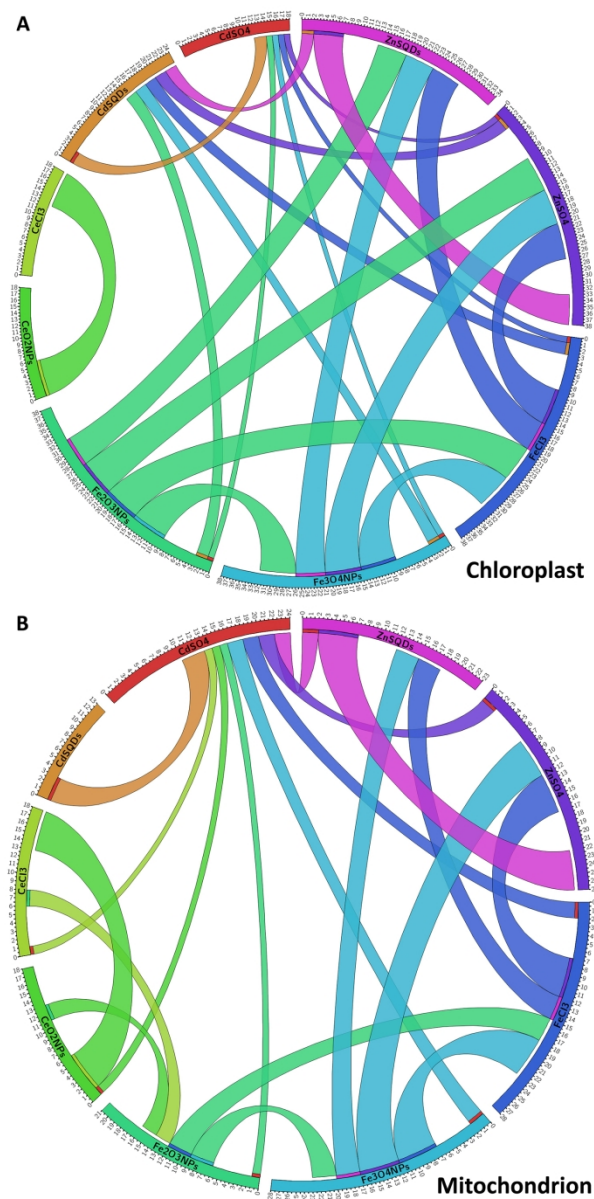


Figure 2. Chord diagram graphical comparison between the relative gene abundance under treatment with ENM or relative metal salts in the chloroplast (a) and mitochondrion (b). Ribbons show the overlap between over- and under-abundant genes in term of copy number, normalized on the untreated control in the different treatments performed.

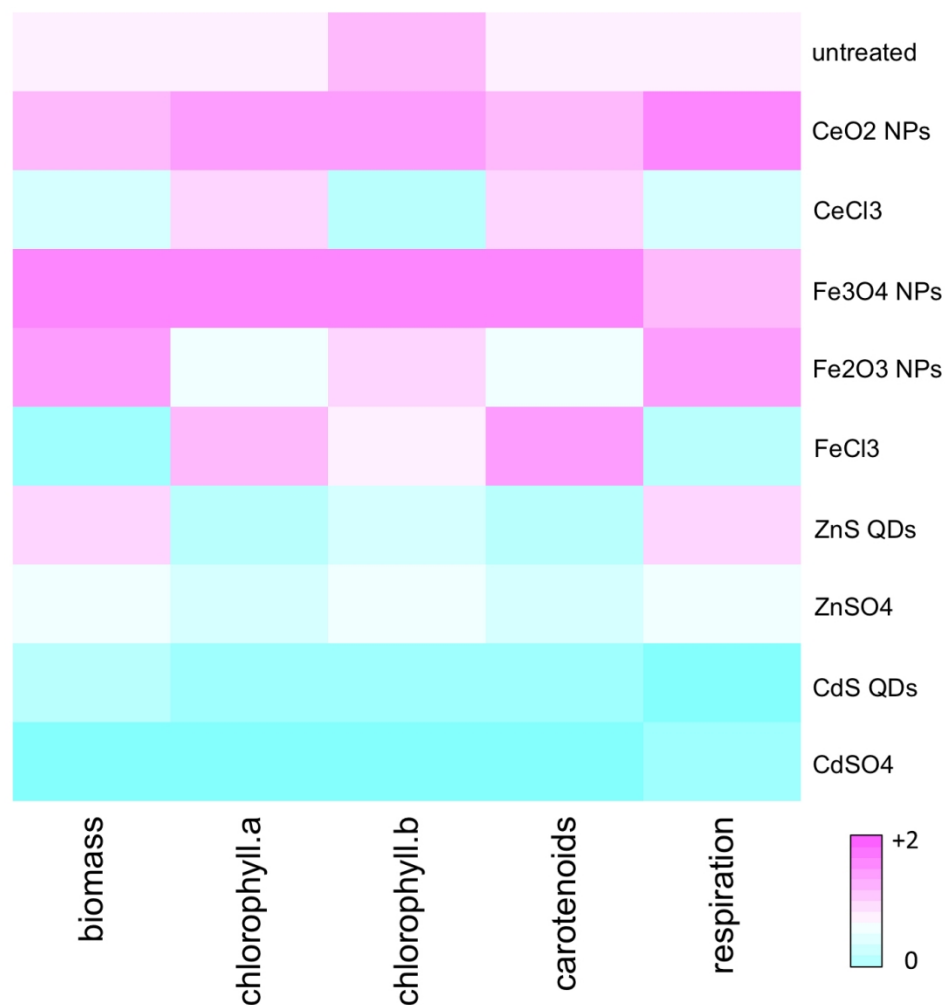


Figure 3. Heatmaps representing the physiological parameters measured: fresh biomass (mg); photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids absorbance) and cellular respiration (formazan absorbance). Data are reported in shades from lilac (higher values) to light blue (lower values). Data and statistics are reported in Tables S3-S5

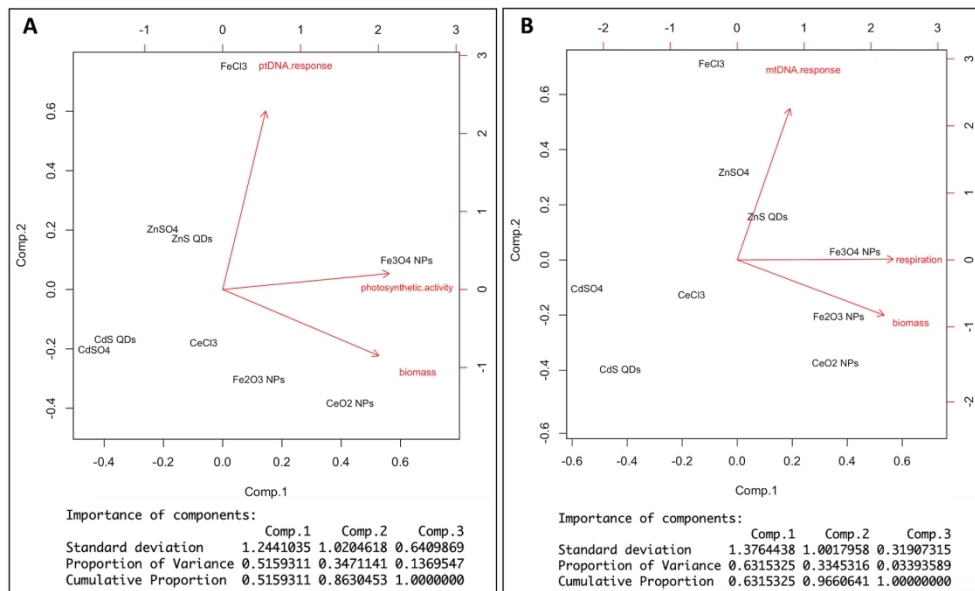


Figure 4. Principal Component Analysis (PCA) related to the effects on the chloroplast (a) and mitochondrion (b). For the chloroplast, parameters taken into account are the relative target gene copy number (ptDNA response), photosynthetic activity and biomass; for mitochondrion, the relative target gene copy number (mtDNA response), cellular respiration and biomass. The first component, representing 51.59% and 63.15% of the total variance for chloroplast and mitochondrion, respectively, is related to differential effects between the ENMs and their metal ion counterparts, as supported by biomass indices and DNA analyses.

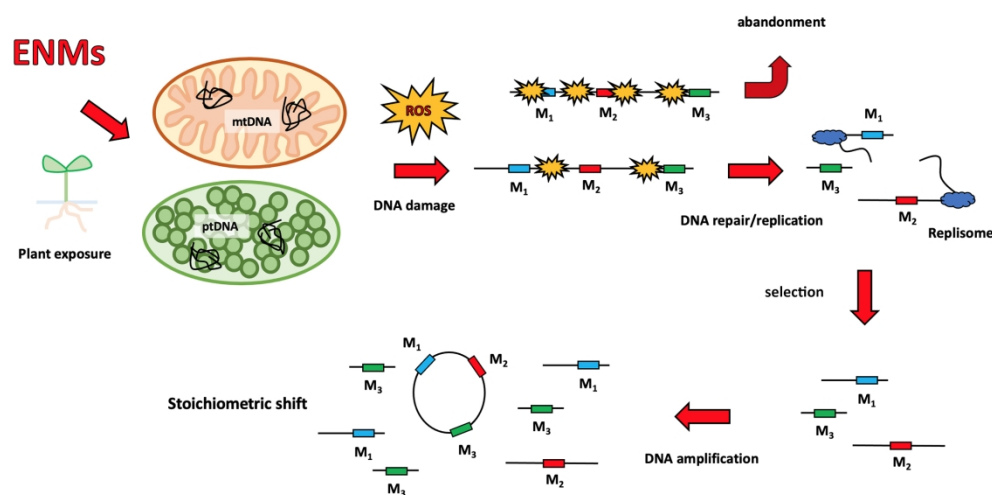
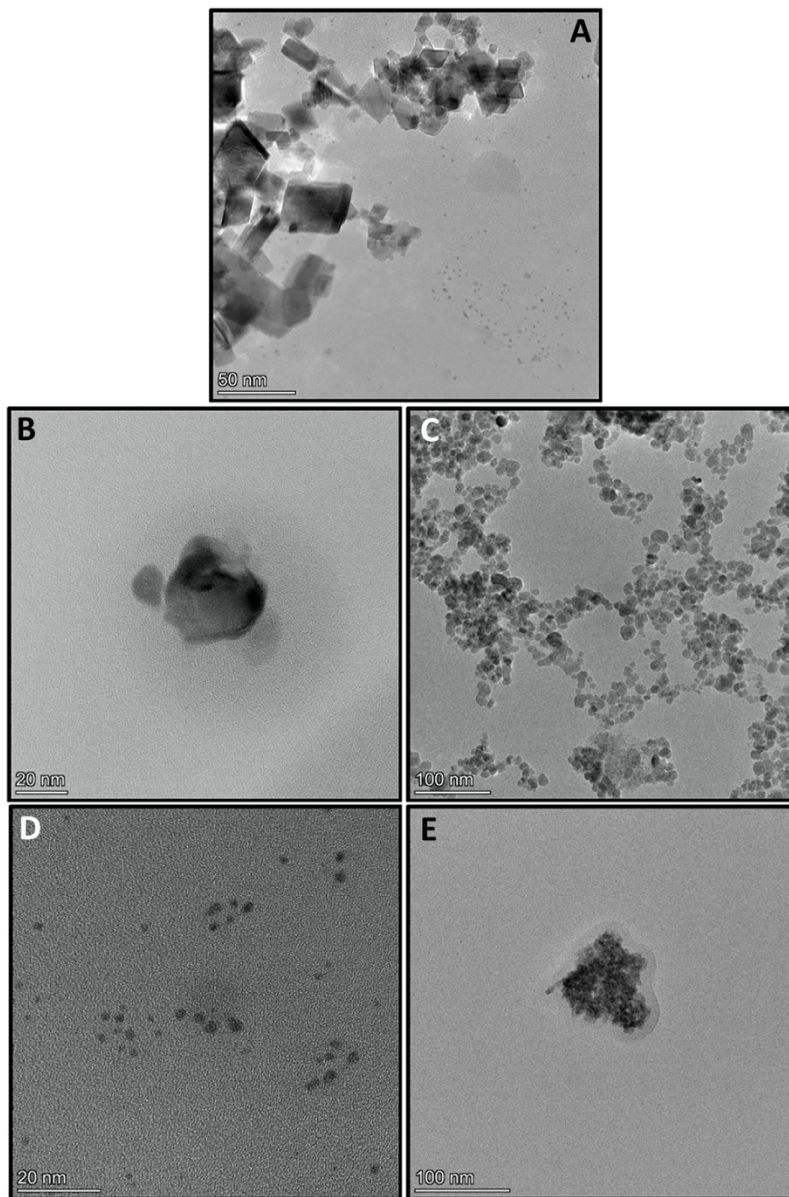
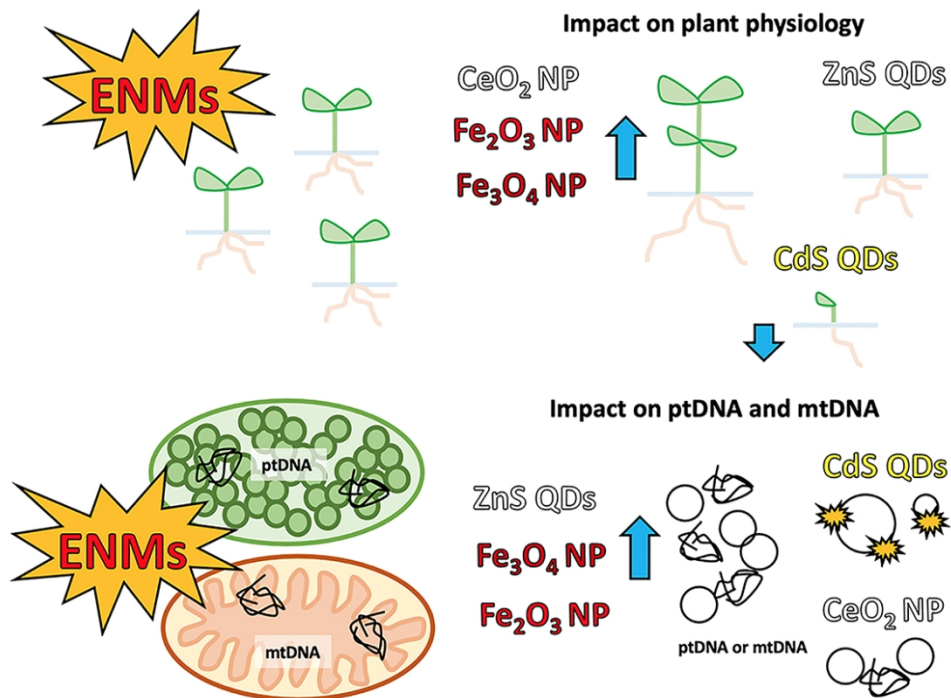


Figure 5. Representation of mechanisms involved in the organellar DNA replication in response to oxidative stress due to ENMs exposure, with particular regard to CdS QDs. Molecular integrity can change drastically as a consequence of inhibition of respiration and photosynthesis, as well as from activation of photorespiration and ROS production. Abandonment of unrepaired organellar DNA molecules and replication of smaller DNA fragments have the role of blocking lesion transmission and modulating the availability of several gene functions.



45 Figure 6. ENMs characterization by transmission electron microscopy (TEM): (a) CeO<sub>2</sub> NPs, (b) Fe<sub>2</sub>O<sub>3</sub> NPs,  
46 (c) Fe<sub>3</sub>O<sub>4</sub> NPs, (d) ZnS QDs, (e) CdS QDs. Additional characterization data are reported in Table 1 and in  
47 the Supporting Information (SI).  
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