JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Agricultural and Environmental Chemistry

Enhanced properties of chitosan microparticles over bulk chitosan on the modulation of auxin signaling pathway with beneficial impacts on root architecture in plants

Maria Jose Iglesias, Silvana L Colman, Maria Cecilia Terrile, Ramiro Paris, Sergio Martín-Saldaña, Alberto Antonio Chevalier, Vera Alejandra Alvarez, and Claudia Casalongue

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b00907 • Publication Date (Web): 03 Jun 2019

Enhanced properties of chitosan microparticles over bulk chitosan on the modulation of auxin signaling pathway with beneficial impacts on root architecture in plants

María José Iglesias[1], Silvana Lorena Colman[1], María Cecilia Terrile[1], Ramiro París[1], Sergio Martín-Saldaña[2], Alberto Antonio Chevalier[2], Vera Alejandra Álvarez[3] and Claudia Anahí Casalongué[1]*

[1]UNMdP,CONICET-, Instituto de Investigaciones Biológicas, UE-CONICET-UNMdP, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata. Funes 3250, B7600, Mar del Plata, Argentina

[2] Gihon Laboratorios Químicos SRL, Calle 4 y 5 Parque Industrial Gral. Salvio, B7600, Mar del Plata, Argentina

[3]UNMdP, CONICET, Instituto Investigación de Ciencia & Tecnología de Materiales INTEMA, UE-CONICET-UNMDP, Grupo Materiales Compuestos Termoplásticos, Facultad de Ingeniería, Universidad Nacional de Mar del Plata. Av. Colón 10850, B7600, Mar del Plata, Argentina.

*Corresponding author: Casalongué CA: casalong@mdp.edu.ar; **telephone** +54223753030; **fax:** +542234753030

ABSTRACT

Improving the root system architecture (RSA) under adverse environmental conditions by using biostimulants is emerging as a new trait to boost crop productivity. Recently, we have reported the characterization of novel chitosan-based microparticles (CS-MPs) with promising biological properties as rooting agent in lettuce. In this work, we demonstrated that in contrast to bulk chitosan (CS) which exerts root growth inhibition, CS-MPs promoted root growth and development from 1 to 10 µg. ml^{-1} without cytotoxicity effects at higher doses in Arabidopsis and lettuce seedlings. In addition, we studied the mechanistic mode of action of CS-MPs in the development of early RSA in the Arabidopsis model. CS-MPs unchained an accurate and sustained spatio-temporal activation of the nuclear auxin signaling pathway. Our findings validated a promising scenario for the application of CS-MPs in the modulation of RSA to respond to changing soil environment and improved crop performance.

Key words: Arabidopsis, auxin, chitosan microparticles, lettuce, root system architecture.

INTRODUCTION

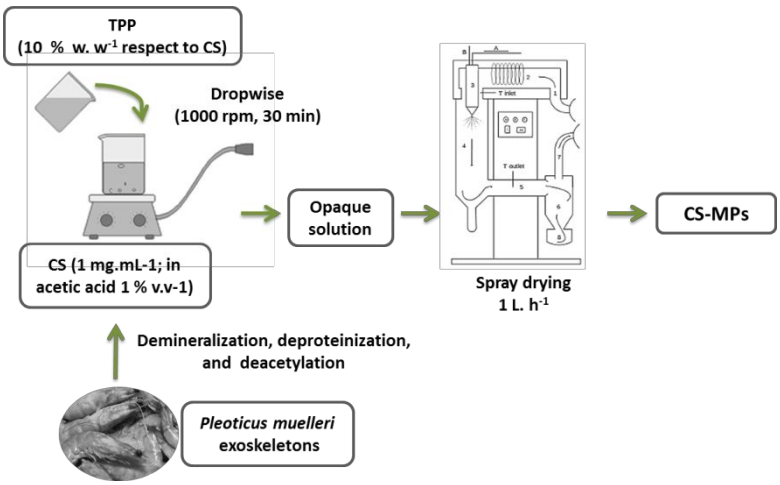
The root system architecture (RSA) involves the coordinated growth and development of primary root (PR), lateral root (LR) and adventitious roots in order to improve soil exploration and resource acquisition, being pivotal for plant fitness and crop productivity.[1] Since the development of RSA is a crucial factor in determining plant survival particularly under adverse environmental conditions, its modulation is emerging as a strategy to generate improvement in crop yields.[2,3] In this context, the development of new bioactive materials with emerging properties fits with the actual challenge of augmenting crop productivity with reduced environment impact.[4,5] Chitosan (CS) are composed by β-1,4-linked glucosamine and N-acetyl glucosamine residues and are generated by the partial deacetylation of chitin polymer. Due to its

unique properties such as biodegradability, biocompatibility, ubiquity and low cost, CS has several applications in several fields including agriculture.[6,7] CS action in the protection of plants against biotic stress by inhibiting microorganism growth and eliciting plant innate immunity has been extensively studied in multiple species.[8-10] Although CS has been suggested as biostimulant promoting plant growth in several horticultural plants, a delicate unbalance from optimal concentrations leads to growth inhibition with high impact on root development.[11,12]

The patterns of plant development are consolidated by the action of hormonal regulation mechanisms. The dynamic and versatile modulation of auxin biosynthesis, transport and signaling has been found to be required for RSA development under changing environmental conditions.[13,14] Auxin regulates root development mainly through the nuclear signaling pathway mediated by the TIR1/AFBs receptors.[15] Auxin binding to TIR1/AFBs receptors triggers the degradation of Aux/IAAs repressors with the consequent activation of auxin response genes.[16] The early response genes involved Aux/IAAs, SAUR and GH3 gene families.[17] The inhibition of auxin-induced growth and the repression of auxin gene expression required for root development has been evidenced in CS-treated wheat coleoptiles and sweet orange plants, respectively.[18,19] Recently, Lopez-Moya, et al. reported the inhibition of PR and LR development in barley and tomato plants.[20] The same authores demonstrated that CS modulates RSA in Arabidopsis plants through the reppresion of the transcription factor *WUSCHEL RELATED HOMEOBOX 5* (*WOX5)* which is a major regulator gene of root stem cell activity. *WOX5* repression was associated to alterations in auxin biosynthesis and transport leading to an over accumulation of auxin in the root tip with detrimental impact on root development, suggesting that doses, frequency and formulation of CS should be adjusted to prevent negative effects on plant development.

Another point to take into account for the application of CS in the field is its limited solubility in water. The complex behavior of bulk CS on plant physiology as a

consequence of chemically heterogeneous copolymers preparations has slowed down its promissory potential use in agriculture.[21,22] Therefore, the development of CS particulated systems is an emerging alternative to the complex problems of bulk CS. They are easy to obtain, and also to modify their water solubility and interactive biological ability.[23] Nevertheless, it is necessary to demonstrate how type, concentration and particle size impact in organs and tissues of plants. We have previously reported the characterization of CS-MPs developed with high molecular weight CS obtained from *Pleoticus mulleri* fishing industry waste from Argentine Sea (Scheme 1). Preliminary assays have shown that CS-MPs stimulate PR elongation in lettuce seedlings suggesting a novelty potential use as rooting agents.[24] In this work we studied CS-MPs properties as biostimulant of root development compared to bulk CS and the hormonal mechanism by which CS-MPs impact on the modulation of RSA in Arabidopsis. Arabidopsis is suggested as an adequate model for dicot plant root research since its root system fits with the typical eudicot root topography.[25]



Scheme 1: Synthesis of CS-MPs.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana (Arabidopsis) wild-type (WT), *pMSG2/IAA19:GUS* , *DR5:GUS*, *BA3:GUS* and *DII-VENUS* are in the Columbia (Col-0) ecotype.[26-29] Butterhead lettuce

(*Lactuca sativa L)* cv. Reina de Mayo seeds were purchased from "El Colono" local seed market, Mar del Plata, Argentina. Arabidopsis and lettuce seeds were surface-sterilized in 30% sodium hypochlorite and 0.2% Tween-20 solution for 10 min, followed by 3 washing steps in sterilized distilled water and stratified at 4 °C for 2-3 d in the dark. Seeds were placed on half-strength Murashige and Skoog medium (½ MS) (SIGMA-Aldrich, USA) plus 0.8% agar in Petri plates and grown vertically at 23 °C under 250 µmol photons m^{-2} s^{-1} with 16:8 h light:dark cycles until analysis.

Chitosan-based materials and treatments

CS-MPs and CS used in this study were described and characterized by Martín-Saldaña, et al.[24] CS exhibited a Mass average molecular weight (Mw) of 1531 ± 372 kDa, a number average molecular weight (Mn) of 559 ± 95 kDa, a polydispersity index (PI= Mw/ Mn) of 1.95 ± 0.32 determined by gel permeation chromatography and a deacetylation degree (DD) higher than 87% determined by Fourier-transform infrared (FTIR) spectroscopy.[30] CS-MPs were prepared by the gelation method with modifications using sodium tripolyphosphate (TPP) as crosslinker and have a mean diameter of 2.10 ± 0.78 µm and a PDI of 0.14 determined by scanning electron microscopy (SEM;JEOL JSM-6100) with 15 kV. Samples were previously coated with metallic gold for 30 s with an Auto Sputter Coater 108 (Cressington, England). Micrographs were analyzed with ImageJ software (USA National Institutes of Health, (http://rsb.info.nih.gov/ij/)).[24,31] CS-MP also present a zeta potential value (ζ) of 27.65 ± 1.22 mV at pH 6.8 determined by a laser particle sizer (Z-sizer 3000 HS, Malvern, UK) . Materials were developed and characterized by Gihon Laboratorios Químicos SRL, Argentina. Figure S1 a and b show the morphology of CS-MP by SEM and FTIR spectra and relevant peaks assigned to CS-MP and bulk CS, respectively. FTIR was performed on an IRAffinity-1S FTIR spectrophotometer (Shimadzu, Japan) in the attenuated total reflection mode (ATR-FTIR). To analyze the efficacy of CS-MPs on root growth parameters, the dry CS-MPs were resuspended in water from 0.1 to 100 µg

111 mL^{-1}. Bulk CS was diluted in 0.1% acetic acid. The pH of each assayed dilution of both
112 bulk CS and CS-MP was in the range of 6.0-6.5.

113 **Fresh weight, primary root and lateral root measurements**

114 Five days post-germination (dpg) Arabidopsis and lettuce seedlings were transferred to
115 ½ MS medium supplemented with CS-MPs or CS and grown vertically in a growth
116 chamber at 25 °C under 250 µmol photons m^{-2} s^{-1} with 16:8 h light:dark cycles until
117 analysis. Root and aerial Fresh weights (FWs) were weighed on a laboratory scale
118 (Sartorius, Germany). Seedlings were photographs after 3 d for PR length and after 5 d
119 for LR number and LR length. PR and LR lengths were quantified using the ImageJ
120 image-analysis software (USA National Institutes of Health, http://rsb.info.nih.gov/ij/).

121 **Measurements of root hair length and density**

122 Five dpg seedlings were transferred to liquid ½ MS medium supplemented with CS-
123 MPs or CS for 48 h. Bright-field images from Arabidopsis roots were taken using a
124 Zeiss Axioplan imaging 2 microscope with an Axiocam HRC CCD camera (Zeiss, USA)
125 using the Axiovision program (version 4.2). Root hair (RH) density and length were
126 analyzed in a 5 mm section from the beginning of the PR differentiation zone.[32] RH
127 length was analyzed using Image-analysis software (USA National Institutes of Health,
128 http://rsb.info.nih.gov/ij/).

129 **Root gravitropic assay**

130 Three dpg seedlings were transferred to fresh ½ MS medium supplemented with 10 µg
131 mL^{-1} CS-MPs. To ensure homogeneous absorption and action, liquid medium was also
132 poured at the surface of each root. The plates were mounted vertically on a scanner
133 (Epson Perfection V600) and let sit for 60 min. After root gravistimulation, images were
134 taken every 15 min for 8 h. Root growth and tip angle were measured by using FIJI
135 software bundle.[33]

136 **Treatment of DII-VENUS transgenic sensor plants with CS-MPs**

137 DII-VENUS Arabidopsis transgenic sensor seedlings were designed to map auxin
138 signaling response at a high resolution in plant cells.[29] Five dpg seedlings were

transferred to fresh plates with the addition of 10 µg mL^{-1} CS-MPs. Liquid solution of CS-MPs (200 µL) was poured at the surface of each root to ensure homogeneous absorption. Seedlings were grown for 24 h. Fluorescence from VENUS protein was detected in root cells using a 20 x objective, a 0.5 numerical aperture; and 470/40-525/50 nm as excitation and detection in a Zeiss Axioplan imaging 2 microscope with an Axiocam HRC CCD camera (Zeiss, USA). Images were analyzed by using FIJI software bundle.[33]

Glucuronidase (GUS) staining

Five dpg transgenic *BA3:GUS*, *DR5:GUS* and *pMSG2/IAA19:GUS* seedlings were transferred into liquid ½ MS medium containing 1, 10 or 100 µg mL^{-1} CS-MPs and then incubated with mild shaking for 24, 48, 72 or 96 h at 23 °C. For *BA3:GUS* line, CS-MPs particles were applied together with 100 nM indole acetic acid (IAA) and incubated for 6 h. After treatment, *BA3:GUS*, *DR5:GUS* and *pMSG2/IAA19:GUS* seedlings were fixed in 90% acetone for 1 h at 20 °C, washed twice in 50 mM sodium phosphate buffer pH 7.0 and incubated in staining buffer [50 mM Na phosphate (pH 7.0), 5 mM EDTA, 0.1% Triton X-100, 5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$ and 1 mg mL^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt) (Gold Biotechnology, USA)] from 2 h to overnight at 37 °C. Bright-field images were taken using a Zeiss Axioplan imaging 2 microscope (Zeiss, USA).

Measurement of nitric oxide (NO) production. Five dpg Arabidopsis seedlings were loaded in the dark with 5 mM of the specific NO dye DAF-FM-DA (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate; Calbiochem, USA) in 20 mM HEPES–NaOH Buffer at pH 7.5 for 30 min. After three washes, seedlings were examined by epi-fluorescence by using a Nikon DS-Fi 1 digital camera coupled to a Nikon Eclipse Ti (Nikon, Japan) epifluorescence microscope (excitation 495 nm; emission 515–555 nm).

RNA extraction and quantitative real-time RT-qPCR

Five dpg seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPs or 10 µg mL^{-1} CS and H_2O as controls. After 24

h, total RNA from Arabidopsis seedlings was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendations. Samples were treated with RQ1 RNase-free DNase (Promega, USA) for DNA contamination removal. For cDNA synthesis, 1 µg of total RNA was reverse transcribed by IMPROM II (Thermo Fisher Scientific, USA) using random primers (Biodynamics, Argentine). The expression of a subset of early auxin response genes was analyzed by Real Time (qPCR), using the following primers: IAA5F: 5´-CCGGAGAAAGAACAGTCTCG-3´; IAA5R: 5´-AGCATCCGAACAGAATTTGC-3´; IAA14F: 5´-GAAGCAGAGGAGGCAATGAG-3´; IAA14R: 5´-CCCATGGTAAAGGAGCTGAA-3´; GH3.5F: 5´-CCATCTCTGAGTTCCTCACAAGC-3´; GH3.5R: 5´-TCCTCTTCGATTGTTGGCATTAGC-3´; GH3.17F:5´-ACGCAGACACGTCATCAATCCC-3´; GH3.17R: 5´-TGCTGTGACGTGGCTTTAGCTC-3´; ACTINF: 5´-GCCATCCAAGCTGTTCTCTC-3´; ACTINR: 5´- GAAACCCTCGTAGATTGGCA-3´. qPCR reactions were conducted in triplicates (95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s) in a Step One real-time PCR system (Applied Biosystems, USA) using FastStart Universal SYBR Green Master Rox (Roche, Germany) following manufacturer´s instructions. Results were normalized to the expression level of the gene actin and expressed as fold-change over controls using the comparative cycle threshold (CT) method.[34] PCR products were analyzed by melting curve analysis to confirm the presence of a single product.

Statistical Analysis

The values shown in figures are mean values +/- standard error (SE) of at least 3 experiments. The data were subjected to analysis of t-Test or ANOVA with Dunnet post hoc comparisons against control by Graphpad Prism version 5.01 software (*$p<0.05$ **$p<0.01$ *** $p<0.001$).

RESULTS

CS-MPs modulate root architecture in Arabidopsis and lettuce plants

To deep on the potential action of CS-MPs on RSA, we performed a complete analysis of root growth parameters. PR elongation, LR and RH development were studied in 5 dpg Arabidopsis seedlings transferred to ½ MS medium supplemented with 0.1, 1, 10 and 100 μg mL^{-1} CS-MPs, or CS as control. After 3 d, seedlings grown in 1 μg mL^{-1} CS-MPs supplemented medium evidenced the higher and significant increment in PR length compared to untreated seedlings (Figure 1 a). In contrast to CS which at higher doses (10 and 100 μg mL^{-1}) severely arrested PR elongation, MP-CSs did not show a detrimental effect on PR length in a wide range of assayed doses (Figures 1a and e). In addition, supplementation of the growing medium with 1 and 10 μg mL^{-1} CS-MPs resulted after 5 days of treatment in a 40% and 60% of increment in the number and length of LRs, respectively compared with control (Figures 1b and c). The promotion of LR development was accompanied by slight reduction in PR elongation (Figure 1e). Again, CS-MPs showed a better performance on LR development compared with bulk CS treatment (Figures 1b and c). In addition to PR inhibition, seedlings exposed to the highest doses of CS evidenced a reduce number of LR, while no cytotoxicity was detected under CS-MPs treatments at the studied concentrations (Figures 1b and e). The reduced cytotoxicity of CS-MPs could be explained by the fact that CS-MPs present a reduce number of exposed -NH3+ positive charges which modified the interactive ability with cell membranes.[35]

In concordance, the rearrangement of RSA triggered by 1 and 10 μg mL^{-1} CS-MPs resulted in approximately 25% improvement on root and aerial FW after 9 d of treatment suggesting a positive effect on plant biomass (Figure 1d). Next, we also analyzed the effect of selected CS-MPs concentrations on RH development (Figure 2). Again, due to its emerging physico-chemical properties, CS-MPs at 1 and 10 μg mL^{-1} resulted in an increment of Arabidopsis RH density (Figure 2a), and RH length (Figure 2b), while no positive effect was detected under 1 μg mL^{-1} CS treatment. To analyze if the gravitropism as a key root growth process is affected by CS-MPs, the root tip angle

was quantified after turning the 3 dpg Arabidopsis seedling 90 degrees as described in París, et al.[36] However, compared with control, CS-MPs treated seedlings did not show changes in root bending, suggesting that CS-MP has specific cell-tissue action (Figure S2). In addition, CS-MPs also promote root development in lettuce (Figure S3). These findings demonstrate that compared with bulk CS, CS-MPs resulted in an improve material to enhance early root growth in Arabidopsis and lettuce seedlings (Figures 1, 2 and S3).

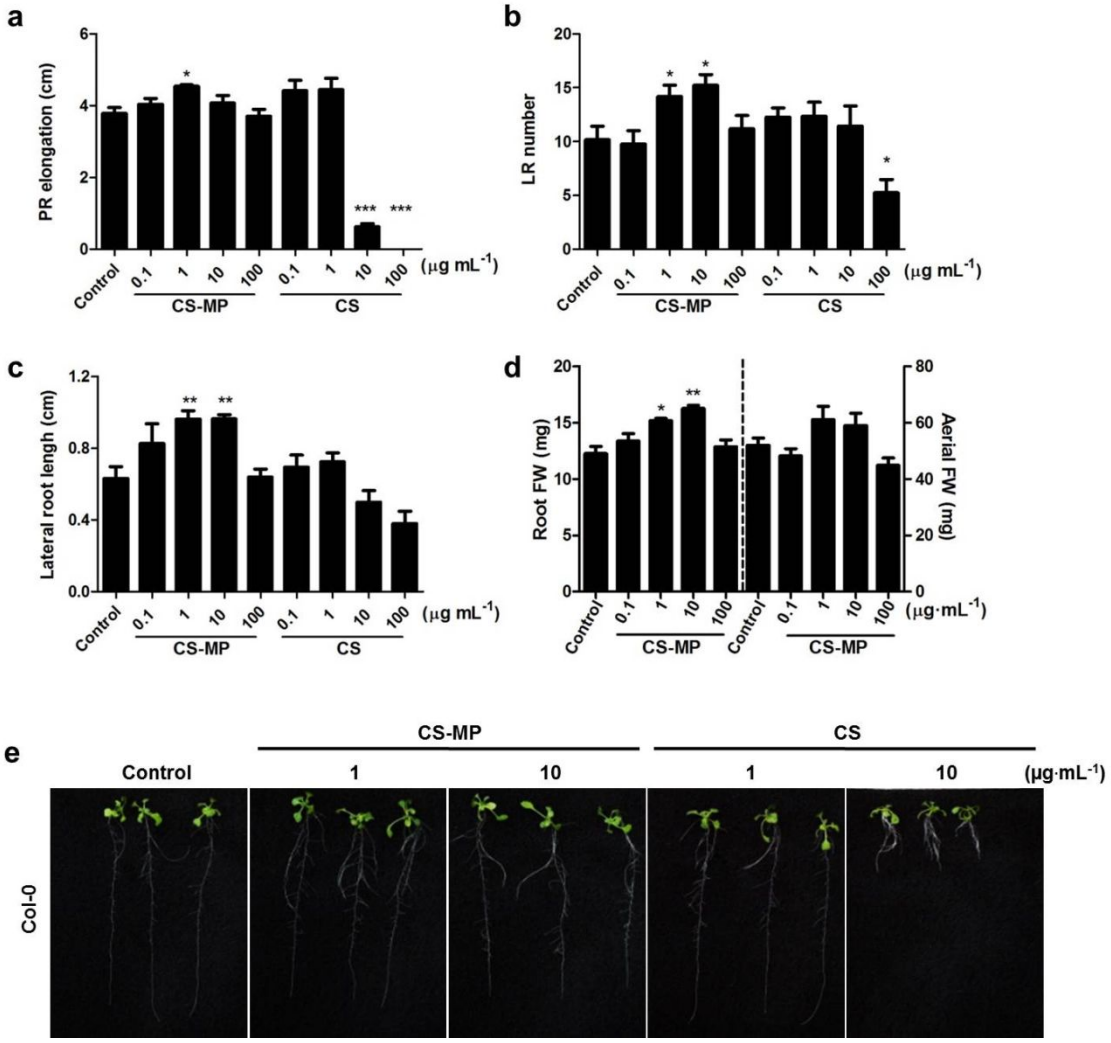


Figure 1. CS-MPs promote root development in Arabidopsis. Five dpg Col-0 Arabidopsis seedlings grown in ½ MS medium were treated with increasing concentrations of CS-MPs or CS as control. PR elongation (a) was quantified 3 d post-treatment. LR number (b) and LR length (c) were analyzed 5 d post treatment. Seedlings FW was quantified 9 d post- treatment (d). Representative images 9 d post

treatments are shown in (e). Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test against control,* p< 0.05 ** p<0.01 ***p<0.001).

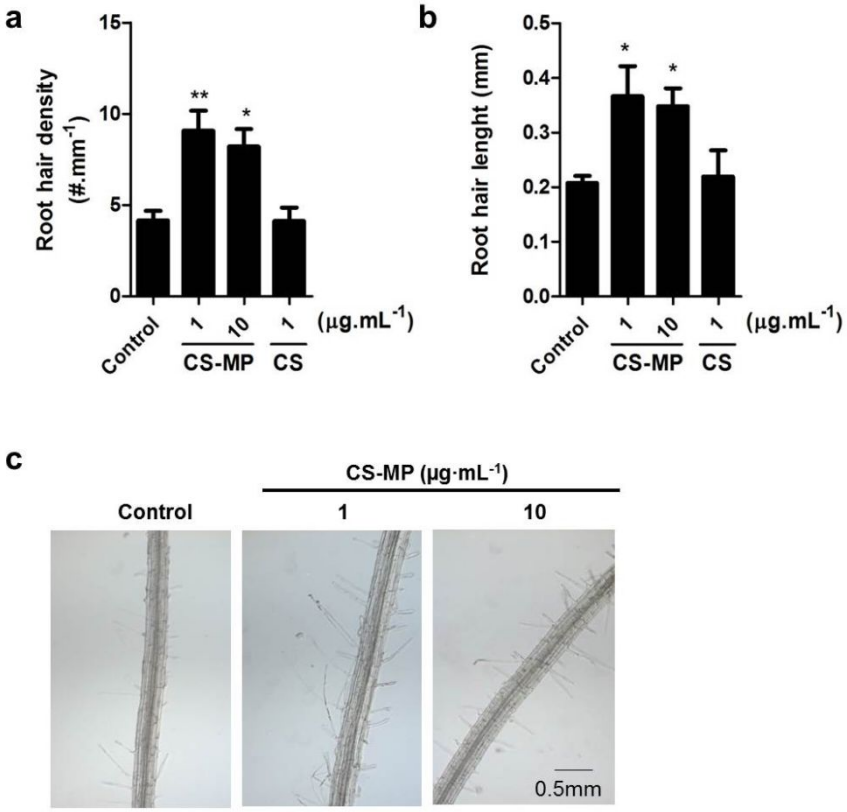
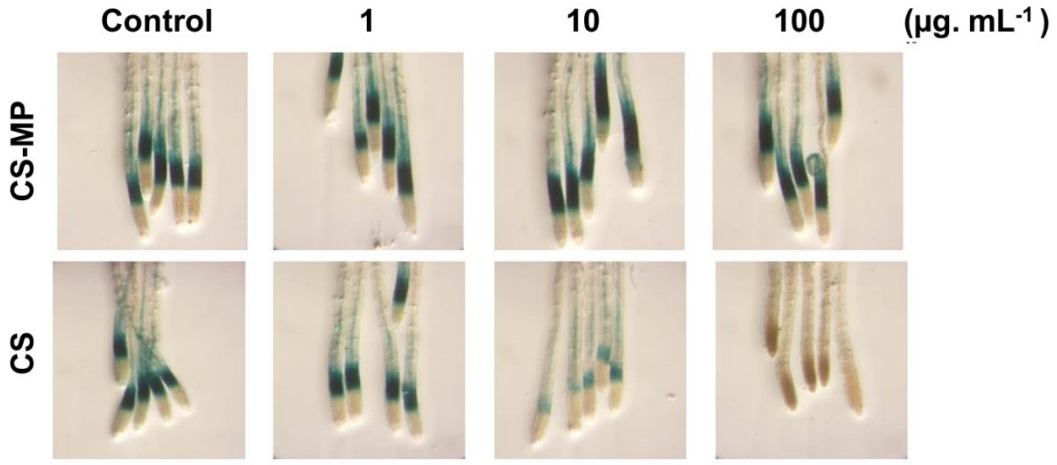


Figure 2. Promotion of RH upon CS-MPs exposure. Five dpg Col-0 Arabidopsis seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPS for 48h. RH density (a) and RH length (b) were analyzed in a 5 mm section from the beginning of the differentiation zone. Representative images are shown in (c). Data are mean values of 4 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control, * p< 0.05).

Auxin response is activated by CS-MPs in Arabidopsis roots

Since auxin is a key regulator of root growth and development, we studied if the modulation of auxin signaling constitutes a mechanism of action downstream MP-CSs application in Arabidopsis plants by using the auxin reporter transgenic seedlings, *BA3:GUS* and *DR5:GUS*. These lines consist of artificial promoters based on auxin response elements which drive the expression of GUS gene. The activation or repression of auxin response is correlated with GUS activity levels.[27,28]



265

266 **Figure 3. Differential effect of CS-MPs and CS on auxin *BA3:GUS* reporter gene**
267 **activity**. Five dpg Arabidopsis *BA3:GUS* seedlings grown in ½ MS medium were
268 treated with 100 nM IAA and increasing concentrations of CS-MPs or CS. Seedlings
269 were subjected to GUS staining after 5 h of treatment. Representative images of PR
270 tips are shown.

271 We studied the effects of CS and CS-MPs on the early activation of auxin response
272 analyzing *BA3:GUS* activity on the tip of PR of 5-dpg seedlings treated with the natural
273 auxin IAA, in combination with increasing concentrations of CS or CS-MPs for 6 h.
274 While bulk CS repressed BA3 auxin-responsive promoter activity in a dose-dependent
275 manner, CS-MPs did not show an effect on auxin response at all analyzed doses (0.1,
276 1, 10 and 100 µg mL^{-1}) in an early period of treatment (Figure 3). Repression of auxin
277 response by CS correlated with the cytotoxicity effects on root growth shown in Figure
278 1e. However, analyzing auxin response after 24 h of treatment, 1 and 10 µg mL^{-1} CS-
279 MPs triggered the activation of DR5 auxin responsive promoter in the tip of PR of 5 dpg
280 *DR5:GUS* seedlings (Figures 4a and b). In concomitance with CS-MPs action on LR
281 (Figure1), an increment in the number of LR primordia showing DR5 activity was
282 detected in 1 and 10 µg mL^{-1} CS-MP treated seedlings after 48 h of treatment (Figures
283 4c and d). In order to evaluate the dynamics of LR induction, a time-course analysis of
284 stained *DR5:GUS* roots was performed. Figure 4e shows statistically higher and faster
285 induction of lateral root development by CS-MPs since CS-MPs treated seedlings

showed an increased number of GUS-stained LR primordia compared with control after 48 h and 72 h treatment. However, after 96 h no significant difference between treatments was found. This temporarily advance in auxin activation was also observed in PR where CS-MPs treated seedlings reached similar activation of DR5 promoter than control 48 h post-treatment (Figure 4b). The early and sustained activation of auxin response activity in DR5 reporter line fits with the promotion of root growth and development described in Figure 1.

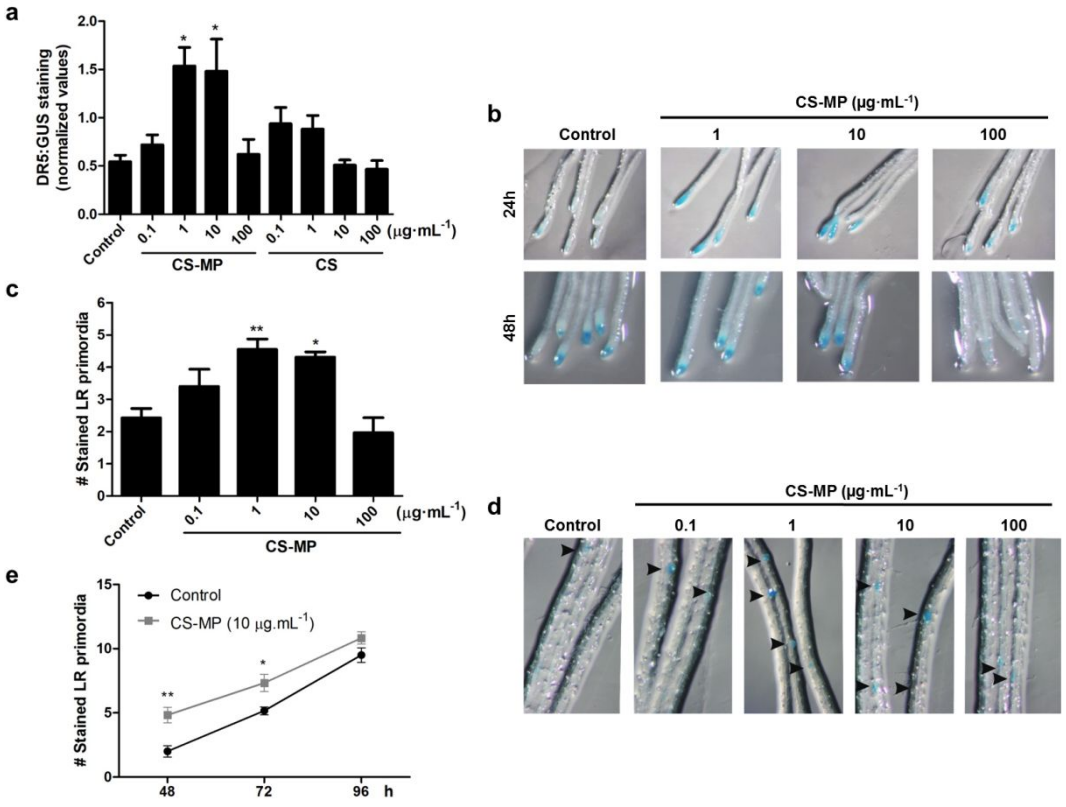


Figure 4. CS-MPs promote an early activation of auxin response in Arabidopsis roots. Five dpg *DR5:GUS* seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of MP-CS. GUS activity was revealed after incubation with X-Gluc at 37 °C. GUS activation in PR was analyzed 24 h post-treatment (a). GUS staining in representative root tip segments after 24 h and 48 h is shown in b. Stained LR primordia were quantified after 48h (c). GUS staining in representative root segments of the differentiation zone are shown in (d). Time-course analysis of stained primordia following 48, 72 and 96 h of treatment with 10 µg mL^{-1} is shown in (e) .Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test,* $p< 0.05$ ** $p<0.01$).

CS-MPs trigger the activation of nuclear auxin signaling pathway

To add evidence on the activation of the nuclear auxin signaling pathway, we analyzed the level of fluorescence emitted by the auxin sensor DII-VENUS in root cells from control and CS-MPs- treated plants. This auxin reporter line has been engineered to allow the detection of dynamic changes in the levels of Aux/IAA auxin repressor associated to a sensitive activation of nuclear TIR1/AFBs dependent auxin pathway.[29] DII-VENUS sensor is rapidly degraded resulting in a decrease of the fluorescence when the auxin pathway is activated. A decrease in the DII-VENUS fluorescence signal was detected in the nucleus of epidermic cells in PR and LR of 10 µg mL^{-1} CS-MPs-treated seedlings compared to control (Figures 5a and b).

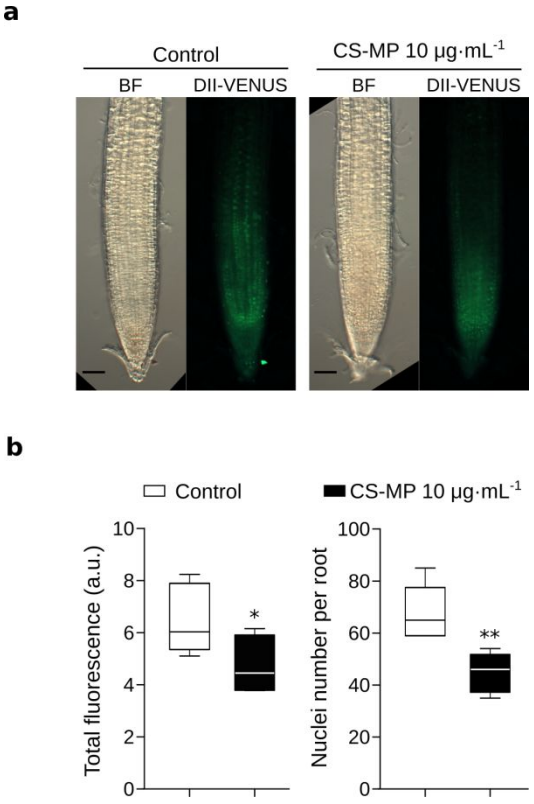


Figure 5. CS-MPs enhance auxin sensitive in *DII-VENUS* Arabidopsis seedlings. Five dpg DII-VENUS seedlings were transferred to liquid ½ MS medium supplemented with 10 µg mL^{-1} CS-MPs (a) DII-VENUS expression (green) in PR of control and CS-MPs treated seedlings. Bright field images (BF). (b) Quantification of total DII-VENUS signal and DII-VENUS positive nuclei number per root. Box plot showing median, minimum and maximum values of 2 independent experiments (n= 24). P-values in

comparison to control were calculated with two-tailed Student's t-test, *p ≤ 0.05 **p ≤ 0.01. Scale bars, 50 μm.

Next, the expression of a set of auxin-response genes, *Aux/IAAs* (*IAA5* and *IAA14*) and *GH3s* (*GH3.5* and *GH3.17*) was analyzed upon 1, 10 and 100 μg mL^{-1} CS-MPs treatment by quantitative qPCR (Figure 6). It is known that auxin response genes have differential patterns of expression under auxin stimuli.[37] Although each gene evidenced a particular expression pattern, most of them were up-regulated in seedlings exposed to 1 and 10 μg mL^{-1} CS-MPs 24 h post treatment (Figures 6a-d). *MSG2/IAA19* constitutes an early auxin response gene associated to root development.(26) Therefore, the activation of IAA19 promoter in roots of *pMSG2/IAA19:GUS* reporter line was analyzed in 1 and 10 μg mL^{-1} CS-MPs treated seedlings (Figure 6e). Both concentrations of CS-MPs led to a significant increment of GUS staining in the PR (Figure 6f).

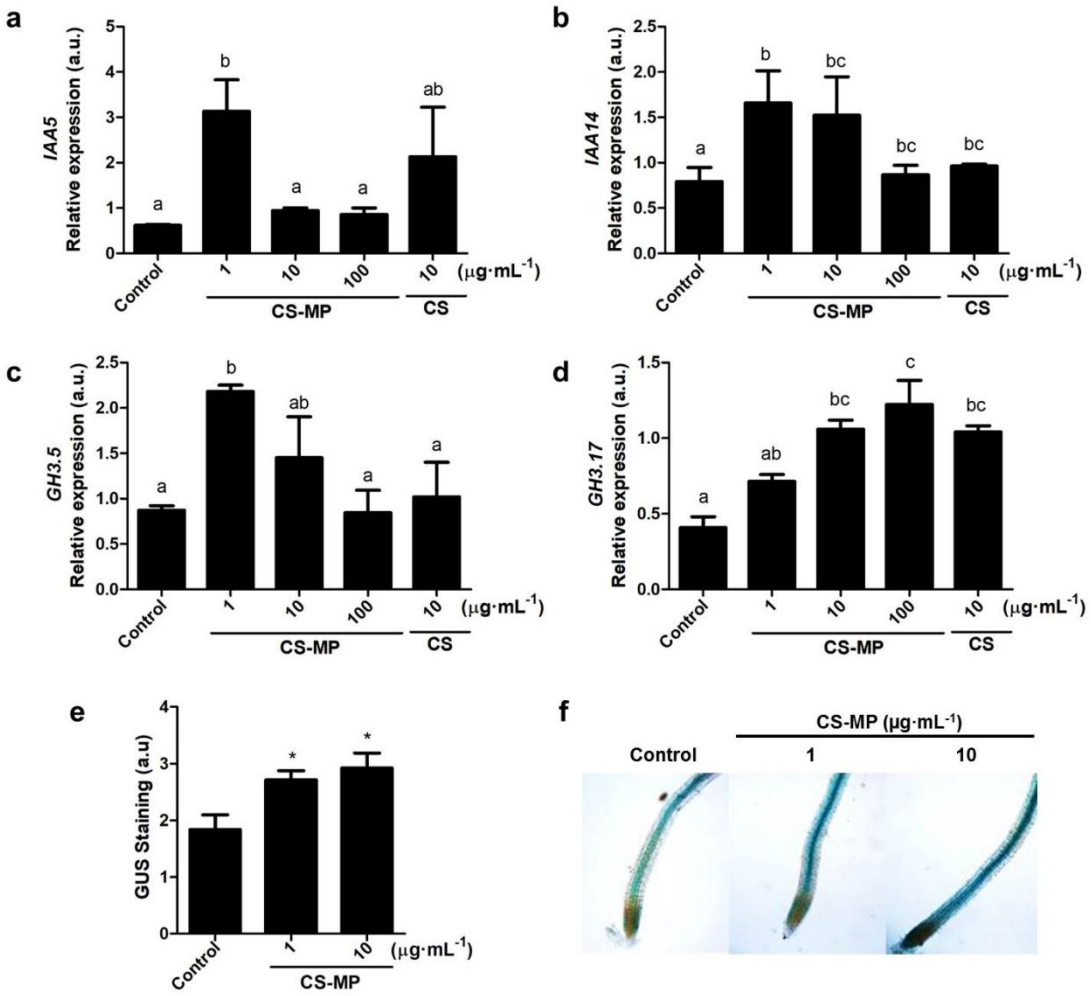


Figure 6. CS-MPs promote the activation of early auxin response genes in Arabidopsis seedlings. Five dpg Col-0 seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPs or CS as control. (a-d) show the expression of a subset of early auxin response genes analyzed by qPCR after 24 h of CS-MPs treatment. (e) GUS activity in representative PR of 5 dpg *MSG2/IAA19:GUS* Arabidopsis seedlings treated with 1 and 10 μg mL^{-1} CS-MPs for 24 h. Quantification of GUS staining in representative root tip segments is shown in (f). Data are mean values of 3 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control,* $p< 0.05$ ** $p<0.01$).

DISCUSSION

CS action seems to be complex in the dynamic and versatile modulation of plant developmental programs since a narrow change from optimal concentrations can lead from a promotion of growth to detrimental effects on plant biomass.[12] CS-MPs

modulate RAS inducing an early promotion of PR elongation, and a subsequent increment in the number and elongation of LRs and RHs compared to untreated seedlings root architecture in Arabidopsis and lettuce plants (Figures 1, 2 and S3). CS-MPs exert its positive effect in a wide range of concentrations (1-10 μg mL^{-1}), resulting in a very beneficial property to recommend doses of application in the field. This fact also represents improved properties compared to bulk CS and CS-nanoparticles recently described which inhibit root growth or alternatively induce root growth at specific doses showing cytotoxicity at higher concentration in several plant species including *Arabidopsis thaliana*, *Solanum licopersicum*, *Hordeum vulgare*, *Capsicum annuum and Ipomoea purpurea* treated with the same range of concentrations than in our study.[20,38,39] Main differences in our results could be attributed to physico-chemical properties of CS that become drastically changeable according to the biological sources and the synthesis methods. In our study CS exhibited a Mw of 1531 ± 372 kDa which is significantly higher than CS used in other published papers.(20, 38, 39) Interestingly, the interaction between TPP^- and -NH3+ groups of CS during the formation of the microstructure organization by the gelation method, confers new properties to the CS-MPs if compared to bulk CS macrostructure. CS present biological properties associated to its cationic nature under acidic pH. The protonated amino groups of the glucosamine could interact by electrostatic interactions with anionic groups of the lipids of cell membrane, causing impairment on its physicochemical equilibrium.[40] In CS-MP, these charged groups are partially neutralized and allow reorganization of the polymeric chains providing new properties to the CS-MP, which might have different interactions between the remaining free cationic groups and the cells. Assuming that the crosslinking efficiency is not 100% (TPP ratio was only 10% in the CS-MP), these remaining positively charge amino groups could interact with plasma membrane phospholipids as well as chelation of metal elements.[11] Therefore, this new microstructure increases the surface of contact reducing the exposed -NH3+ positive charges of CS which could disrupt cell membrane potential. In addition, CS-

MPs present a regular shape and medium size around 2.10 μm which favors a better interaction with cells if compared with bulk CS.[24] Then, the polymeric microstructure properties of CS-MPs make them an improved material for root growth promotion in a wider range of application doses compared with bulk CS.

In addition, multiple evidences from genetic, molecular, and cellular approaches demonstrate the relevance of maintaining auxin gradients which ensure a proper activation of TIR1/AFBs- dependent auxin signaling during root development in Arabidopsis.[15] It was recently reported that Arabidopsis seedlings react to sensible changes in auxin concentrations by extremely rapid adaptation of root growth rate. [41] Therefore, compounds which exert an effect on auxin metabolism should be well characterized prior application. The fact that CS is able to induce a rapid and strong accumulation of auxin in Arabidopsis fits with the reports where plant growth is affected by it application.[20] Although we used a CS of higher Mw than these authors, our results also demonstrated that CS triggers auxin signaling repression (Figure 3). However, CS-MPs unchain an accurate and coordinated spatio-temporal induction of the nuclear auxin signaling in CS-MPs-treated seedlings evidenced by the activation of DR5 promoter, the repression of DII-VENUS activity, and the expression of early auxin response genes, Aux/IAAs and GH3s (Figures 4,5 and 6). A counter balance of nitric oxide (NO) concentrations appears to be essential for the control of the auxin action during root growth and development.[42] The induction of NO levels by CS-MP (Figure S4) could contribute to the enhancement in auxin sensitivity which promotes root growth. NO exert its action, in part, through the S-nitrosylation of multiple components of the nuclear TIR1-dependent auxin signaling.[43,44] Despite the differences in the dimensions of the particles with an average diameter of 90 ± 5 nm compared to 2.10 ± 0.78 μm exhibited by the particles described in this work and in concordance with our results, Chandra, et al.[45] demonstrated that CS nanoparticles also induce NO accumulation in addition to antioxidant enzymes as part of the defense response mechanism in *Camellia sinensis* tea plants suggesting that particles might mediate

different physiological processes sharing, at least partially, the same signaling mechanisms.

In addition, main differences in our results and previously reported papers could be attributed to physicochemical properties of CS including the molecular weight since it has been suggested that it has more influence on the biological activity than the DD. [46] The electrostatic interaction between TPP and -NH3+ groups of CS during the formation of the MP allow to a new organization of the molecules of the polymer and also a new way to exhibit the cationic charges reflected in the ζ of the CS-MP. This new conformation confers new properties to the material if compared to bulk CS macrostructure. This microstructure increases the surface of contact when compared to bulk CS reducing the exposed -NH3+ positive charges of CS which could disrupt cell membrane potential. [24]

Although auxin is considered an omnipotent regulator of root development cytokinin and jasmonate hormonal pathways and the crosstalk auxin-ethylene have been extensively described in the regulation of LR initiation, emergence and positioning in Arabidopsis. [47,48] The fact that the application of CS-MPs to the double mutant in the auxin receptors TIR1 and AFB2, *tir1afb2* was able to promote root FW and the number and length of LRs (Figure S5) suggests that CS-MPs enhance the sensitivity of remaining auxin receptors of TIR1/AFBs family or alternatively that CS-MPs exert its action through additional pathways.

Curiously, CS-MPs-induced phenotype resembles RSA of plants exposed to soil with low phosphate (Pi) availability where modulation of auxin sensitivity leads to augmented density and length of LRs and RHs.[49] However, CS-MPs enhanced root branching without a drastic effect on aerial organs in contrast to plants grown under low Pi which allocate more carbon to roots increasing their root-to-shoot ratio.[50] Due to the relevance of soil Pi level for crop yield, Ham, et al. proposed the bio-engineering of agricultural species for improved Pi acquisition and utilization in plants. [51] Although the

mode of action of CS has not been completely deciphered yet, CS exhibits several reactive amino side groups which enhance its applicability. For instance, it has been demonstrated that CS stimulates the activity of plant symbiotic microbes affecting the homeostasis of microbial rhizosphere and also promoting the nutrient uptake by plant. [52,53] Then, CS-MPs could participate in the modulation of Arabidopsis root interphase and/or the microbiome and associated mineral nutritional compounds. In this context, new CS biomaterials with improve biological performance like CS-MPs may constitute an overcome alternative to transgenic plants for the promotion of plant growth under soil with nutrient deficiency. However, further studies are necessary in order to decipher the cellular uptake and biodistribution of CS-MPs in root cells.

FUNDING

This work was supported by grants from ANPCyT (PICT start up0008, RAICES 0959), CONICET and Universidad Nacional de Mar del Plata and Gihon Laboratorios Químicos SRL, Mar del Plata Argentina.

ABBREVIATIONS

RSA, root system architecture; CS, chitosan; CS-MPs, chitosan microparticles; DPG, days post-germination; FW, fresh weight; IAA, Indole acetic acid; NO, nitric oxide; LR, lateral root; PR, primary root; RH, root hair; SE, standard error; TPP, sodium tripolyphosphate;

SUPPORTING INFORMATION CONTENT

Figure S1. Characterization of CS-MP by SEM and FTIR.

Figure S2. Analysis of CS-MPs effect on root gravitropism in Arabidopsis.

Figure S3. Analysis of CS-MPs effect on root development in lettuce.

Figure S4. Analysis of CS-MPs effect on nitric oxide accumulation in Arabidopsis roots.

Figure S5. Analysis of CS-MPs effect on root development in *tir1afb2* double mutant.

REFERENCES

1. Rogers, E. D.; Benfey, P. N., Regulation of plant root system architecture: implications for crop advancement. *Current Opinion in Biotechnology* **2015**, *32*, 93-98.
2. Meister, R.; Rajani, M.; Ruzicka, D.; Schachtman, D. P., Challenges of modifying root traits in crops for agriculture. *Trends in Plant Science* **2014**, *19*, 779-788.
3. Rogers, C.; Oldroyd, G. E., Synthetic biology approaches to engineering the nitrogen symbiosis in cereals. *Journal of Experimental Botany* **2014**, *65*, 1939-1946.
4. Anderson, J. A.; Gipmans, M.; Hurst, S.; Layton, R.; Nehra, N.; Pickett, J.; Shah, D. M.; Souza, T. L. P.; Tripathi, L., Emerging agricultural biotechnologies for sustainable agriculture and food security. *Journal of agricultural and food chemistry* **2016**, *64*, 383-393.
5. Tittonell, P., Ecological intensification of agriculture—sustainable by nature. *Current Opinion in Environmental Sustainability* **2014**, *8*, 53-61.
6. Pirbalouti, A. G.; Malekpoor, F.; Salimi, A.; Golparvar, A., Exogenous application of chitosan on biochemical and physiological characteristics, phenolic content and antioxidant activity of two species of basil (Ocimum ciliatum and Ocimum basilicum) under reduced irrigation. *Scientia horticulturae* **2017**, *217*, 114-122.
7. Malerba, M.; Cerana, R., Recent Advances of Chitosan Applications in Plants. *Polymers* **2018**, *10*, 118.
8. Yin, H.; Zhao, X.; Du, Y., Oligochitosan: a plant diseases vaccine—a review. *Carbohydrate Polymers* **2010**, *82*, 1-8.
9. Mansilla, A. Y.; Albertengo, L.; Rodríguez, M. S.; Debbaudt, A.; Zúñiga, A.; Casalongué, C., Evidence on antimicrobial properties and mode of action of a chitosan obtained from crustacean exoskeletons on Pseudomonas syringae pv. tomato DC3000. *Applied microbiology and biotechnology* **2013**, *97*, 6957-6966.
10. Iriti, M.; Varoni, E. M., Chitosan-induced antiviral activity and innate immunity in plants. *Environmental Science and Pollution Research* **2015**, *22*, 2935-2944.
11. Malerba, M.; Cerana, R., Chitosan effects on plant systems. *International journal of molecular sciences* **2016**, *17*, 996.
12. Pichyangkura, R.; Chadchawan, S., Biostimulant activity of chitosan in horticulture. *Scientia Horticulturae* **2015**, *196*, 49-65.
13. Vanneste, S.; Friml, J., Auxin: a trigger for change in plant development. *Cell* **2009**, *136*, 1005-1016.
14. Lavenus, J.; Goh, T.; Roberts, I.; Guyomarc'h, S.; Lucas, M.; De Smet, I.; Fukaki, H.; Beeckman, T.; Bennett, M.; Laplaze, L., Lateral root development in Arabidopsis: fifty shades of auxin. *Trends in plant science* **2013**, *18*, 450-458.
15. Overvoorde, P.; Fukaki, H.; Beeckman, T., Auxin control of root development. *Cold Spring Harbor perspectives in biology* **2010**, a001537.
16. Chapman, E. J.; Estelle, M., Mechanism of auxin-regulated gene expression in plants. *Annual review of genetics* **2009**, *43*, 265-285.

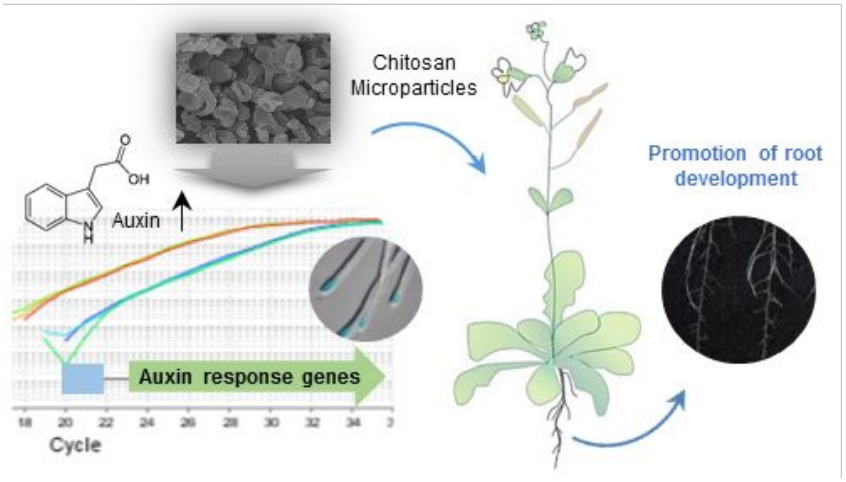
519 17.Hagen, G.; Guilfoyle, T., Auxin-responsive gene expression: genes, promoters and
520 regulatory factors. *Plant molecular biology* **2002**, *49*, 373-385.
521 18.Khairullin, R.; Akhmetova, I.; Yusupova, Z., Inhibition of IAA-induced growth of wheat
522 coleoptile fragments by chitin-chitosan oligomers. *Biology Bulletin of the Russian Academy of*
523 *Sciences* **2002**, *29*, 135-138.
524 19.Coqueiro, D. S. O.; de Souza, A. A.; Takita, M. A.; Rodrigues, C. M.; Kishi, L. T.; Machado, M.
525 A., Transcriptional profile of sweet orange in response to chitosan and salicylic acid. *BMC*
526 *genomics* **2015**, *16*, 288.
527 20.Lopez-Moya, F.; Escudero, N.; Zavala-Gonzalez, E. A.; Esteve-Bruna, D.; Blázquez, M. A.;
528 Alabadí, D.; Lopez-Llorca, L. V., Induction of auxin biosynthesis and WOX5 repression mediate
529 changes in root development in Arabidopsis exposed to chitosan. *Scientific reports* **2017**, *7*,
530 16813.
531 21.Divya, K.; Smitha, V.; Jisha, M., Antifungal, antioxidant and cytotoxic activities of chitosan
532 nanoparticles and its use as an edible coating on vegetables. *International journal of biological*
533 *macromolecules* **2018**, *114*, 572-577.
534 22.Sreekumar, S.; Goycoolea, F. M.; Moerschbacher, B. M.; Rivera-Rodriguez, G. R., Parameters
535 influencing the size of chitosan-TPP nano-and microparticles. *Scientific reports* **2018**, *8*, 4695.
536 23.Anitha, A.; Rani, V. D.; Krishna, R.; Sreeja, V.; Selvamurugan, N.; Nair, S.; Tamura, H.;
537 Jayakumar, R., Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, O-
538 carboxymethyl and N, O-carboxymethyl chitosan nanoparticles. *Carbohydrate Polymers* **2009**,
539 *78*, 672-677.
540 24.Martin-Saldaña, S.; Chevalier, M. T.; Iglesias, M. J.; Colman, S. L.; Casalongué, C. A.; Álvarez,
541 V. A.; Chevalier, A. A., Salicylic acid loaded chitosan microparticles applied to lettuce seedlings:
542 Recycling shrimp fishing industry waste. *Carbohydrate Polymers* **2018**, *200*, 321-331.
543 25.Zobel, R. W., Arabidopsis: An Adequate Model for Dicot Root Systems? *Frontiers in plant*
544 *science* **2016**, *7*, 58.
545 26.Tatematsu, K.; Kumagai, S.; Muto, H.; Sato, A.; Watahiki, M. K.; Harper, R. M.; Liscum, E.;
546 Yamamoto, K. T., MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions
547 together with the transcriptional activator NPH4/ARF7 to regulate differential growth
548 responses of hypocotyl and formation of lateral roots in Arabidopsis thaliana. *The Plant Cell*
549 **2004**, *16*, 379-393.
550 27.Ulmasov, T.; Murfett, J.; Hagen, G.; Guilfoyle, T. J., Aux/IAA proteins repress expression of
551 reporter genes containing natural and highly active synthetic auxin response elements. *The*
552 *Plant Cell* **1997**, *9*, 1963-1971.
553 28.Oono, Y.; Chen, Q. G.; Overvoorde, P. J.; Köhler, C.; Theologis, A., age mutants of
554 Arabidopsis exhibit altered auxin-regulated gene expression. *The Plant Cell* **1998**, *10*, 1649-
555 1662.
556 29.Brunoud, G.; Wells, D. M.; Oliva, M.; Larrieu, A.; Mirabet, V.; Burrow, A. H.; Beeckman, T.;
557 Kepinski, S.; Traas, J.; Bennett, M. J., A novel sensor to map auxin response and distribution at
558 high spatio-temporal resolution. *Nature* **2012**, *482*, 103.
559 30.Duarte, M.; Ferreira, M.; Marvao, M.; Rocha, J., An optimised method to determine the
560 degree of acetylation of chitin and chitosan by FTIR spectroscopy. *International Journal of*
561 *Biological Macromolecules* **2002**, *31*, 1-8.
562 31.Cerchiara, T.; Abruzzo, A.; Di Cagno, M.; Bigucci, F.; Bauer-Brandl, A.; Parolin, C.; Vitali, B.;
563 Gallucci, M.; Luppi, B., Chitosan based micro-and nanoparticles for colon-targeted delivery of
564 vancomycin prepared by alternative processing methods. *European Journal of Pharmaceutics*
565 *and Biopharmaceutics* **2015**, *92*, 112-119.
566 32.Jones, A. R.; Kramer, E. M.; Knox, K.; Swarup, R.; Bennett, M. J.; Lazarus, C. M.; Leyser, H. O.;
567 Grierson, C. S., Auxin transport through non-hair cells sustains root-hair development. *Nature*
568 *cell biology* **2009**, *11*, 78.

33.Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B., Fiji: an open-source platform for biological-image analysis. *Nature methods* **2012**, *9*, 676.
34.Pfaffl, M. W., A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic acids research* **2001**, *29*, e45-e45.
35.Shahidi, F.; Arachchi, J. K. V.; Jeon, Y.-J., Food applications of chitin and chitosans. *Trends in food science & technology* **1999**, *10*, 37-51.
36.París, R.; Vazquez, M. M.; Graziano, M.; Terrile, M. C.; Miller, N. D.; Spalding, E. P.; Otegui, M. S.; Casalongué, C. A., Distribution of endogenous NO regulates early gravitropic response and PIN2 localization in Arabidopsis roots. *Frontiers in plant science* **2018**, *9*, 495.
37.Goda, H.; Sawa, S.; Asami, T.; Fujioka, S.; Shimada, Y.; Yoshida, S., Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. *Plant physiology* **2004**, *134*, 1555-1573.
38.Acemi, A.; Bayrak, B.; Çakır, M.; Demiryürek, E.; Gün, E.; El Gueddari, N. E.; Özen, F., Comparative analysis of the effects of chitosan and common plant growth regulators on in vitro propagation of Ipomoea purpurea (L.) Roth from nodal explants. *In Vitro Cellular & Developmental Biology-Plant* **2018**, *54*, 537-544.
39.Asgari-Targhi, G.; Iranbakhsh, A.; Ardebili, Z. O., Potential benefits and phytotoxicity of bulk and nano-chitosan on the growth, morphogenesis, physiology, and micropropagation of Capsicum annuum. *Plant Physiology and Biochemistry* **2018**, *127*, 393-402.
40.Elgadir, M. A.; Uddin, M. S.; Ferdosh, S.; Adam, A.; Chowdhury, A. J. K.; Sarker, M. Z. I., Impact of chitosan composites and chitosan nanoparticle composites on various drug delivery systems: A review. *Journal of food and drug analysis* **2015**, *23*, 619-629.
41.Fendrych, M.; Akhmanova, M.; Merrin, J.; Glanc, M.; Hagihara, S.; Takahashi, K.; Uchida, N.; Torii, K. U.; Friml, J., Rapid and reversible root growth inhibition by TIR1 auxin signalling. *Nature Plants* **2018**, *4*, 453.
42.Correa-Aragunde, N.; Foresi, N.; Lamattina, L., Auxin and nitric oxide: a counterbalanced partnership ensures the redox cue control required for determining root growth pattern. In *Advances in Botanical Research*, Elsevier: 2016; Vol. 77, pp 41-54.
43.Terrile, M. C.; París, R.; Calderón-Villalobos, L. I.; Iglesias, M. J.; Lamattina, L.; Estelle, M.; Casalongué, C. A., Nitric oxide influences auxin signaling through S-nitrosylation of the Arabidopsis TRANSPORT INHIBITOR RESPONSE 1 auxin receptor. *The Plant Journal* **2012**, *70*, 492-500.
44.Iglesias, M. J.; Terrile, M. C.; Correa-Aragunde, N.; Colman, S. L.; Izquierdo-Álvarez, A.; Fiol, D. F.; París, R.; Sánchez-López, N.; Marina, A.; Calderón Villalobos, L. I. A.; Estelle, M.; Lamattina, L.; Martínez-Ruiz, A.; Casalongué, C. A., Regulation of SCFTIR1/AFBs E3 ligase assembly by S-nitrosylation of Arabidopsis SKP1-like1 impacts on auxin signaling. *Redox Biology* **2018**, *18*, 200-210.
45.Chandra, S.; Chakraborty, N.; Dasgupta, A.; Sarkar, J.; Panda, K.; Acharya, K., Chitosan nanoparticles: a positive modulator of innate immune responses in plants. *Scientific reports* **2015**, *5*, 15195.
46.Goy, R. C.; Britto, D. d.; Assis, O. B., A review of the antimicrobial activity of chitosan. *Polímeros* **2009**, *19*, 241-247.
47.Olatunji, D.; Geelen, D.; Verstraeten, I., Control of endogenous auxin levels in plant root development. *International journal of molecular sciences* **2017**, *18*, 2587.
48.Liu, J.; Moore, S.; Chen, C.; Lindsey, K., Crosstalk complexities between auxin, cytokinin and ethylene in Arabidopsis root development: from experiments to systems modelling, and back again. *Molecular plant* **2017**.
49.Pérez-Torres, C.-A.; López-Bucio, J.; Cruz-Ramírez, A.; Ibarra-Laclette, E.; Dharmasiri, S.; Estelle, M.; Herrera-Estrella, L., Phosphate availability alters lateral root development in Arabidopsis by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. *The Plant Cell* **2008**, *20*, 3258-3272.

621 50.Hermans, C.; Hammond, J. P.; White, P. J.; Verbruggen, N., How do plants respond to
622 nutrient shortage by biomass allocation? *Trends in plant science* **2006**, *11*, 610-617.
623 51.Ham, B.-K.; Chen, J.; Yan, Y.; Lucas, W. J., Insights into plant phosphate sensing and
624 signaling. *Current opinion in biotechnology* **2018**, *49*, 1-9.
625 52.Sharif, R.; Mujtaba, M.; Ur Rahman, M.; Shalmani, A.; Ahmad, H.; Anwar, T.; Tianchan, D.;
626 Wang, X., The multifunctional role of chitosan in horticultural crops; a review. *Molecules* **2018**,
627 *23*, 872.
628 53.Agbodjato, N. A.; Noumavo, P. A.; Adjanohoun, A.; Agbessi, L.; Baba-Moussa, L., Synergistic
629 effects of plant growth promoting rhizobacteria and chitosan on in vitro seeds germination,
630 greenhouse growth, and nutrient uptake of maize (Zea mays L.). *Biotechnology research*
631 *international* **2016**, *2016*, 1-11.

632

633


Chitosan
Microparticles
Promotion of root
development
Auxin
Auxin response genes
Cycle

634

635

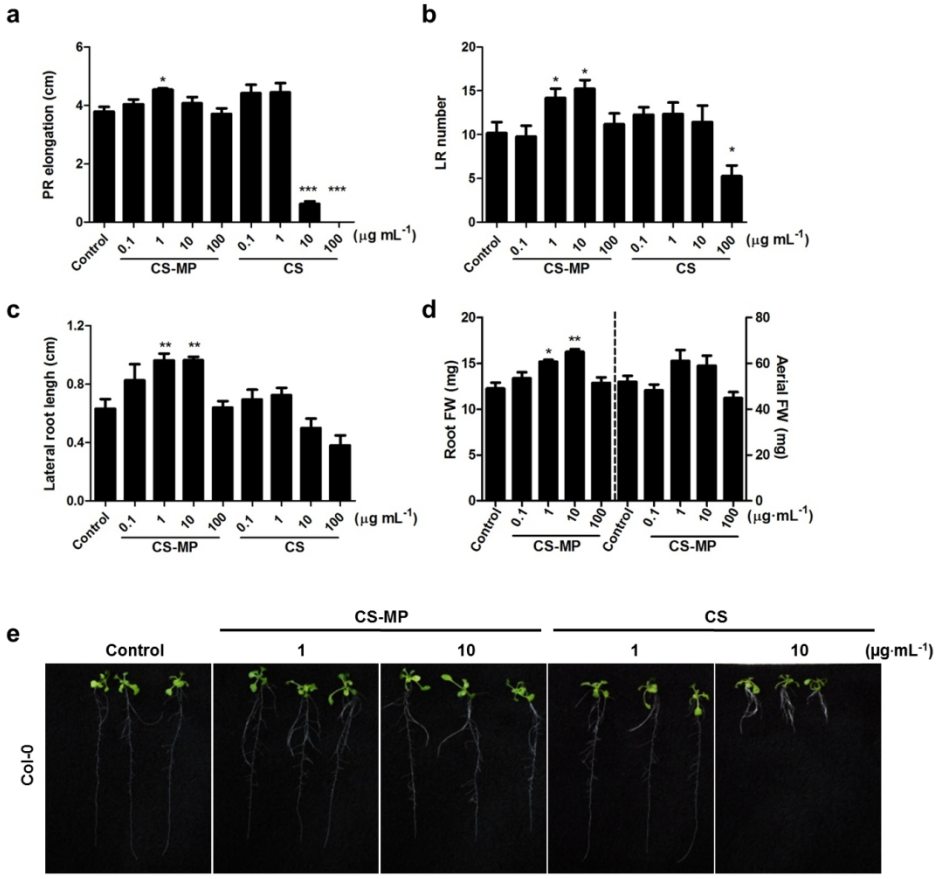


Figure 1. CS-MPs promote root development in Arabidopsis. Five dpg Col-0 Arabidopsis seedlings grown in ½ MS medium were treated with increasing concentrations of CS-MPs or CS as control. PR elongation (a) was quantified 3 d post- treatment. LR number (b) and LR length (c) were analyzed 5 d post treatment. Seedlings FW was quantified 9 d post- treatment (d). Representative images 9 d post treatments are shown in (e). Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test against control,* $p< 0.05$ ** $p<0.01$ ***$p<0.001$).

252x236mm (150 x 150 DPI)

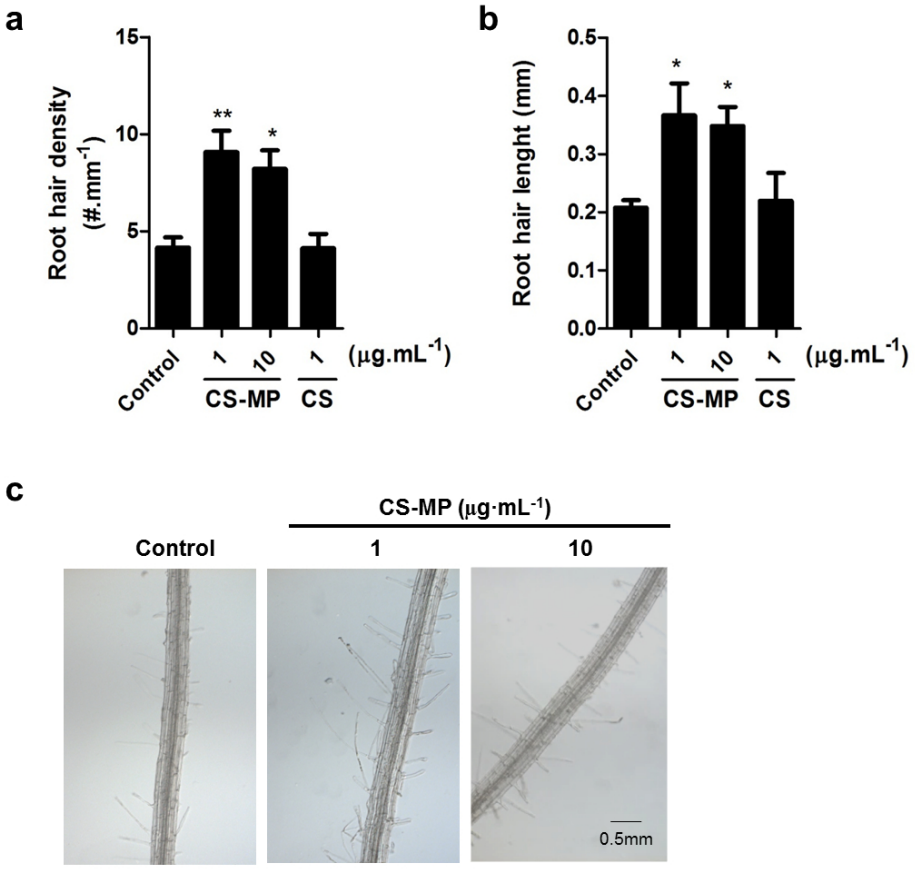


Figure 2. Promotion of RH upon CS-MPs exposure. Five dpg Col-0 Arabidopsis seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPS for 48h. RH density (a) and RH length (b) were analyzed in a 5 mm section from the beginning of the differentiation zone. Representative images are shown in (c). Data are mean values of 4 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control, * $p < 0.05$).

186x173mm (150 x 150 DPI)

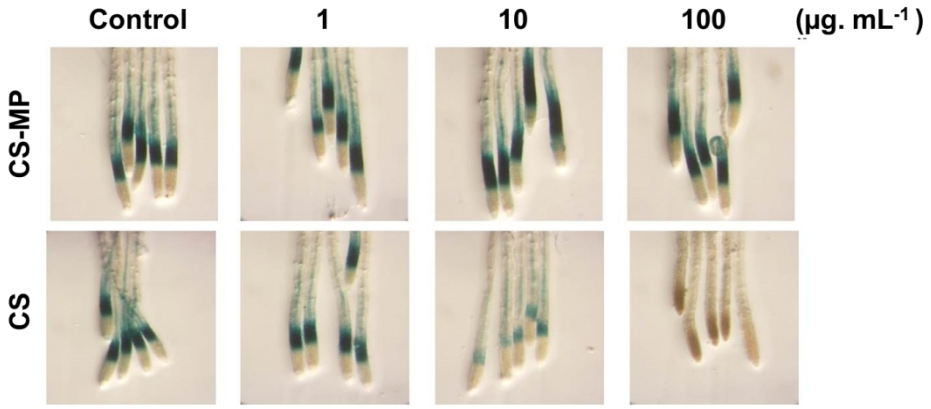


Figure 3. Differential effect of CS-MPs and CS on auxin BA3:GUS reporter gene activity. Five dpg Arabidopsis BA3:GUS seedlings grown in ½ MS medium were treated with 100 nM IAA and increasing concentrations of CS-MPs or CS. Seedlings were subjected to GUS staining after 5 h of treatment. Representative images of PR tips are shown.

255x120mm (150 x 150 DPI)

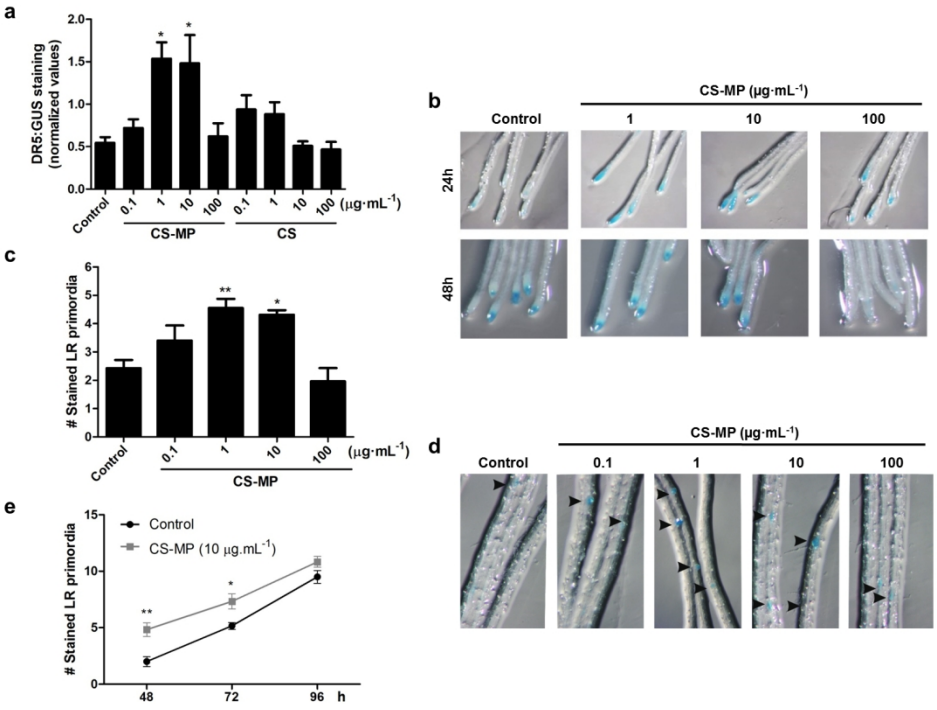


Figure 4. CS-MPs promote an early activation of auxin response in Arabidopsis roots. Five dpg DR5:GUS seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of MP-CS. GUS activity was revealed after incubation with X-Gluc at 37°C. GUS activation in PR was analyzed 24 h post-treatment (a). GUS staining in representative root tip segments after 24 h and 48 h is shown in b. Stained LR primordia were quantified after 48h (c). GUS staining in representative root segments of the differentiation zone are shown in (d). Time-course analysis of stained primordia following 48, 72 and 96 h of treatment with 10 µg mL-1 is shown in (e) .Data are mean values of 5 independent experiments (n= 60; ANOVA, Dunnet post-hoc test,* p< 0.05 ** p<0.01).

297x224mm (150 x 150 DPI)

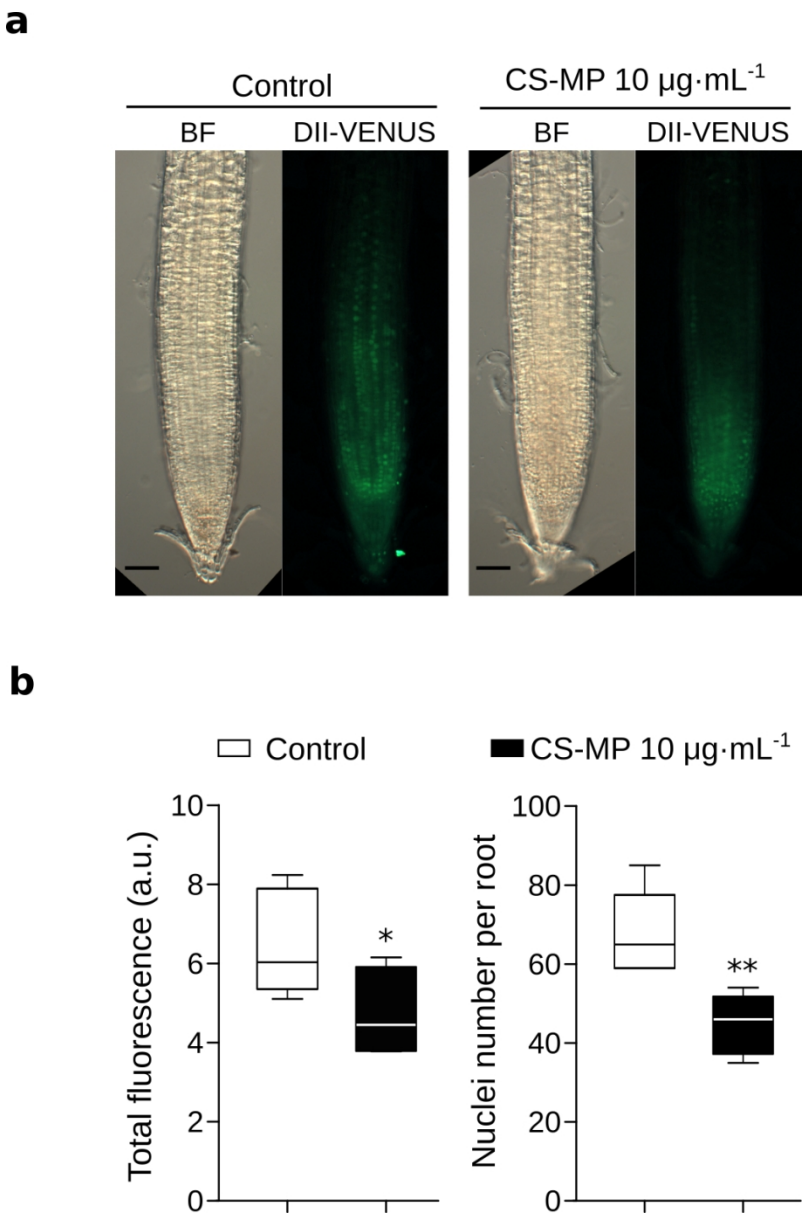


Figure 5. CS-MPs enhance auxin sensitive in DII-VENUS Arabidopsis seedlings. Five dpg DII-VENUS seedlings were transferred to liquid ½ MS medium supplemented with 10 µg mL-1 CS-MPs (a) DII-VENUS expression (green) in PR of control and CS-MPs treated seedlings. Bright field images (BF). (b) Quantification of total DII-VENUS signal and DII-VENUS positive nuclei number per root. Box plot showing median, minimum and maximum values of 2 independent experiments (n= 24). P-values in comparison to control were calculated with two-tailed Student's t-test, *p ≤ 0.05 **p ≤ 0.01. Scale bars, 50 µm.

372x561mm (72 x 72 DPI)

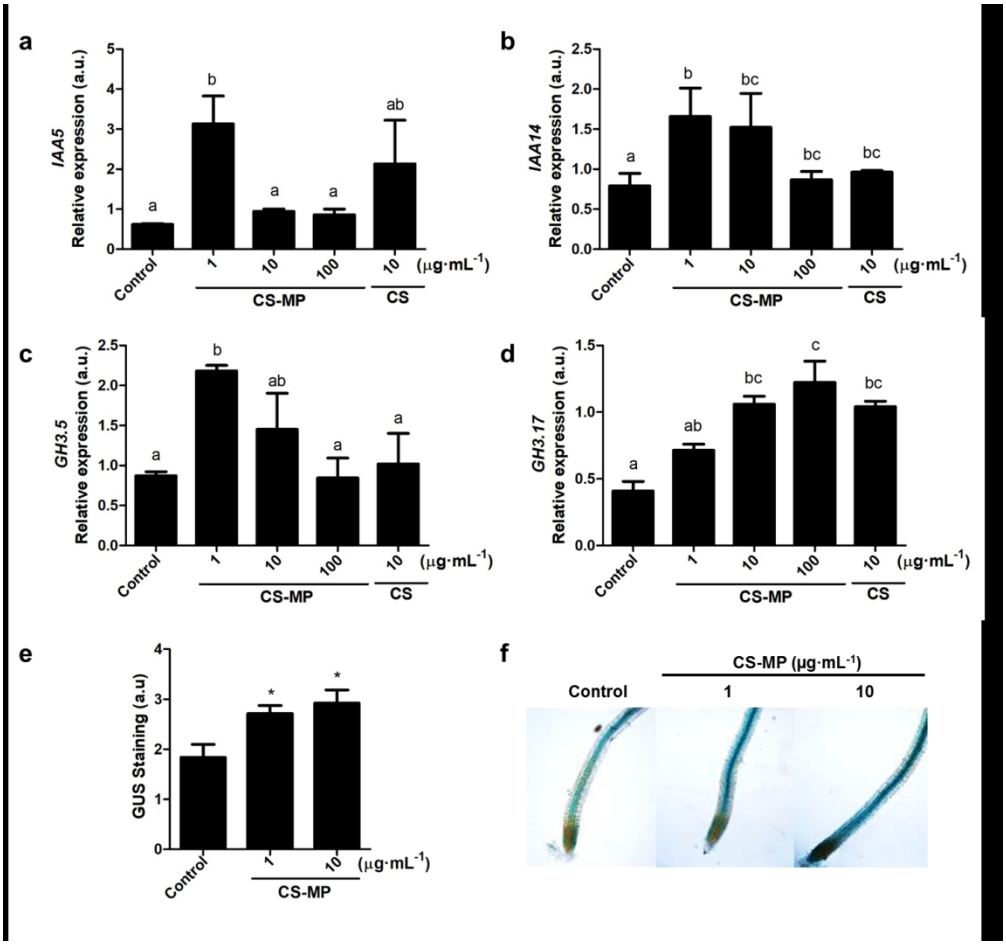
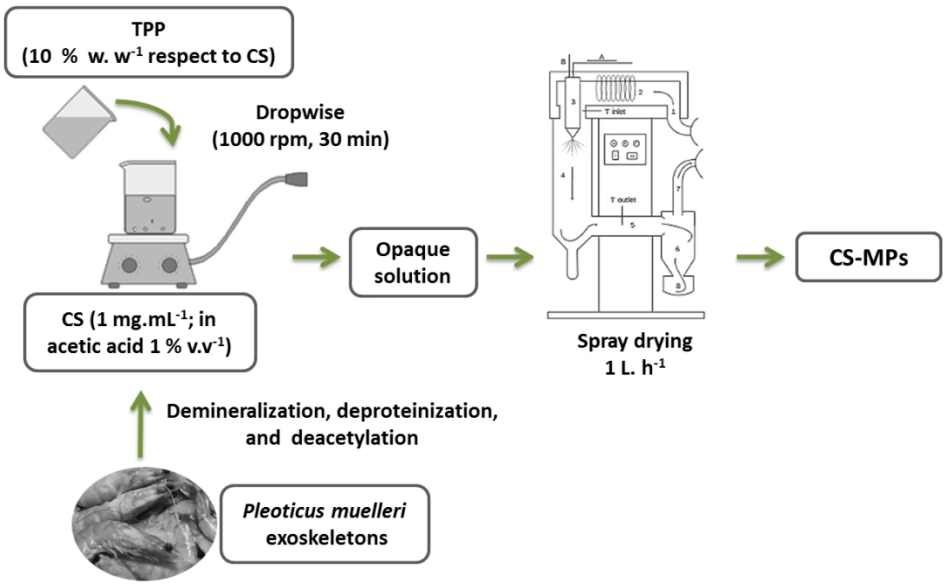


Figure 6. CS-MPs promote the activation of early auxin response genes in Arabidopsis seedlings. Five dpg Col-0 seedlings were transferred to liquid ½ MS medium supplemented with increasing concentrations of CS-MPs or CS as control. (a-d) show the expression of a subset of early auxin response genes analyzed by qPCR after 24 h of CS-MPs treatment. (e) GUS activity in representative PR of 5 dpg MSG2/IAA19:GUS Arabidopsis seedlings treated with 1 and 10 µg mL-1 CS-MPs for 24 h. Quantification of GUS staining in representative root tip segments is shown in (f). Data are mean values of 3 independent experiments (n= 30; ANOVA, Dunnet post-hoc test against control,* p< 0.05 ** p<0.01).

251x235mm (150 x 150 DPI)



Scheme 1: Synthesis of CS-MPs

219x137mm (150 x 150 DPI)