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Chlortetracycline inhibits seed germination and seedling growth in *Brassica campestris* by disrupting H_2O_2 signaling

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Abstract

Antibiotics have been identified as a new type of environmental contaminant because of their increased use in farm animal production systems. Those drugs that animals are not absorbed mostly are excreted in the feces and urine and contaminates soils. However, the effects of antibiotics on crop plants are still largely unknown. In this study, we determined the effects of chlortetracycline (CTC), a veterinary drug released into the agricultural field by grazing animals or through manure application, on the growth and physiology of *Brassica campestris* seedlings. Differently from animals, *Brassica campestris* seedlings have accumulated 5–10-fold higher CTC during cultivation rather than excretion. Morphologically, CTC delays seed germination and inhibits seedling growth such as shortening primary root length and decreasing chlorophyll level. At the molecular level, CTC accumulation in plants downregulated the expression of *superoxide dismutase* (SOD) genes and decreased the production of hydrogen peroxide (H_2O_2). Since H_2O_2 is one of the signaling components involved in the regulation of root growth, exogenous application of H_2O_2 partially restored the growth and physiology of CTC-treated seedlings. These results suggest that application of CTC-containing manure or compost to soil delays seed germination and inhibits plant growth.

Keywords: *B. campestris*, Plant growth, Chlortetracycline, Superoxide dismutase (SOD), Hydrogen peroxide (H_2O_2)

Introduction

Plants are sessile organisms that respond to external environments by altering their growth and development, which affects crop yield and quality. Environmental stresses including contaminants such as organic pollutants cause a variety of physiological or oxidative stresses in plants characterized by the inhibition of growth and chlorosis of leaves [1–3].

The Brassicaceae family includes a number of important vegetable crops that are a rich source of oil, minerals, vitamins, and dietary fiber [4]. Among the *Brassica* species, Napa cabbage (*Brassica campestris* L. ssp. *pekinensis*), also known as Kimchi cabbage, is a nutritionally rich vegetable cultivated worldwide, especially in Asia [5]. More than two million tons of cabbage is produced and consumed in Korea each year, accounting for 20–25% of the Korean vegetable consumption [6]. The early growth and development of cabbage seedlings influence the growth of the leafy head of cabbage, thus affecting crop yield and quality [7]. Additionally, fertilizer application is required to maintain crop quality and quantity, which are liable to decline because of the depletion of nutrients during the short cultivation time of Kimchi cabbage [7, 8].

Composting is a natural process of aerobic decomposition or fermentation of manure by microorganisms, which improves soil health. Composting is the most common method of recycling resources and livestock manure [8, 9]. In farm animal production systems, the use of veterinary drugs including antibiotics for disease treatment or prevention has increased over time. However, animals are excreted most of these veterinary drugs without absorption, and these antibiotics are released

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into the agricultural field through manure [9–12]. The environmental fate of these byproducts after composting and field application, and their effects on plant growth, development, and physiology remain largely unknown [10, 13, 14].

Plant roots are important plant organs, as they provide structural support to plants in the soil and enable the uptake of nutrients and water. Physiologically, roots are involved in the perception of and response to environmental fluctuations [15, 16]. Reactive oxygen species (ROS) are required for root growth and development [17] and are produced in plant roots by the activity of various enzymes such as NADPH oxidases [18], also known as respiratory burst oxidase homologs (RBOHs), and superoxide dismutase (SOD) [19, 20].

NADPH oxidase produces superoxide radical (O_2^-) by reducing oxygen (O_2) [18]. Since a high level of O_2^- is toxic to cells, it is rapidly converted to H_2O_2 and O_2 by the catalytic activity of SOD [19–21]. Therefore, SOD enzymes are important for the tight regulation of O_2^- level and maintenance of redox balance in plant cells. SOD isoenzymes are classified into three groups, depending on their metal cofactor: iron SOD (FeSOD), manganese SOD (MnSOD), and copper/zinc SOD (Cu/ZnSOD); all of these isozymes function in different subcellular organelles [21].

Chlortetracycline (CTC) is one of the most commonly used veterinary antibiotics [13, 26]. In this study, we investigated the environmental fate of CTC in the agricultural field and its impact on the growth and development of *B. campestris* seedlings.

Materials and methods

Plant growth conditions and CTC treatment

Seeds of Kimchi cabbage (*Brassica campestris* L. ssp. *pekinensis* Rupr.) were purchased from ASIA seed company (Seoul, Korea). To conduct the seedling growth assay, 45 seeds were placed on 1.2% agar media in the presence of CTC and incubated vertically at 23 °C under 16-h light/8-h dark photoperiod for 4–7 days. The number of seeds with a ruptured seed coat (indicating radicle emergence) were counted daily at 24-h intervals, and germination rate (%) was calculated. Cotyledon opening (%) and greening (%) were calculated based on the number of seedlings with open cotyledons and over 50% green cotyledons, respectively. Photographs of seedlings were captured, and primary root length was measured using ImageJ (https://imagej.nih.gov/ij/download.html). Chlorophyll was extracted from the detached cotyledonary leaves using 95% ethanol, and chlorophyll content was calculated as described previously [22].

To conduct the seedling growth assay, seeds with a ruptured seed coat were transferred to 1.2% agar media containing CTC, and/or diphenyleneiodonium (DPI), hydrogen peroxide (H_2O_2), and grown vertically for 4–7 days more. Length of the primary root and chlorophyll contents were determined using ImageJ software and 95% ethanol [22], respectively.

To detect CTC in *B. campestris* seedlings, seeds were sown on half-strength Murashige and Skoog (1/2 MS) agar medium containing 0 or 5 ppm CTC, and the plates were incubated in a growth chamber maintained at 22 °C temperature, 16-h light/8-h dark photoperiod, and 200 μE m^{-2} s^{-1} light intensity using fluorescent lamps. Shoots of 20-day-old seedlings were harvested and washed three times with 50% methanol. To analyze a CTC level, surface washed the ground tissues of seedlings were frozen-dried and measured the weight (dry weight).

Detection of CTC residue in *B. campestris* seedlings

The shoots of *B. campestris* seedlings were freeze-dried and ground in liquid nitrogen. Then, 0.1 g of each milled sample was extracted using McIlvain buffer (0.2 M Na_2HPO_4, 0.1 M citric acid, and 2.5 mM EDTA), and CTC content was determined as described by Kim and Carlson [23].

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from *B. campestris* seedlings using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions, and 1.0 μg of RNA was used for cDNA synthesis. Then, qRT-PCR was performed on a CFX Connect Real-time PCR Detection System (Bio-Rad) using the cDNA template, gene-specific primers (Additional file 1: Table S1), and SYBR Green Real-time PCR Master Mix (Toyobo Co., Ltd.). *Actin7* was used as the internal reference gene for data normalization, and average gene expression levels were determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

H_2O_2 staining

Seedlings grown in the presence of 0 or 5 ppm CTC were incubated in 3,3′-diaminobenzidine (DAB) solution (Sigma-Aldrich) in a 6-well plate and covered with aluminum foil [24]. After overnight incubation, the foil was removed, and the DAB staining solution was replaced with distaining solution (ethanol:acetic acid: glycerol = 3:1:1 [v/v/v]) until dark brown color at the plant tissues such as roots is visualized and brightened.

Statistical analysis

Statistical analysis of data was performed using one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test for multiple comparisons. Differences between means were considered statistically significant at $p < 0.05$.

Results

CTC inhibits the growth of *B. campestris* seedlings

To understand the effect of CTC on crop physiology, we examined *B. campestris* seed germination and seedling growth in the presence of CTC (Fig. 1). *B. campestris* seeds were placed on 1.2% agar media in the presence 0, 1, 5, 10, and 25 (mg/L) of CTC. The number of seeds with a ruptured seed coat and those with open cotyledons and > 50% green cotyledons (post-germination) were counted and determine the seed germination, cotyledon opening, and cotyledon greening data (%), respectively (Additional file 1: Fig. S1). On day 4, primary root length (Fig. 1b), and chlorophyll content in cotyledonary leaves (Fig. 1c) were showed. These results conclusively indicated that physiological development of *B. campestris* seeds and seedlings was delayed and/or diminished with the increase in CTC concentration (Fig. 1). To confirm that CTC affects plant growth, we next examined the growth of *B. campestris* seedlings after transferring the seeds with a ruptured seed coat onto CTC-containing agar media (Fig. 2).

CTC reduced the growth and chlorophyll content of germinated seeds and significantly decreased lateral root growth (Figs. 1a, 2). Together, these data suggest that

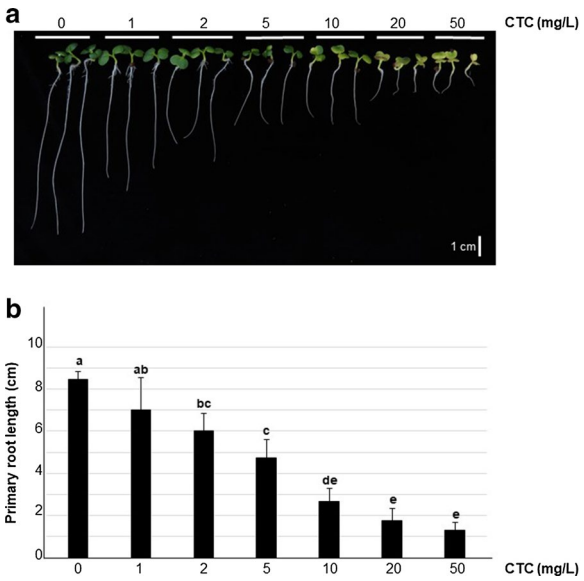

Fig. 2 Effects of external CTC treatment during B. campestris seedling growth. Germinated ruptured seeds were transferred onto CTC-containing media and grown 6-days more vertically. **a** The morphological phenotype of *B. campestris* seedlings. **b** Primary root length. Values are the mean ± SD (n = 36). Different alphabet letters indicate significantly statistical difference (Turkey's test, p < 0.05). Experiment was repeated three times with similar results

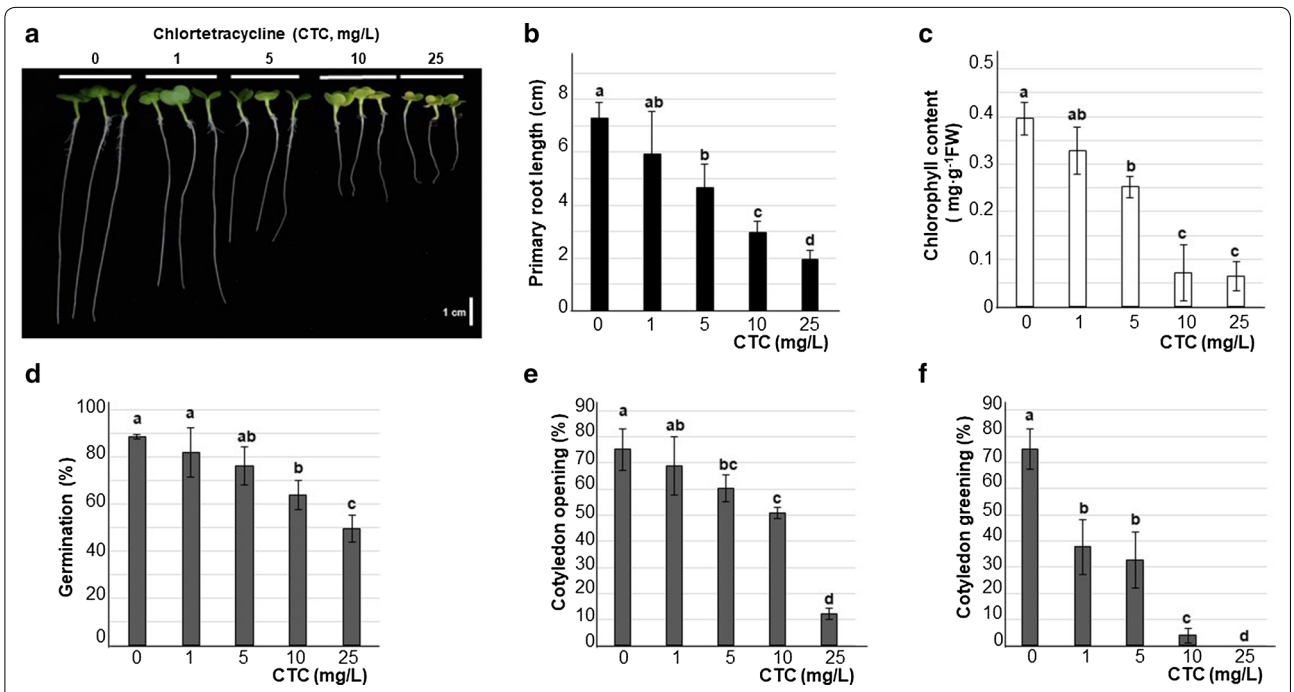

Fig. 1 Effects of CTC treatment on seed germination and seedling growth in *Brassica campestris*. Seeds were cultured in deionized and distilled water containing chlortetracycline (CTC) at 0, 1, 5, 10, and 25 mg/L for 4 days. **a**–**c** Photograph (**a**), primary root length, and chlorophyll content (**c**) of *B. campestris* seedlings on day 4. **d** Germination rate (%) on day 1. **e** Cotyledon opening (%) on day 3. **f** Cotyledon greening (%) on day 2. All experiments were performed in triplicate with similar results. Data represent mean ± standard deviation (SD; *n* = 39). Different lowercase letters indicate statistically significant differences (Turkey's test; $p < 0.05$)

CTC inhibits seed germination and seedling growth in *B. campestris*.

To verify plant growth inhibition by CTC, we further monitored seed germination and seedling growth in *Arabidopsis thaliana*, which is generally used as a model plant, in the presence of CTC. The results obtained in Arabidopsis were similar to those obtained in *B. campestris* (Additional file 1: Fig. S2). Collectively, these results indicate that CTC inhibits plant growth and development, suggesting that the use of CTC as an antibiotic in farm animal production systems should be controlled.

Accumulation of CTC in vegetative tissues

We found that CTC inhibits plant growth, both seed germination and early growth of seedlings biologically (Figs. 1, 2; Additional file 1: Fig S2). Before understanding the role of CTC in the plant physiological level, we have a question how much CTC is accumulated in plants. Animals generally do not absorb CTC through their intestine but mostly excrete it in feces and urine as an intact chemical [25].

To examine CTC level *in planta*, we used the *B. campestris* seedings, which seeds were geminated on media containing CTC (5 mg/L) and grown for 15 to 20 days. In addition, we harvested the shoot aerial part of seedlings to confirm the absorbed CTC transport and accumulation in the plants from CTC of growth media. Although CTC application (5 mg/L) was once during seed sowing at the beginning, CTC influx may last until harvest. The CTC residual level of vegetative tissues from 15- to 20-day-old seedlings exhibits 12.6 ± 4.17 and 95.26 ± 2.36 mg/kg, respectively. These results reveal that CTC can be absorbed from the soil, transported from root to shoot, and accumulated in the shoots. In addition, this result supports that CTC residues are accumulated in the edible plant parts during cultivation rather than metabolic degradation or secretion through plant root [10], thus suggesting that CTC residues enters the human food chain, which can pose serious health risks.

CTC plays a role in a RBOH-mediated signaling pathway to inhibit root growth

Oxidative stress is typically characterized by leaf chlorosis and root growth inhibition [15, 26, 27]. Given that *B. campestris* seedlings treated with CTC contained less chlorophyll and exhibited shorter primary root than untreated seedlings (Fig. 1), we hypothesized that CTC induced oxidative stress and disrupts ROS signaling. To test our hypothesis, we observed the phenotype of *B. campestris* seedlings treated with CTC and DPI, a pharmacological chemical that inhibits NADPH oxidases and blocks RBOH-mediated early-stage of ROS signaling [18, 19]. Interestingly, no differences were observed between seedlings treated with both DPI and CTC and those treated with DPI alone (Fig. 3a, b). This suggests that CTC may disrupt in RBOHs-mediated signaling pathway as DPI inhibits seedling growth.

Based on our physiological observation that growth inhibition under the DPI treatment is prior than those by CTC (Fig. 3a, b), we investigated the expression of *RBOHD* and *RBOHF* genes in CTC treated plants by qRT-PCR. The sequence and information of these genes

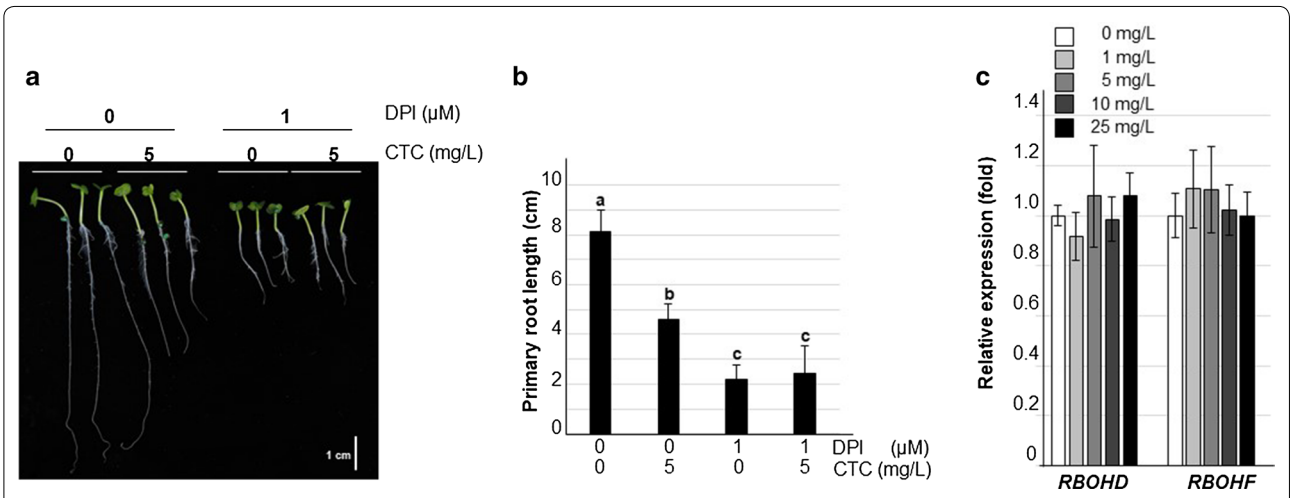

Fig. 3 Effects of CTC and diphenyleneiodonium (DPI) on the growth and physiology of *B. campestris* seedlings. Photograph of seedlings (**a**) and quantification of primary root length in seedlings (**b**) treated with CTC alone or in combination with DPI. **c** Quantitative real-time PCR (qRT-PCR) analysis of *RBOHD* and *RBOHF* genes at the seedling stage. Expression of genes was normalized to that of *Actin7*. The qRT-PCR experiments were replicated four times with similar results. Data represent mean ± SD ($n = 12$). Asterisks indicate statistically significant differences relative to the control (0 mg/L, CTC) (Student's *t*-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

were obtained from Brassica Database BRAD (http://www.brassicadb.org) and Arabidopsis Database TAIR (http://www.arabidopsis.org) (Additional file 1: Table S1). Surprisingly, the mRNA expression of *RBOHD* and *RBOHF* genes, which are direct targets of not only DPI but also regulatory genes during various environmental stress affecting root growth and development [17, 28, 29], was not altered during CTC stress (Fig. 3c). This result speculated that CTC may control downstream components such as *SODs* of *RBOHD* and *RBOHF* in RBOH-mediated signaling.

CTC controls the expression of *SOD* genes

We found that CTC treatment did not affect the expression of *RBOH* genes despite of functioning on DPI-dependent ROS signaling (Fig. 3). Several other studies have supported this speculation that chemical treatment not only led to gene expression but also alters growth and development [30]. Next, we further performed qRT-PCR to determine the expression of *SOD* genes in *B. campestris* treated with or without CTC at different concentrations. We figured out seven different types of *SOD* genes and twelve *B. campestris* genes (Additional file 1: Table S1). Differently from gene expression of *RBOHD* and *RBOHF*, six of the seven *SOD* genes were down-regulated by CTC treatment (Fig. 4a). Different from manganese SOD (MnSOD; MSD1), iron SODs (FeSODs; FSDs), and copper/zinc SODs (Cu/ZnSODs; CSDs) were down-regulated by CTC treatment in a dose- dependent manner.

To confirm down-regulation of SOD, we performed a DAB staining assay. As H_2O_2 is a product of SOD enzyme activity, the oxidized form of 3,3′-diaminobenzidine (DAB) by H_2O_2 generates a dark brown precipitate [24]. As shown Fig. 3b, DAB staining showed that dark brown color in the root tips was diminished in CTC (5 mg/L) treated seedlings. This result indicated that the H_2O_2, which a signal molecule induces root growth and development during early seedling growth [26, 31], has less accumulated at the root tip on the seedlings grown in the presence of CTC (5 mg/L). Notably, these results further indicated that reduction in *SOD* expression limits the accumulation of H_2O_2 and suggested that CTC blocks H_2O_2-mediated signaling during plant growth and development.

H_2O_2 treatment restored the morphology of CTC treated seedlings

Given that CTC treated seedlings showed less accumulation of H_2O_2 in the root tip (Fig. 4b), we next hypothesized that exogenous H_2O_2 supply would reverse the inhibitory effect of CTC on root growth. To test this hypothesis, we examined the growth of seedlings on media containing H_2O_2 and CTC. As expected, the primary root length and chlorophyll content of CTC-treated seedlings recovered to normal levels upon the application of 0.5 mM H_2O_2 (Fig. 5). This result supported that CTC reduces the level of H_2O_2 in the cell, suggesting that H_2O_2 signaling is important for CTC-mediated regulation of seedling growth in *B. campestris*.

To verify the effect of 0.5 mM H_2O_2 on seedling morphology, we further examined the growth of *B. campestris* seedlings in the presence of different concentrations of H_2O_2. The results showed that CTC-induced reduction in primary root length was rescued by 1 and 2 mM H_2O_2 (Additional file 1: Fig. S3). Additionally, H_2O_2 application

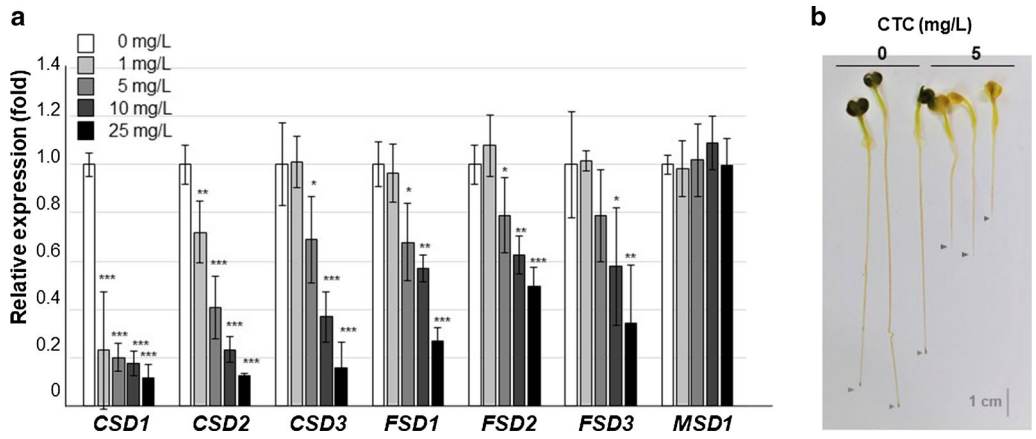

Fig. 4 Analysis of *SOD* expression and H_2O_2 accumulation in *B. campestris* seedlings. **a** Expression of *SOD* genes was normalized relative to that of *Actin7*. Experiments were replicated four times with similar results. Data represent mean ± SD ($n = 12$). Asterisks indicate statistically significant differences relative to the control (0 mg/L) (Student's *t*-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). **b** CTC-induced reduction in H_2O_2 accumulation in root tips of 4-day-old seedlings, as shown by DAB staining

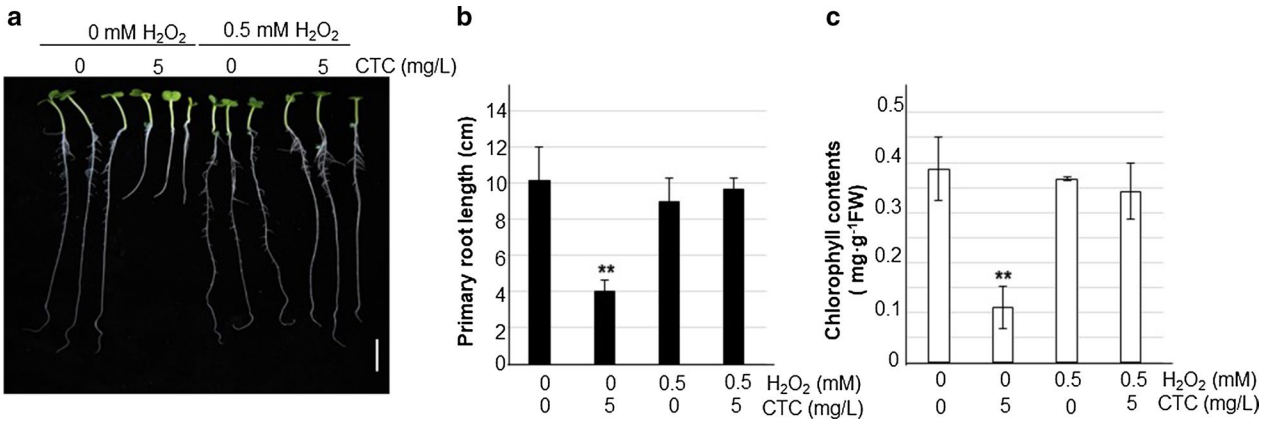

Fig. 5 The growth phenotype of CTC treated seedlings upon H_2O_2 treatment. *B. campestris* seeds were cultured in deionized and distilled water containing indicated CTC (5 mg/L) and/or H_2O_2 (0.5 mM) for 7 days. Morphology (**a**), primary root length (mean ± SD; $n = 39$) (**b**), and chlorophyll content (mean ± SD; $n = 12$) (**c**) of seedlings on day 7. Experiments were performed in four independent biological replicates

also recovered the phenotype of seedlings treated with tetracycline and oxytetracycline, which belong to the same class of antibiotics and cause similar morphological changes in plants as CTC (Additional file 1: Fig. S3).

Next, we analyzed the expression of *RBOHD* and *RBOHF* in plants treated with CTC and/or H_2O_2. The expression of both these genes was not altered by even H_2O_2 application in the presence of CTC (Fig. 3c, Additional file 1: Fig. S4), indicating that CTC and/or H_2O_2 act downstream of *RBOHD* and *RBOHF*. Interestingly, unlike the effect of H_2O_2 treatment on root growth in CTC-treated plants, the expression of *SOD* genes did not recover to normal levels (Fig. 6) [32]. Thus, the expression of *SOD* genes in plants treated with both CTC and H_2O_2 was similar to that in plants treated with CTC alone. This suggests that CTC inhibits plant growth and development by downregulating the expression of *SOD* genes and inhibiting H_2O_2-mediated signaling (Fig. 7).

Discussion

The organic matter content of soil in an agricultural field decreases over time because of intensive cultivation, and application of manure and other organic fertilizers is needed to not only increase the nutrient content of soil but also improve its physiochemical properties for crop production [5, 8]. Animal manure is rich in nutrients [9]. However, approximately 70–80% of veterinary antibiotics supplied in animal feed are released into the environment through the application of manure in the agricultural field [10, 33]. As high as 0.1 mg/kg CTC, one of the widely used antibiotics in animal farming, has been reported in the surface soil and manure [34]. Recent reports indicate that veterinary antibiotics are phytotoxic and affect the growth and yield of crop plants [7, 13]. However, the molecular basis of the phytotoxicity of antibiotics has been reported only in a few studies.

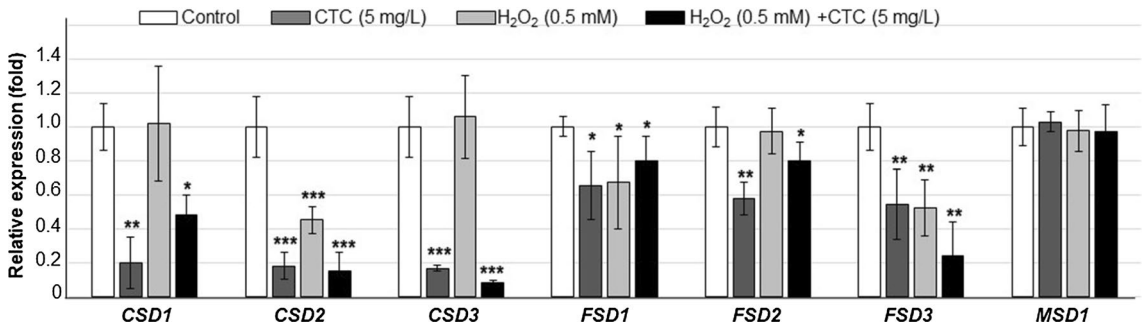

Fig. 6 Expression analysis of *SOD* genes of CTC treated seedlings upon H_2O_2 treatment. Gene expression was normalized relative to *Actin7*. Asterisks indicate statistically significant differences relative to the control (Student's *t*-test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Experiments were performed in three independent biological replicates with similar results. The qRT-PCR experiments were replicated three times with similar results (mean ± SD; $n = 9$)

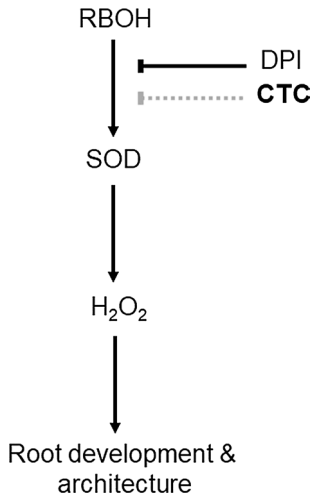

Fig. 7 A CTC working model during root growth of *B. campestris*. CTC down-regulates gene expression of *SODs* rather than *RBOH* and inhibits H_2O_2 production which is a signaling component for controlling root development and architecture

In this study, we analyzed the effect of CTC on seed germination and seedling growth in *B. campestris,* and evaluated the effect of H_2O_2 treatment on the recovery of CTC treated seedings. CTC delayed seed germination, inhibited root growth, and decreased chlorophyll level in *B. campestris* (Figs. 1, 2; Additional file 1: Figs. S1, S2). Moreover, CTC downregulated the expression of *SOD* genes and reduced the accumulation of H_2O_2 (Fig. 4). On the other hand, exogenous supply of H_2O_2 to CTC treated seedlings resulted in the recovery of seedling growth (Fig. 6, Additional file 1: Fig. S4). These results and the finding that H_2O_2 is important for seedling growth and development suggest that CTC blocks H_2O_2-mediated signal transduction (Fig. 7), thus affecting plant growth and development. However, how H_2O_2 treatment facilitated the recovery of damage caused by CTC needs further investigation. It is possible that exogenously supplied H_2O_2 restored H_2O_2-mediated signaling, which was blocked by CTC, thus resuming normal plant growth and development. The other possibility is that H_2O_2 decreases the concentration of CTC in the plant cell. Nonetheless, our results suggest that the use of veterinary antibiotics in the farm animal production systems should be reduced to minimize the contamination of soil and consequently agricultural produce.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13765-019-0484-7.

Additional file 1: Table S1. Primer sequence for qRT-PCR. **Figure S1.** Effects of external CTC treatment on *B. campestris* growth for 4 days. **Figure S2.** Effects of external CTC treatment of *Arabidopsis thaliana*. **Figure S3.** The H_2O_2 effects on tetracycline type antibiotics-mediated growth inhibition. **Figure S4.** The expression analysis of RBOHD and RBOHF genes by qRT-PCR.

Abbreviations

CTC: chlortetracycline; SOD: superoxide dismutase; DPI: diphenyliodonium; RBOHD: respiratory burst oxidase homolog D; RBOHF: respiratory burst oxidase homolog F; SOM: soil organic matter.

Acknowledgements

Not applicable.

Authors' contributions

MSC and YBL contributed to design, data acquisition, and analysis of the study. MSC, Y-EY, JWK, and YKH performed experiments and analyzed the data. MSC and YKH wrote manuscript. SCK and YBL revised the manuscript. All authors agree to be accountable for all aspects of the work. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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