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Physiological and molecular responses of tomato and citrus to chromium (III) stress at early growth stage

Min Wang[1†], Hangfei Li[1†], Kai Xu[1], Jiaying Fang[1], Chao Yu[1], Weiwei Zheng[1*] and Haijie Ma[1*]

Abstract

Chromium (Cr) contamination poses food safety and environmental challenges, yet the early-stage physiological and molecular responses to Cr(III) stress remain unclear. Citrus and tomato are economically important crops representing woody and herbaceous species, making them valuable models for studying heavy metal toxicity in plants. This study investigates the impact of Cr (III) exposure on citrus and tomato seedlings, with a focus on physiological phenotypes and transcriptional response. Citrus seed germination declines with increasing Cr(III) concentrations, while low Cr(III) levels promote tomato germination, with inhibition occurring above 1 g/L. Under hydroponic conditions, Cr (III) severely hampers root and leaf growth in both citrus and tomato plants, accompanied by decreased net photosynthetic rate. Using a GFP-based confocal microscopy system, we observed reduced fluorescence intensity within three days of Cr(III) exposure (100 mg/L and 500 mg/L), indicating early cellular damage. Biochemical assays revealed oxidative stress, marked by increased H_2O_2, malondialdehyde (MDA), and antioxidant enzyme activity. Additionally, low Cr (III) concentrations could result in the death of various microorganisms, including *Escherichia coli*, *Agrobacterium rhizogenes*, and *Agrobacterium tumefaciens*. Transcriptomic analysis identified differentially expressed genes related to "MAPK signaling pathway" and "Plant hormone signal transduction pathway". Transcription of many transcription factors, such as bHLH, WRKY, and MYB, also underwent significant changes.

Keywords Heavy metal, Cr (III), Horticultural plants, Genetic transformation, Gene transcription

Introduction

Rapid urbanization, industrialization, extensive agrochemical use, unsustainable mining, and poor waste management have significantly exacerbated heavy metal (HM) contamination [1–3]. HM are non-biodegradable, persistent inorganic substances with an atomic mass over 20 and a density above 5 g/cm³ [4]. They exhibit cytotoxic, genotoxic, and mutagenic effects on humans, animals, and plants by contaminating food chains, soil, irrigation sources, drinking water, aquifers, and the atmosphere [5–8]. Globally, there are over 10 million contaminated sites, with more than 50% affected by HM [9]. The China Ministry of Environmental Protection reports that

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exceedance in agricultural soils exceeds 19.4%. Among them, the average contents of Cd, Hg, As, Cu, Pb, Cr, Zn and Ni in the soil exceed the standard by 7.0%, 1.6%, 2.7%, 2.1%, 1.5%, 1.1%, 0.9% and 4.8%, respectively [10]. HM in nature fall into two categories: essential micronutrients for plant growth (e.g., Zn, Fe, Mn, Ni, Mg, Cu, Mo) and non-essential metals with unclear biological functions (e.g., Cd, Hg, Sb, Cr, As, Ag, Se, Co, Pb) [6, 11–13]. While plants require certain HM for growth, their ability to absorb essential metals also leads to the uptake of nonessential metals, which can become toxic at excessive levels and pose risk to food safety [14]. Toxic levels of HM can interact with various cellular biomolecules, including nuclear proteins and DNA [15].

Recently, HM contamination has garnered public attention due to its frequent detection in crops. Heavy metals available for plant uptake are those soluble in the soil solution or solubilized by root exudates [14]. Excessive metal concentrations in plants, which cannot be degraded, cause direct and indirect adverse effects, such as cytoplasmic enzyme inhibition and oxidative stress-induced cell damage [16, 17]. HM stress impairs plant growth and indirectly affects human health through the food chain [18]. Unlike organic pollutants, HM pollution is often irreversible, persisting long-term in soils and posing serious threats to plants through high-rate soil-to-plant transfer [19, 20]. Depending on their oxidation states, HMs can be highly reactive and cause various defects in plant cells [21]. HM in soil adversely affect crop growth, photosynthesis, biomass, productivity, and restrict the uptake and translocation of essential nutrients [22, 23]. HM also adversely impacts soil microorganisms, thereby reducing beneficial microorganism populations, hindering organic matter decomposition and soil fertility, ultimately indirectly affecting plant growth.

Chromium (Cr) significantly contaminates groundwater, soil, and sediments through industrial discharge and sewage disposal [24]. Cr is the 17th most abundant element in the Earth's mantle [25]. In *Salix viminalis*, metal toxicity to new root primordia follows the order Cd > Cr > Pb, with root length being more affected by Cr than by other HM [26]. In nature, chromium is found as ore, not as a free element. High Cr concentrations can disrupt chloroplast ultrastructure, thereby impairing photosynthesis. Since seed germination is the first physiological process affected by Cr, a seed's ability to germinate in a Cr-containing medium indicates its tolerance level to this metal [27]. At 200 μM Cr, seed germination of the weed *Echinochloa colona* was reduced to 25% [28]. Oliveira (2012) identified chromate, dichromate, and CrO_3 as highly toxic forms of hexavalent Cr [Cr (VI)] in nature [29]. High levels (500 ppm) of Cr (VI) in soil reduced germination by up to 48% in bush bean (*Phaseolus vulgaris*) [30]. In addition to germination, root growth is often impacted by HM. Cr (VI) concentrations up to 200 mg/L decreased the growth of *Oryza sativa* [31]. Additionally, roots of *Zea mays* treated with Cr (VI) were shorter, brownish, and had fewer root hairs [32]. Adverse effects of Cr on plant height and shoot growth have been reported, with Cr transport to the shoots directly impacting cellular metabolism and reducing plant height. Cr-induced oxidative stress causes lipid peroxidation, damaging cell membranes, and degrading photosynthetic pigments, leading to reduced growth. The addition of 0.5 mM Cr to the nutrient solution reduced the leaf number per wheat plant by 50% [33]. Thus, leaf growth traits could serve as bioindicators of HM pollution and aid in selecting tolerant species [32]. Plant yield hinges on leaf growth, area, and count. Since Cr disrupts numerous biochemical and physiological processes in plants, it also impacts productivity and yield. Under 200 mg/L of Cr (VI) in irrigation water, grain weight and yield (kg/ha) of *Oryza sativa* decreased significantly, up to 80% [31]. Cr stress significantly impacts photosynthesis by affecting CO_2 fixation, electron transport, photophosphorylation, and enzyme activities [34]. Prior research indicates a more pronounced effect of Cr (VI) on PS I activity compared to PS II activity in isolated chloroplasts [35, 36]. HM-induced chlorosis often correlates with reduced plant Fe content, indicating effects on Fe mobilization and uptake. Cr is reported to impact Fe uptake in dicots by inhibiting Fe (III) to Fe (II) reduction or by competing with Fe (II) at absorption sites [32]. Cr stress can induce three types of metabolic modifications in plants: (I) changes in pigment production crucial for plant vitality (e.g., chlorophyll, anthocyanin) [37]; (II) heightened production of metabolites (e.g., glutathione, ascorbic acid) as a direct response to Cr stress, potentially damaging plants [24]; and (III) adjustments in the metabolic pool to generate new biochemically related metabolites, possibly conferring resistance or tolerance to Cr stress (e.g., phytochelatins, histidine). Activation of superoxide dismutase (SOD) and catalase serve as key metal detoxification mechanisms in plants [38]. Cr toxicity in tomato (*Lycopersicon esculentum*) leads to reduced plant nutrient acquisition [39, 40]. In onion (*Allium cepa*), it inhibits germination and reduces plant biomass [41]. Also, in wheat (*Triticum* sp.), reductions in shoot and root growth are observed [33, 42].

Trivalent Cr [Cr (III)] is the predominant stable oxidation state found in living organisms. It cannot easily cross cell membranes and has low reactivity, distinguishing it significantly from Cr (VI) [43]. However, research on the physiological and molecular effects of Cr (III) on horticultural plants remains limited. In this study, citrus and tomato, representing woody and herbaceous crops, were selected for their economic significance and distinct

growth traits, allowing for the analysis of Cr(III) toxicity. The impact of Cr (III) on plant physiology and gene transcription, as well as its toxicity to bacteria, were analyzed.

Materials and methods

Plant materials and growth condition

The fruits and plants of citrus (*Citrus medica* L. *var. sarcodactylis* Swingle) were obtained from the Citrus Research Institute, Chinese Academy of Agricultural Sciences (CRICAAS). The seeds and plants of dwarf red tomatoes (*Solanum lycopersicum* cv. Micro-Tom) were sourced from the National Vegetable Engineering and Technology Research Centre (NVETRC). All plants with an age of one month were maintained in a growth chamber or greenhouse at temperatures ranging from 22 to 26 °C, under a 16-hour light/8-hour dark photoperiod. The Li-6800 photosynthesis analyzer was used to measure the net photosynthetic rate. Before the detection, the instrument was set as follows: Fow = 500 μmol/s, RH = 65%, CO_2_S = 400 ppm, Speed = 10,000 rpm, and Setpoint = 25 °C.

Plasmids and bacterial strains

Binary vectors of MT-gb (GFP localized to Mitochondria, http://nebenfuehrlab.utk.edu/marke-rs/default.htm) and pDSK-GFP (Table. S1, Fig. S1) were used in this study. *A. rhizogenes* strain K599 (NCPPB2659) were obtained from Shanghai Weidi Biotechnology Co., Ltd. (http://www.weidibio.com/). All bacterial strains were stored in 15% glycerol at -80℃. *A. rhizogenes* was transformed with MT-gb, and pDSK-GFP, respectively.

Reagents

LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH = 7.0). TY medium (5 g/L tryptone, 3 g/L yeast extract, 10 mM $CaCl_2$, pH = 7.0). Infiltration solution (100 μM acetosyringone, 10 mM $MgCl_2$, 10 mM MES, pH = 5.8).

Str (Streptomycin sulfate, CAS 3810-47-0, stock solution 50 mg/mL, working concentration 50 μg/mL), kana (Kanamycin sulfate, CAS 13292-46-1, stock solution 50 mg/mL, working concentration 50 μg/mL), Agar (A8190, Solarbio), Vermiculite (LP-ZS, GREEN HOPE), MES (MES sodium salt, A610611, BBI), Yeast extract (A505245, Sangon), Tryptone (A505250, Sangon), Acetosyringone (A601111, BBI).

SOD activity, H_2O_2, and MDA content detection

SOD activity detection: weigh 0.1 g fresh tissue in a 2 mL centrifuge tube, and quickly freeze with liquid nitrogen; Grind the sample and suspend it uniformly in phosphate buffer (0.1 M, pH = 7.0-7.4, 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4,0.0018 M K_2HPO_4), 10,000 rpm for 10 min and collect the supernatant; detect SOD activity using SOD detection kit (A001-1-1, NJJC). H_2O_2 content detection: weigh 0.1 g fresh tissue in a 2 mL centrifuge tube, and quickly freeze with liquid nitrogen; Grind the sample and suspend it uniformly in physiological saline (0.9 % NaCl), 10,000 rpm for 10 min and collect the supernatant; detect H_2O_2 content using a hydrogen peroxide detection kit (A064-1-1, NJJC). MDA content detection: weigh 0.1 fresh tissue in a 2 mL centrifuge tube, and quickly freeze with liquid nitrogen; Grind the sample and suspend it uniformly in phosphate buffer, 10,000 rpm for 10 min and collect the supernatant; detect MDA content using an MDA detection kit (A003-1-1, NJJC).

K599 mediated hairy roots genetic transformation of citrus and tomato

K599 preparation

K599 strains carrying the corresponding plasmids were cultured in liquid TY medium at 200 rpm and 28℃ overnight until OD_{600} reached 0.6–1.2; the bacteria were collected by centrifugation at 5,000 rpm for 10 min at room temperature (RT), resuspended in infiltration solution, and adjusted to OD_{600} = 0.8, the infiltration solution was then incubated at RT for 3–5 h.

Citrus agroinfiltration

Leaf wounds were immersed in the infiltration solution in a vacuum tank, evacuated to vacuum, and maintained for 30 min. The agroinfiltrated explants were then incubated in sterilized vermiculite at 20–26℃ with a 16 h/8 h (light/dark) photoperiod under high humidity.

Tomato agroinfiltration. The lower part of the 4-week-old tomato plants was scratched with a blade dipped in K599 bacterial suspension and then incubated under humid conditions.

Transformants identification

Transgenic hairy roots formed 15–40 days post transformation and were initially identified GFP visualization with a flashlight (LUYOR-3280). Laser confocal microscopy and PCR were used for subsequent verification of transformed roots.

RNA extraction and qRT-PCR analysis

Hairy roots were cut into 2–3 mm segments and placed in 2 mL RNase-free tubes with two sterilized grinding beads. After snap-freezing in liquid nitrogen, the tubes were transferred to a pre-cooled grinding adapter and ground into a fine powder using two 30-second cycles at 60 Hz. RNA was extracted using the FastPure Universal Plant Total RNA Isolation Kit (RC411, Vazyme) and assessed for concentration and quality via Nanodrop and agarose gel electrophoresis. RT-qPCR was performed with the AceQ Universal SYBR qPCR Master Mix

(Q511-02, Vazyme) on a Q2000A real-time PCR system (LongGene).

Transcriptome sequencing and analysis

Healthy citrus and tomato plants were cultivated in Hoagland's nutrient solution (pH = 5.15) at 26℃, 12,000 lx, with a 16 h/8 h (light/dark) photoperiod for 7 days. The treatment group was then transferred to a solution containing 100 mg/L Cr (III), while the untreated group served as the control. After 3 days, roots from each group were flash-frozen in liquid nitrogen for RNA extraction. RNA samples meeting quality standards were used for library preparation and sequencing on the BGI DNBSEQ high-throughput platform. Raw data were filtered with fastp, and quality control was performed using FastQC. The filtered transcriptome sequences were aligned to the reference genome using STAR, and statistical analysis was conducted. RSEM quantified reads mapped to each transcript, followed by FPKM conversion. Differential expression analysis was performed with DESeq2 using read count data, with thresholds set at padj < 0.05 and|log2FoldChange| > 1. GO and KEGG enrichment analyses were carried out using clusterProfiler, and transcription factors were annotated with PlantTFDB.

Results

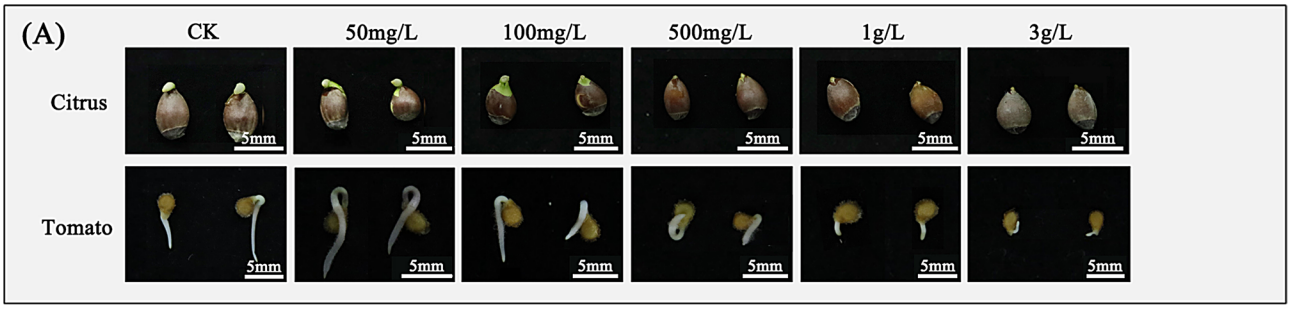
Effect of Cr(III) on seed germination rates

Cr(III) treatment concentrations were 0 mg/L (CK), 50 mg/L, 100 mg/L, 500 mg/L, 1 g/L, and 3 g/L. Citrus seeds were obtained from fresh fruit, and tomato seeds were soaked in cold water for 10 min, followed by a 30-minute incubation at 55 °C before treatment. The germination rate of citrus seeds progressively decreased with increasing Cr(III) concentration, decreased to 41.9% at 500 mg/L, demonstrating a significant inhibitory effect of Cr(III) on citrus seed germination (Fig. 1). Conversely, the germination rate of tomato seeds increased with Cr(III) concentration in the range of 0-500 mg/L (Fig. 1). Additionally, tomato radicle growth accelerated at 50 mg/L Cr(III), but was significantly inhibited at concentrations of 500 mg/L and above (Fig. 1).

Effects of Cr(III) on plant growth and development

To precisely regulate Cr concentration, citrus seedlings with intact root systems were hydroponically cultured in Hoagland nutrient solution to investigate the impact of varying Cr(III) concentrations on plant growth. At 50 mg/L Cr(III), citrus growth was reduced by 50% at day 14, but no mortality was observed (Fig. 2A and C). At 100 mg/L Cr(III), citrus root growth ceased, resulting in a 42% mortality rate after 28 days (Fig. 2A and C). Cr(III) concentrations of 500 mg/L and above completely inhibited citrus root and leaf growth, escalating plant mortality (Fig. 2A and C). These findings underscore Cr(III)'s inhibitory effects on citrus root and leaf growth.

Tomato plants without roots grew well under hydroponic conditions and could regenerate new roots, facilitating a clearer assessment of Cr(III)'s impact on root development. After 7 days, the control group tomato plants exhibited robust shoot and leaf growth with



(B)

Species	CK	50mg/L	100mg/L	500mg/L	1g/L	3g/L
S. lycopersicum	0.683±0.057a	0.900±0.050a	0.867±0.104b	0.767±0.029ab	0.633±0.126b	0.616±0.076b
C. medica	0.717±0.076a	0.633±0.153a	0.617±0.1041a	0.417±0.126b	0.367±0.076b	0.250±0.050b

Fig. 1 Effect of Cr(III) on seed germination rates. **(A)** Photograph showing the effect of different Cr(III) concentrations (50 mg/L, 100 mg/L, 500 mg/L, 1 g/L, 3 mg/L; CK: control group) on citrus and tomato seed germination. **(B)** Quantitative analysis of Cr(III) on the germination rates of tomato and citrus seeds

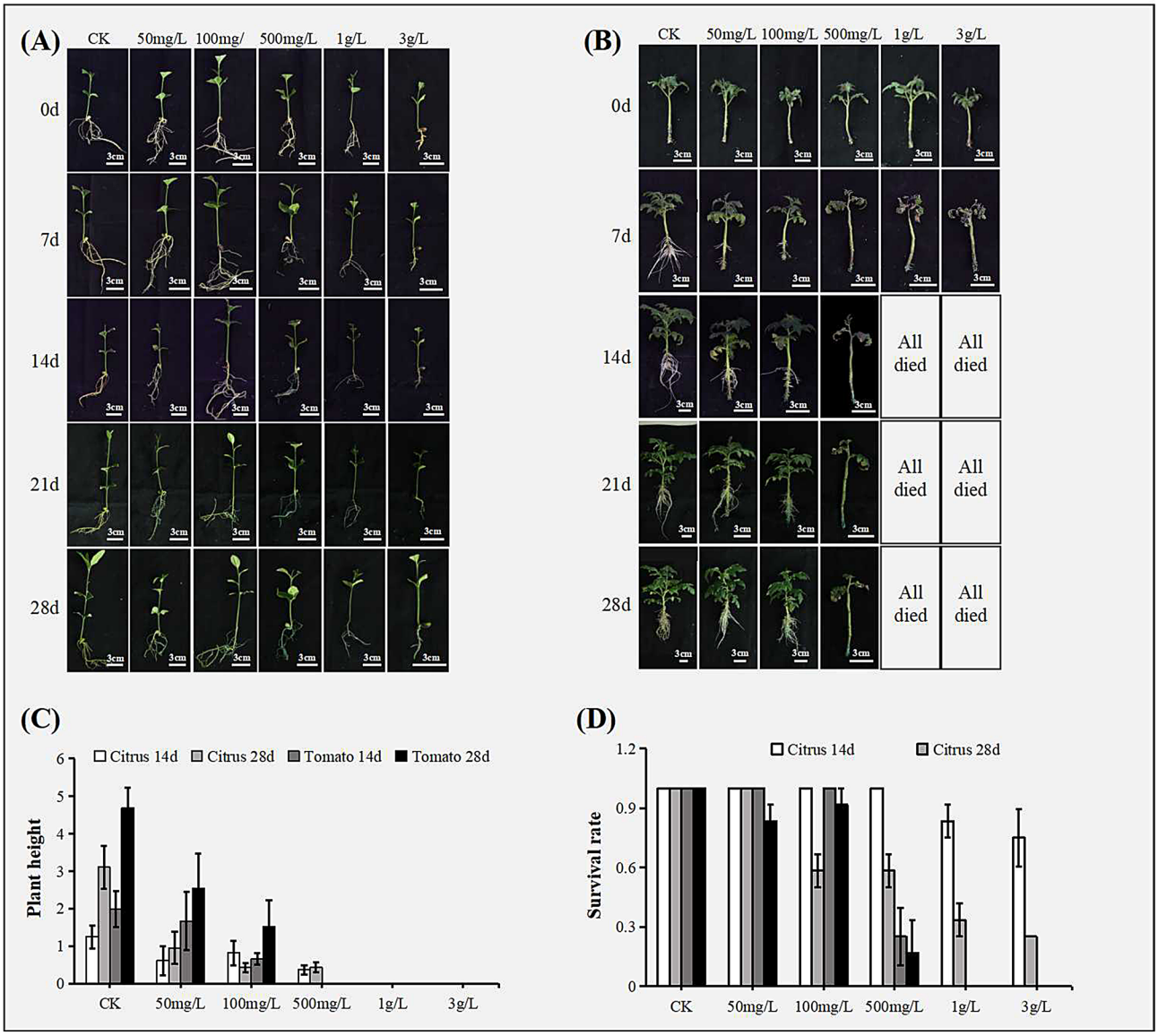


Fig. 2 Effects of Cr(III) on plant growth and development. The effect of different Cr(III) concentrations on the growth and development of citrus **(A)** and tomato **(B)**. **(C)** Quantitative analysis of different Cr(III) concentrations on the growth of tomato and citrus. **(D)** Quantitative analysis of different Cr(III) concentrations on the survival rate of tomato and citrus

abundant root formation, while all Cr(III) treatment groups displayed significant inhibition in tomato root formation, with pronounced leaf wilting observed beyond 500 mg/L Cr(III) (Fig. 2B and D). By day 14, tomato plants under Cr(III) concentrations exceeding 500 mg/L exhibited significant mortality. At a concentration of 500 mg/L, root growth was completely inhibited, stem growth was suppressed, and leaf wilting became more severe. By day 28, the mortality rate reached 83% under 500 mg/L Cr(III), with complete mortality in higher concentration groups. While 50 mg/L and 100 mg/L Cr(III) did not cause tomato mortality, they significantly hindered the growth of roots, stems, and leaves, with hairy root development being particularly inhibited, whereas leaf development and stem coloration were relatively less affected (Fig. 2B and D).

Effects of Cr(III) on SOD activity, H_2O_2, and MDA content

Following previous studies on the effects of Cr on antioxidative enzymes and malondialdehyde content in *Kandelia candel* [44], this study also selected samples 24 h post Cr(III) treatment for biochemical analysis. After 24 h of treatment with 100 mg/L Cr(III), H_2O_2 content in citrus and tomato roots significantly increased, with citrus roots showing a 36.69% increase and tomato roots a 18.64% increase (Fig. 3A). In plant tissues, MDA levels indicate lipid peroxidation and indirectly reflect cellular damage. After 24 h of treatment with 100 mg/L Cr(III),

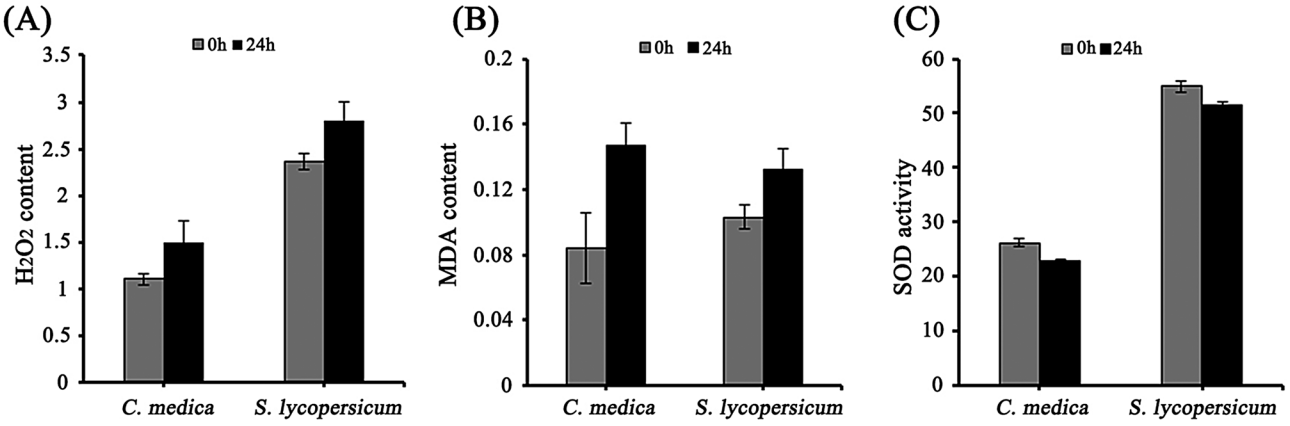


Fig. 3 Effects of Cr(III) on SOD activity, H_2O_2, and MDA content. H_2O_2**(A)** and MDA **(B)** content in citrus and tomato after 0 h and 24 h treatment with 100 mg/L Cr(III). **(C)** SOD activity in citrus and tomato after 0 h and 24 h treatment with 100 mg/L Cr(III)

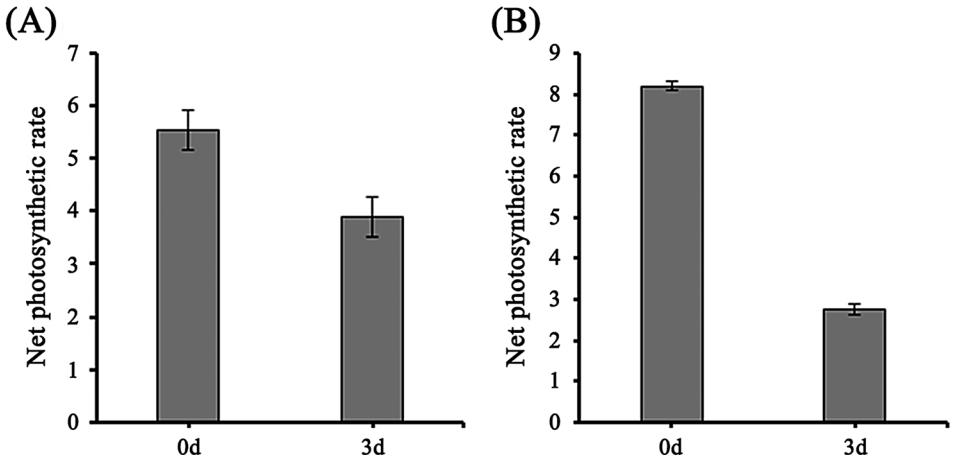


Fig. 4 Effect of Cr(III) on net photosynthetic rate. **(A)** Effect of 100 mg/L Cr(III) treatment for 3d on net photosynthetic rate of citrus. **(B)** Effect of 100 mg/L Cr(III) treatment for 3d on net photosynthetic rate of tomato

MDA levels increased by 75% in citrus roots and 30% in tomato roots, similar to the H_2O_2 trend (Fig. 3B). Additionally, Cr(III) inhibits SOD activity, reducing it by 12.04% in citrus roots and 7.77% in tomato roots after treatment with 100 mg/L (Fig. 3C). Additionally, Cr(III) treatment at a concentration of 100 mg/L resulted in a decrease in net photosynthetic rate by 29.7% in citrus (Fig. 4A) and 66.3% in tomato (Fig. 4B).

Effects of Cr(III) on the activity of different bacteria

To investigate whether the concentration of Cr(III) that exhibits toxicity towards citrus and tomato would likewise exert a toxic impact on other microorganisms. We analyzed the toxicity of different concentrations of Cr(III) on *Escherichia coli* (DH5α), *Agrobacterium tumefaciens* (GV3101), and *Agrobacterium rhizogenes* (K599). Rapid identification of bacterial activity using microscopy is challenging. For precise analysis, we transferred a plasmid (Fig. S1, Table. S1) containing the *GFP*-encoding gene into DH5α, GV3101, and K599. Successfully transformed bacteria were distinctly identified by green fluorescence under a laser scanning confocal microscope (LSCM), indicating GFP protein expression during normal activity. Without Cr(III) treatment, LSCM showed normal fluorescence in bacterial cultures within two days. Following 1 day of exposure to 50 mg/L Cr(III), the survival rates of DH5α and K599 significantly decreased, with observed bacterial adhesion, both increasing in severity with time or Cr(III) concentration (Fig. 5A-D). One day of treatment with 50 mg/L or 100 mg/L Cr(III) did not cause significant death in GV3101. However, extending the treatment duration or increasing the Cr(III) concentration resulted in substantial death in GV3101 (Fig. 5E and F). These results indicate that Cr(III) severely impacts bacterial cell activity.

Establishment of efficient genetic transformation system for tomato and citrus

In previous study, the toxicity of Cr(III) to plants was primarily assessed based on phenotypic changes and tissue

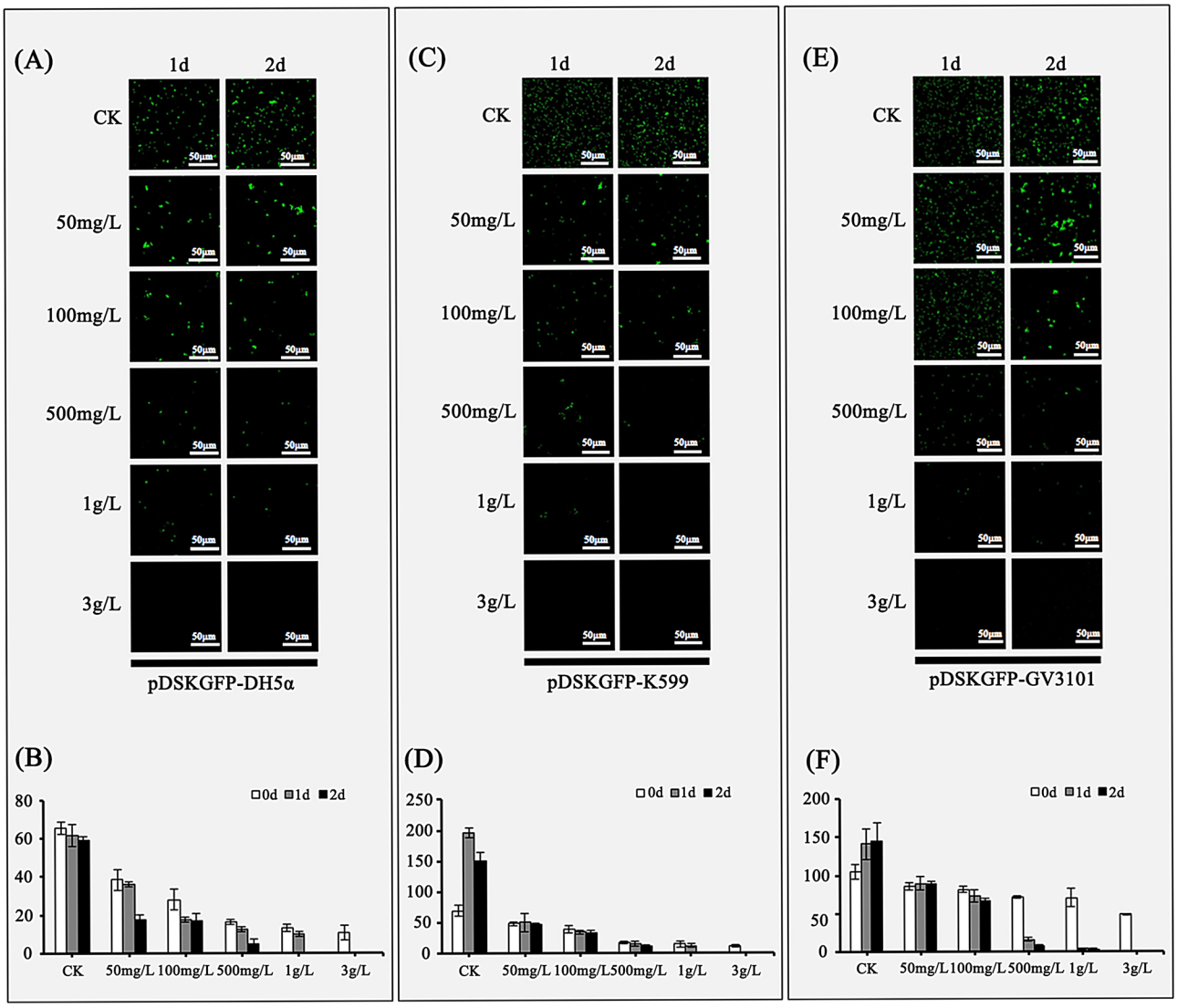


Fig. 5 Effects of Cr(III) on the activity of different bacteria. The effect of different Cr(III) concentrations (50 mg/L, 100 mg/L, 500 mg/L, 1 g/L, 3 mg/L; CK: control group) on the activity of DH5α **(A)**, K599 **(C)**, and GV3101 **(E)**. Quantitative analysis of different Cr(III) concentrations to DH5α **(B)**, K599 **(D)**, and GV3101 **(F)** based on the amount of GFP fluorescence. pDSKGFP-DH5a, pDSKGFP-K599, and pDSKGFP-GV3101 represent DH5a, K599, and GV3101 strains transformed with the pDSKGFP plasmid and normally expressing GFP protein.

color alterations observed days or weeks after treatment, resulting in low evaluation efficiency. To address this issue, we established an efficient, tissue culture-free genetic transformation system for tomato and citrus to generate large batches of GFP-overexpressing transgenic hairy roots. These roots were then used to analyze Cr(III) toxicity based on changes in GFP fluorescence. For citrus genetic transformation, we developed a system using leaves of *C. medica*, leveraging their high cutting survival rate in vermiculite. This method is efficient, time-saving, and cost-effective (Fig. 6A). Two to three weeks after K599 infection of citrus leaves, transgenic fluorescent callus appeared at the leaf base and developed into fluorescent transgenic roots within 3–7 weeks (Fig. 6B). LSCM revealed that most cells in the transgenic roots exhibited green fluorescence (Fig. 6C). PCR, DNA sequencing and gene expression analysis confirmed the successful integration and high expression of the *GFP* in the transgenic roots (Fig. 6D, E and F). Tomato plants, which have a fast growth cycle and are easily infected, were transformed by wounding the base of 4-week-old seedlings with blades dipped in K599 bacterial suspension (Figs. 6A and 7A). Transgenic hairy roots emerged at the wound sites within 2 weeks (Fig. 7B). LSCM, PCR, DNA sequencing, and gene expression analysis demonstrated that this method efficiently produced a large number of transgenic tomato roots (Fig. 7C-F).

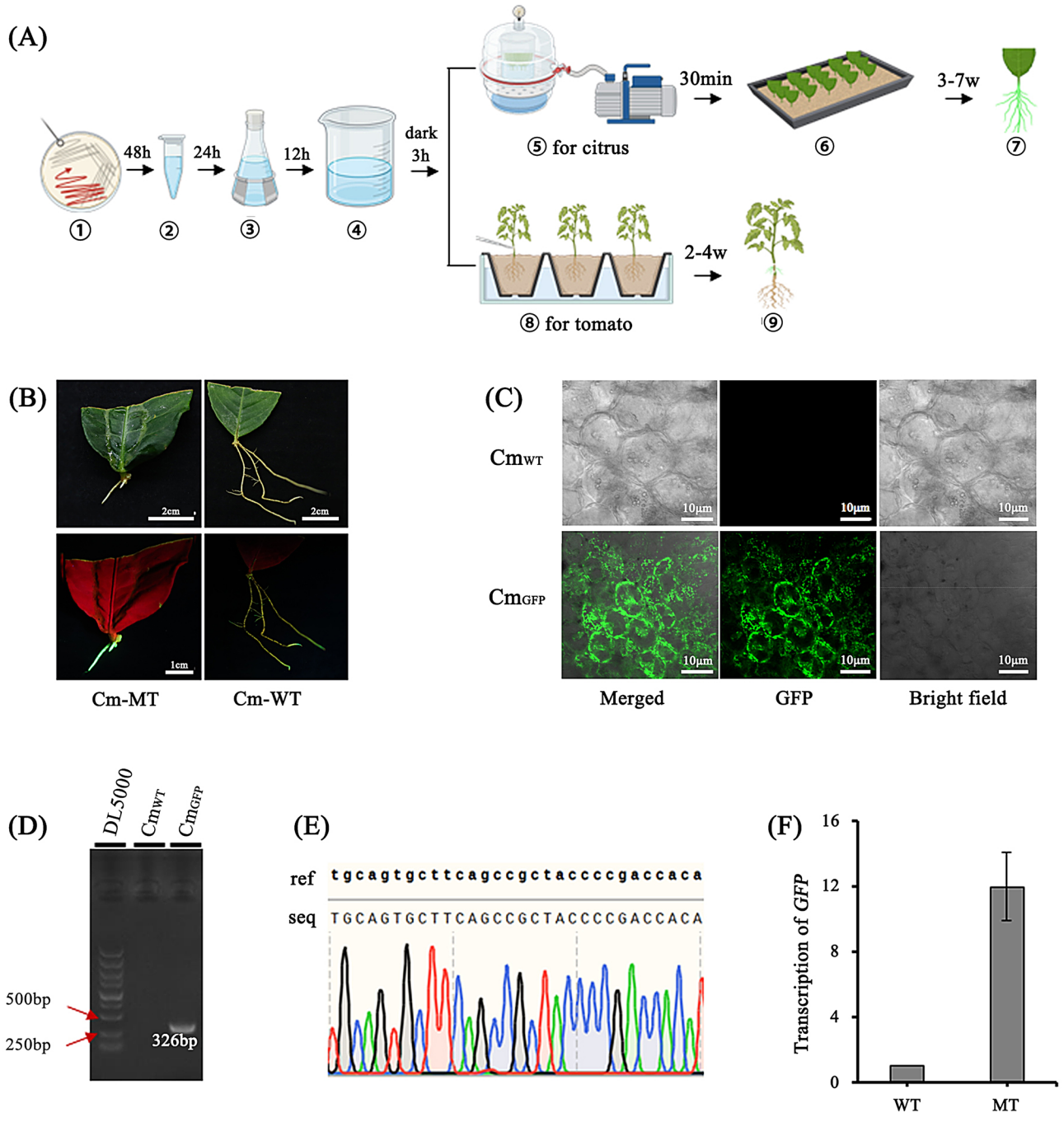


Fig. 6 Establishment of efficient genetic transformation system for citrus. **(A)** Diagram of K599-mediated genetic transformation of *C. medica* leaves and *S. lycopersicum*, bypassing tissue culture. ① Agrobacteria activation, ② culture in 1.5 mL tube, ③ culture in triangular flask, ④ induction in dark, ⑤ vacuum infiltration, ⑥ incubation in vermiculite, ⑦ transformants identification, ⑧ Agrobacteria inoculation, ⑨ transformants identification. **(B)** Identification of transgenic *C. medica* hairy roots with handheld GFP excitation light source. Cm-WT, wild-type of *C. medica*; Cm-GFP, GFP overexpressing transgenic hairy roots of *C. medica*. **(C)** Analysis of transgenic hairy roots using LSCM. **(D)** PCR verification of transgenic hairy roots. **(E)** DNA sequencing verification of transgenic hairy roots. **(F)** Analysis of *GFP* expression using RT-qPCR

Rapid analysis of Cr(III) toxicity to plants mediated by *GFP* Transgenic roots

Under hydroponic conditions, 100 mg/L Cr(III) did not cause severe adverse phenotypes in tomato plants after 3 days of treatment. However, as the concentration increased, adverse phenotypes began to appear and became more severe (Fig. 8A and B). To more accurately analyze the toxicity of low concentrations of Cr(III) on plant cells, transgenic roots expressing mitochondria-targeted GFP were treated with different concentrations

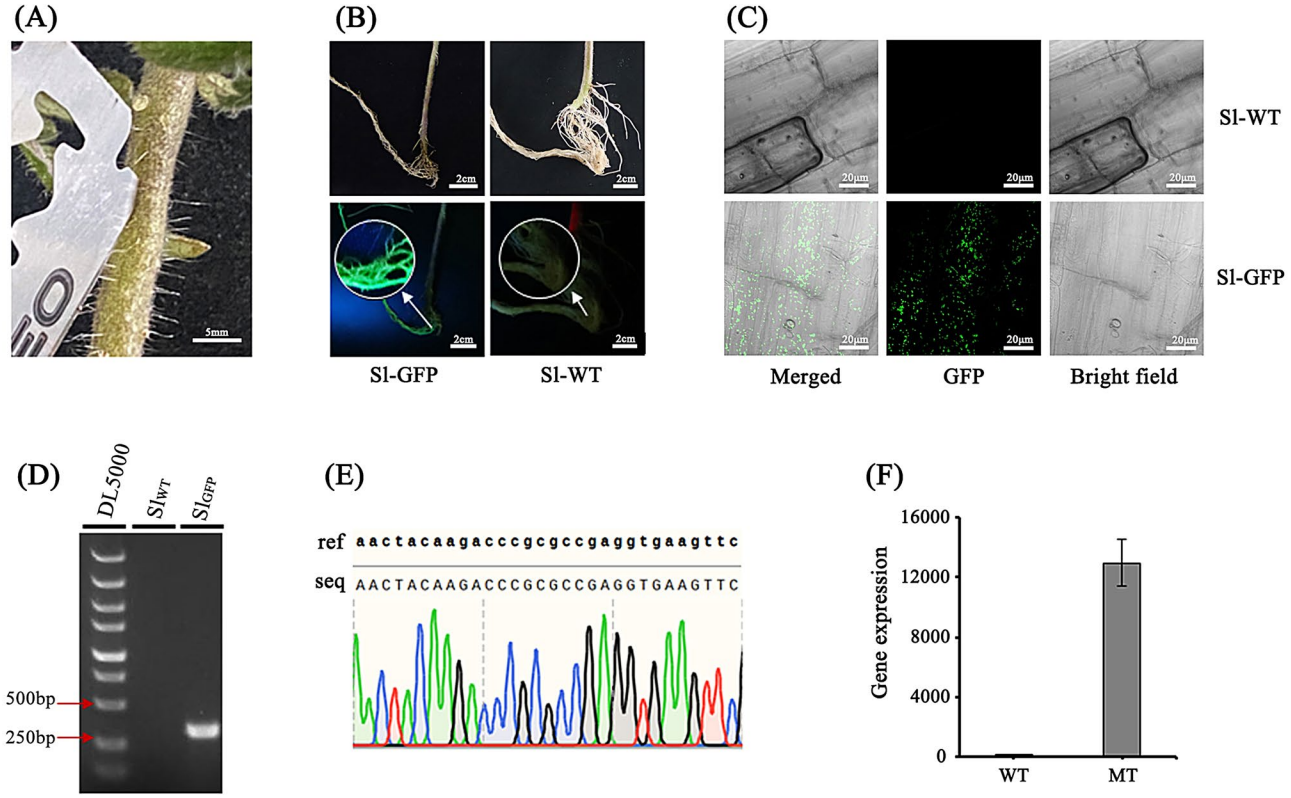


Fig. 7 Establishment of efficient genetic transformation system for tomato. **(A)** Diagram of K599 Agro-inoculation in tomato. **(B)** Identification of transgenic tomato hairy roots with handheld GFP excitation light source. Sl-WT, wild-type of tomato; Sl-GFP, GFP overexpressing transgenic hairy roots of tomato. **(C)** Analysis of transgenic hairy roots using LSCM. **(D)** PCR verification of transgenic hairy roots. **(E)** DNA sequencing verification of transgenic hairy roots. **(F)** Analysis of GFP expression using RT-qPCR

of Cr(III) (Fig. S2). LSCM observation revealed that after 3 days of treatment with 100 mg/L Cr(III), the amount of mitochondria-localized GFP in the transgenic roots significantly decreased. When the Cr(III) concentration increased to 500 mg/L or higher, mitochondria-localized GFP completely disappeared, whereas the control group without Cr(III) treatment still exhibited abundant and normal GFP fluorescence (Fig. 8C). The same experiment on citrus plants showed that treatment with 100 mg/L or higher concentrations of Cr(III) for 3 days also led to the complete disappearance of mitochondria-localized GFP in the transgenic roots (Fig. 8D). These results demonstrate that 100 mg/L Cr(III) can severely affect plant cell viability in a relatively short period.

Effects of Cr(III) on gene transcription in roots

To analyze genes responding to Cr(III) stress, this study conducted transcriptome sequencing on tomato (https://www.ncbi.nlm.nih.gov/bioproject/term=PRJNA1135651) and citrus (https://www.ncbi.nlm.nih.gov/bioproject/term=PRJNA1135332) roots treated with 100 mg/L Cr(III) and H_2O (Fig. S3). Based on preliminary experiments, significant changes in seed germination, net photosynthetic rate, and tissue cell activity were observed after three days of Cr(III) exposure, suggesting this time point is crucial for detecting early molecular responses. Therefore, transcriptome sequencing was performed on samples treated for three days. Transcriptome analysis identified 4,751 differentially expressed genes (DEGs) in Cr(III)-treated tomato roots, with 2,418 upregulated and 2,333 downregulated. In citrus roots, 2,179 DEGs were found, with 1,054 upregulated and 1,125 downregulated (Fig. 9A, Table S2 and S3). Eight DEGs were randomly selected from each transcriptome for validation using qRT-PCR, confirming the reliability of the transcriptome analysis (Fig. 9B). KEGG pathway enrichment analysis revealed that most DEGs were involved in environmental information processing (Fig. 9C and D). Further analysis revealed that lots of genes enriched in hormone response pathways exhibited significant differential expression (Fig. S4). Transcription factors (TFs) play a crucial role in gene regulation under stress. In tomato roots, 322 TF-encoding genes were differentially expressed, with MYB (46 genes), NAC (32 genes), and WRKY (29 genes) being the most affected (Table S4). In citrus roots, 111 TF-encoding genes were differentially expressed, with MYB (21 genes), NAC (13 genes), and WRKY (13 genes) being the most affected (Table S5). Analysis of DEG

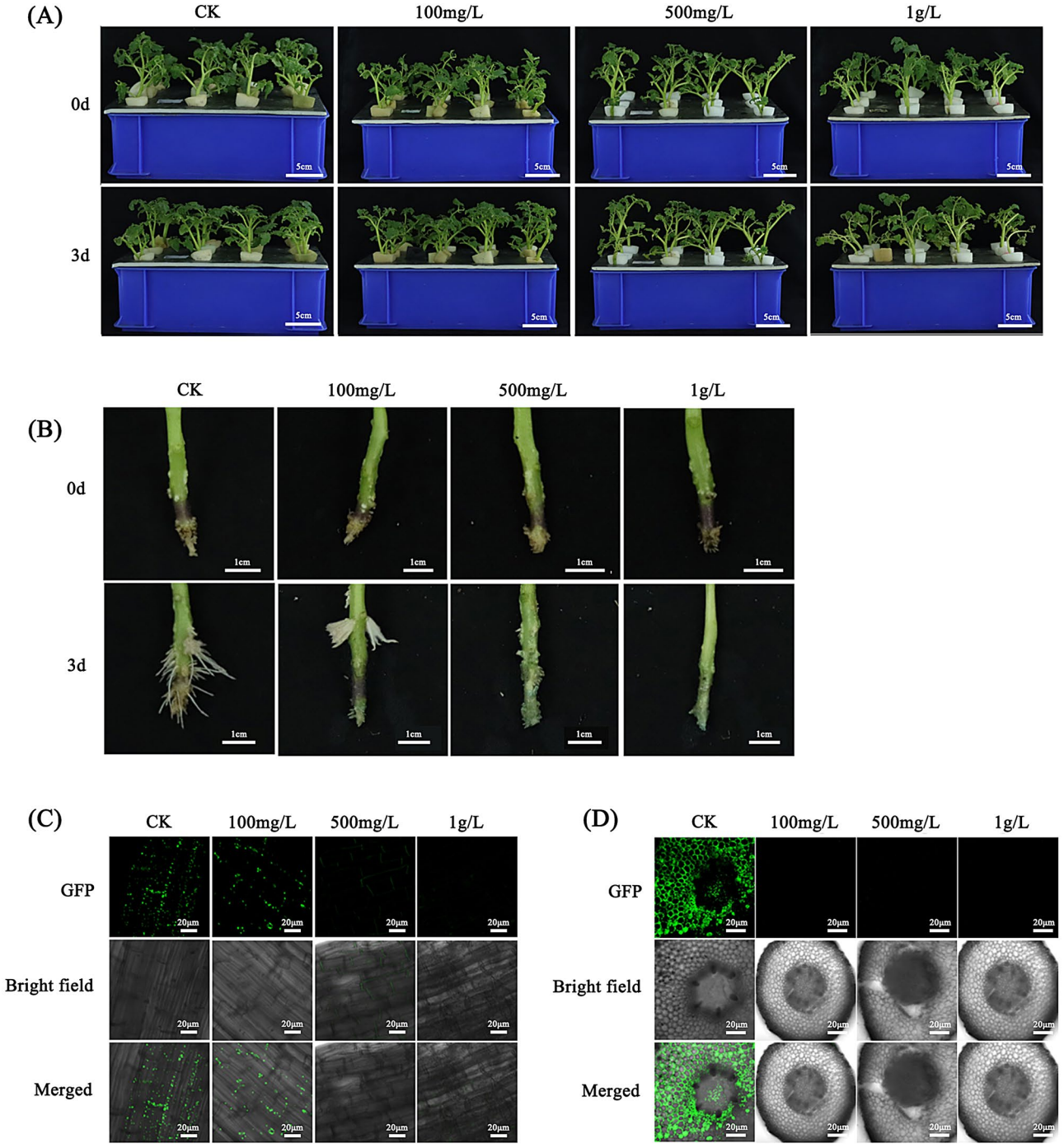


Fig. 8 Rapid analysis of Cr(III) toxicity to plants mediated by GFP transgenic roots. **(A)** Phenotypic changes in tomato plants after 3 days of treatment with different concentrations of Cr(III). **(B)** Phenotypic changes in tomato roots after 3 days of treatment with different concentrations of Cr(III). Changes in GFP fluorescence GFP-overexpressing transgenic tomato roots **(C)** and citrus roots **(D)** after 3 days of treatment with different concentrations of Cr(III)

expression levels revealed significant changes in MYB, WRKY, and bHLH TFs in tomato roots, while bHLH, GATA, and NFYC TFs showed prominent changes in citrus roots (Table S4 and S5). Furthermore, the MAPK signaling pathway and plant hormone signal transduction pathway are closely linked to plant stress responses. Pathway enrichment analysis identified 182 DEGs in the MAPK signaling pathway in tomato, with 100 upregulated and 82 downregulated, and 75 DEGs in citrus, with 38 upregulated and 37 downregulated (Table S6 and S7). In the plant hormone signal transduction pathway, there were 172 DEGs in tomato, with 97 upregulated

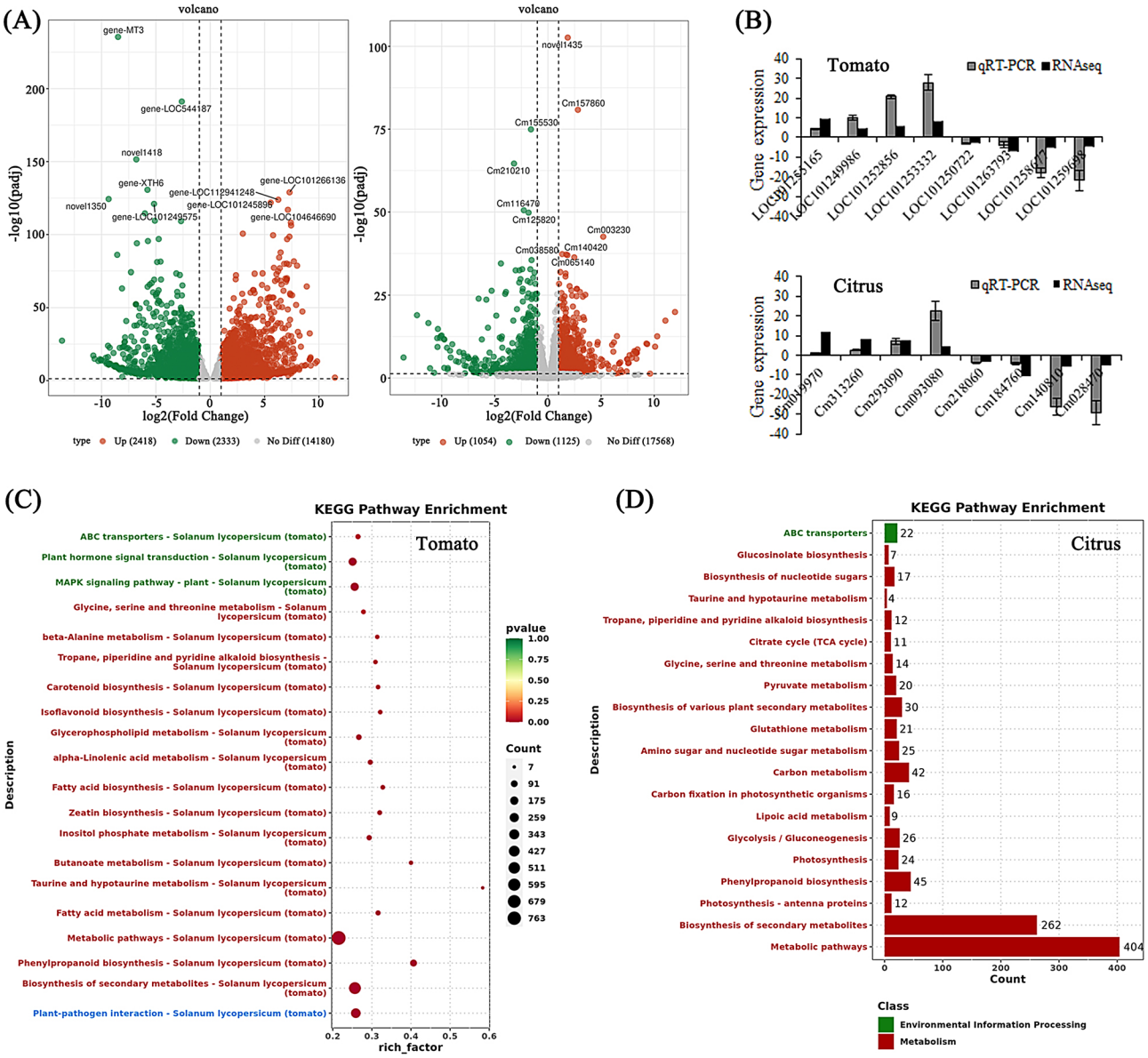


Fig. 9 Effects of Cr(III) on gene transcription in roots. **(A)** Number of DEGs in citrus and tomato roots under 100 mg/L Cr(III) stress. **(B)** RT-qPCR validation of transcriptome data in citrus and tomato. KEGG pathway enrichment analysis of DEGs in the transcriptome of citrus **(C)** and tomato **(D)**

and 75 downregulated, and 44 DEGs in citrus, with 16 upregulated and 28 downregulated (Table S6 and S7). Plant metal tolerance proteins (MTPs) play a crucial role in enhancing resistance to HM stress and maintaining metal homeostasis. The transcriptomic data revealed that *SlMTP2* (LOC101255067) and *SlMTP9* (LOC101261962) were significantly downregulated in tomato under Cr(III) treatment.

Discussion

Heavy metal pollution in soil is a global environmental issue, particularly severe for food safety and agricultural production. Due to their high toxicity and lack of a safe threshold, elements such as As, Cd, Pb, Hg, and Cr are among the most hazardous substances posing significant public health risks [45]. Cr, a common heavy metal contaminant, can adversely affect crop growth and quality, thereby impacting human health. This study selected citrus and tomato to analyze the effects of Cr(III) on seed germination, plant morphology, and the antioxidant enzyme system. Additionally, we established an efficient *A. rhizogenes*-mediated transformation system for citrus and tomato bypassing tissue culture. Using this system, we developed a GFP-mediated rapid analysis technique for Cr(III) toxicity. Finally, we conducted transcriptome sequencing on plants treated with Cr(III) to investigate

the molecular mechanisms underlying plant responses to chromium stress. Analyzing these Cr(III)-responsive genes provides insights into the molecular mechanisms of plant responses to heavy metal stress.

This study provides significant insights into the physiological and molecular responses of horticultural plants to Cr(III) stress. The research highlights several critical aspects of Cr(III) toxicity, from its impact on seed germination and plant growth to its influence on gene transcription and bacterial activity. The differential response of citrus and tomato seeds to Cr(III) underscores the complexity of heavy metal stress in plants. While citrus seeds exhibited a progressive decline in germination rates with increasing Cr(III) concentrations, tomato seeds demonstrated a biphasic response, with low concentrations (50 mg/L) promoting germination and root growth, but higher concentrations (500 mg/L and above) causing significant inhibition. This suggests that low Cr(III) levels may induce a hormetic effect in tomato, a phenomenon where a low dose of a harmful substance can have stimulating effects [46]. However, the detrimental effects at higher concentrations confirm the toxicity of Cr(III), aligning with previous findings on heavy metal stress impacting seed germination and early plant development [27, 47–50]. Hydroponic studies further illustrated Cr(III)'s adverse effects on plant growth, with both citrus and tomato plants showing significant root and leaf growth inhibition at higher Cr(III) concentrations. The severe impact on root development, as evidenced by increased H_2O_2 and MDA levels, indicates oxidative stress and lipid peroxidation, which are common responses to heavy metal toxicity [51–56]. The reduction in SOD activity in roots treated with Cr(III) further supports the occurrence of oxidative stress, as SOD is a crucial enzyme in mitigating ROS in plants [57, 58]. Additionally, studies on various other crops have shown that Cr treatment similarly increases ROS and MDA levels while changing antioxidant enzyme activity in roots or leaves, indicating that Cr has comparable effects on both roots and leaves in these aspects [59–62].

Transcriptome analysis revealed extensive changes in gene expression in response to Cr(III) stress. In both citrus and tomato roots, numerous DEGs were identified, particularly those involved in the MAPK signaling pathway and plant hormone signal transduction pathway. These pathways are integral to plant stress responses, indicating that Cr(III) induces a broad reprogramming of cellular processes. SA is a phenolic derivative widely distributed in the plant kingdom and is known as a regulator of several physiological and biochemical processes such as thermogenesis, plant signaling or plant defense, and response to biotic and abiotic stress [63, 64]. The significant alterations in TFs such as MYB, WRKY, and bHLH in both species highlight the central role of these regulatory proteins in mediating stress responses. MYB and WRKY are known to be involved in modulating ROS-scavenging mechanisms and stress-responsive gene expression [65–68], suggesting their critical role in managing Cr(III)-induced oxidative stress.

The development of an efficient, tissue culture-free genetic transformation system using *GFP*-overexpressing transgenic hairy roots marks a significant advancement for studying Cr(III) toxicity. This system allows for rapid and precise assessment of Cr(III) effects on cellular functions. The observed decrease in mitochondria-localized GFP fluorescence under Cr(III) treatment demonstrates the utility of this method for visualizing and quantifying cellular damage at sub-lethal concentrations. This approach provides a valuable tool for future research on heavy metal stress in plants, enabling more detailed and dynamic studies of stress responses at the cellular level. The study also explored the impact of Cr(III) on bacterial microbiota, showing significant reductions in the survival rates of *E. coli* and *Agrobacterium* species with increasing Cr(III) concentrations. This highlights the broader ecological impact of Cr(III) pollution, as it not only affects plant health but also disrupts beneficial soil microorganisms that play crucial roles in nutrient cycling and soil fertility.

This research underscores the necessity for comprehensive studies on Cr(III) toxicity, given its potential to disrupt plant physiological processes, induce oxidative stress, and alter gene expression patterns. The findings suggest that even trivalent chromium, often considered less harmful than hexavalent chromium, can pose significant risks to horticultural plants and bacterial microbiota at higher concentrations. Future research should focus on exploring the underlying mechanisms of Cr(III) detoxification and tolerance in plants, potentially identifying key genes and pathways that could be targeted for developing Cr(III)-resistant crops. Additionally, investigating the long-term effects of Cr(III) exposure on plant health and productivity, as well as its interactions with other environmental stressors, will be crucial for developing effective strategies to mitigate heavy metal pollution in agriculture.

Conclusion

Briefly, the present study provides valuable insights into the response of horticultural plants to Cr(III) stress in citrus and tomato treated with Cr(III) on growth and development, antioxidant system, photosynthesis and organelle stresses, highlighting both the physiological and molecular adaptations involved. The innovative use of GFP-overexpressing transgenic roots offers a promising approach for future research, potentially leading to the development of strategies to enhance plant resistance to heavy metal stress.

Abbreviations

Cm	*Citrus medica*
Sl	*Solanum lycopersicum*
Cr (III)	Trivalent chromium
Cr (VI)	Hexavalent chromium
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
SOD	Superoxide dismutase
GFP	Green fluorescent protein
DH5α	*Escherichia coli*
K599	*Agrobacterium rhizogenes*
GV3101	*Agrobacterium tumefaciens*
Str	Streptomycin sulfate
Kana	Kanamycin sulfate
RT	Room temperature
OD_{600}	Optical density at 600 nm
Rpm	Revolutions per minute
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
DEG	Differentially expressed gene
LSCM	Laser scanning confocal microscope
DNA	Deoxyribonucleic acid
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
KEGG	Kyoto encyclopedia of genes and genomes
TFs	Transcription factors
MYB	Myeloblastosis
NAC	NAM、ATAF1/2、CUC1/2
WRKY	WRKY DNA-binding domain
bHLH	Basic helix-loop-helix
GATA	Zinc finger DNA-binding proteins
MAPK	Mitogen-activated protein kinase

Supplementary Information

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Supplementary Material 1: Supplementary Fig. S1. Plasmids used in this study. (A) pDSK-GFP plasmid. (B) MT-gb plasmid.

Supplementary Material 2: Supplementary Fig. S2. Diagram of treatments with different concentrations of Cr on transgenic roots of tomato (A) and citrus (B).

Supplementary Material 3: Supplementary Fig. S3. Roots of tomato (A) and citrus (B) were treated with either H_2O (CK, control) or 100 mg/L Cr(III) (Cr) for 3 days. Subsequently, the roots were sampled for transcriptome analysis.

Supplementary Material 4: Supplementary Fig. S4. DEGs of citrus and tomato enriched in "Plant hormone signal transduction" pathways.

Supplementary Material 5: Supplementary Table. S1. Plasmids used in this study. Supplementary Table. S2. DEGs of citrus root in Cr(III) treatment group compared to the wild type. Supplementary Table. S3. DEGs in tomato root of Cr(III) treatment group compared to the wild type. Supplementary Table. S4. Analysis of differentially expressed transcription factors in tomato root when treated with Cr(III). Supplementary Table. S5. Analysis of differentially expressed transcription factors in citrus root when treated with Cr(III). Supplementary Table. S6. KEGG pathway enrichment analysis of citrus roots treated with Cr(III) compared to the wild type. Supplementary Table. S7. KEGG pathway enrichment analysis of tomato roots treated with Cr(III) compared to the wild type. Supplementary Table S8. Primers used in this study.

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Author contributions

H.M contributed to the conception of the research; M.W and H.L carried out the experiments, K.X reviewed data, J.F and C.Y collated the data; H.M and M.W performed the data analyses and manuscript preparation; W.Z helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Data availability

Transcriptome data isavailable under project IDs: PRJNA1135651 (Solanumycopersicum), PRJNA1135332(Citrus medica) alongwith all libraryIDs: SAMN42486116-SAMN42486121(Solanum lycopersicum); SAMN42474588-SAMN42474593(Citrus medica).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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