# Hydrogen-rich water enhanced salt tolerance in tomato seedlings by regulating strigolactone biosynthesis genes SlMAX1 and SlD27

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## Abstract

# Background

Hydrogen gas ($H_2$) and strigolactones (SLs) are involved in various biotic and abiotic stress response in plants. However, the crosstalk between $H_2$ and SLs has not been investigated.

# Methods

Using pharmacological methods and virus-induced gene-silencing, the regulatory roles of $H_2$ and SLs and their interaction in tomato (*Solanum lycopersicum* L. 'Micro-Tom') under salt stress were investigated.

# Results

Both GR24 (a SLs synthetic analog) and hydrogen rich water (HRW, a $H_2$ donor) significantly reversed salt-induced growth retardation as evidenced by promoted root morphological parameters and root activity. SLs might be involved in $H_2$-enhanced salt stress tolerance in tomato seedling roots. Additionally, HRW treatment increased endogenous SLs content in tomato seedling roots under salt stress However, the positive roles of HRW were blocked by TIS108 (a specific SLs synthesis). In addition, HRW and GR24 could effectively maintain the integrity of the internal anatomical structure in roots under salt stress; while TIS108 also inhibited the positive roles of HRW. Thus,. Simultaneously, HRW treatment significantly up-regulated the expression levels of SL biosynthesis-related genes *SlCCD7*, *SlCCD8*, *SlD27* and *SlMAX1* and SL signal transduction genes *SlD14* and *SlMAX2* under salt stress. Further, after silencing *SlD27* and *SlMAX1* genes, the alleviation effect of HRW on tomato roots under salt stress was basically eliminated. HRW did not increase the content of endogenous SLs in *SlD27* and *SlMAX1* silenced seedlings.

# Conclusion

SLs biosynthesis genes *SlMAX1* and *SlD27* may be involved in $H_2$-alleviated salt stress in tomato seedlings.

## Introduction

Roots are the first organ to sense and adapt to stress and they are also critical to plant survival. Roots are usually located below the soil surface where they primarily absorb water and inorganic salts, and store nutrients (Petricka et al. 2012). As the one of the most concerning abiotic stresses, salt stress causes serious toxic effects on the growth and development of various plants all over the world, severely limiting productivity and reducing crop yield and quality (Van Zelm et al. 2020). Numerous studies have found that high salt concentrations accumulate mainly in plant roots, reducing the ability of the root system to

absorb water (Munns. 2002). Under high salinity stress, cell wall of plant roots becomes thicker, the content of hemicellulose and cellulose increases, and the cell division and elongation are reduced, which is not conducive to the elongation of root cells. Meanwhile, salt stress also decreases the hydraulic conductivity of the root system by reducing the water flow along the cross-cellular pathway (Knipfer et al. 2021). In addition, salt stress leads to a decrease in glutathione reductase (GR) activity in the root system of rice varieties, which causes oxidative stress in plants and leads to a reduction in crop growth and yield (Demiral et al. 2005). Thus, salt stress can negative affect the normal growth and development of plants. Therefore, improving the salt tolerance of plants has become an urgent problem that needs to be urgently addressed in the process of agricultural development.

Root growth and development are determined by the synergistic action of multiple plant hormones, including indoleacetic acid (IAA), Gibberellic acid ($GA_3$) and zeatin (ZT) (Wang et al. 2021). Meanwhile, Zhang et al. (2009) found that IAA and nitric oxide (NO) may play a role in $H_2S$-induced formation of adventitious roots in Ipomoea batatas, which significantly increased the number of long roots. In addition, abscisic acid (ABA) promoted root hair elongation by regulating root tip homeostasis through polar auxin transport (Wang et al. 2021). Furthermore, $GA_3$ could regulate lateral root development in cucumber and significantly increased root dry weight (Cai et al. 2022). Therefore, phytohormones play an important role in root growth and structural development under stress. Strigolactone (SLs) are a class of terpene lactone compounds. Originally, it was isolated from cotton roots with germination stimulatory effect. SLs have been identified as novel classes of phytohormones, which not only regulate the early development in Arabidopsis seedlings, but also inhibit the elongation of stem sheaths in *Physcomitrella patens* (Hoffmann et al. 2014). Besides, it was found that SLs could respond to drought (Min et al. 2018), salt (Sun et al. 2014), low temperature (Zhou et al. 2022), nitrogen (Ito et al. 2016). Moreover, crosstalk between SLs and other hormones could regulate the growth and development of plants, such as seed germination (Chesterfield et al., 2020), vegetative meristem growth (Vogel et al. 2010), and mycelial branching of arbuscular mycorrhizal fungi (Aliche et al. 2020). In addition, SLs have been considered as a class of plant growth regulators mainly are synthesized in plant roots. SLs treatment promoted root hair elongation, increased stem thickness and stimulated internode growth in rice and Arabidopsis (Marzec et al. 2015). SLs significantly increased endogenous hormone levels in the root system of cherry rootstocks (Jiu et al. 2022). Therefore, SLs have potential importance in plant root growth and development.

Hydrogen gas ($H_2$), a colorless, odorless, less dense gas, is highly reductive. It is a new kind of beneficial gas molecule that plays a role in plant growth and development, including seed germination (Xu et al. 2013), seedling development (Yan et al. 2022), adventitious root formation (Zhao et al. 2022), and fruit ripening (Hu et al. 2017). Our previous studies showed that root organogenesis is induced by HRW treatment in cucumber (Zhao et al. 2022; Zhu et al. 2016). NO was involved in $H_2$-promoted adventitious root formation in cucumber (Zhu et al. 2016). In addition, $H_2$ might regulate adventitious root formation in cucumber by promoting glucose metabolism (Zhao et al. 2022). Simultaneously, hydrogen rich water (HRW) treatment also improved the tolerance of plants to various adverse environmental stresses, such as paraquat toxicity (Jin et al. 2013), heavy metal stress (Zhao et al. 2017; Cui et al. 2013), heat stress

(Chen et al. 2017), and UV irradiation (Su et al. 2014). The application of HRW promoted root growth in rice under lead (Pb) stress and improved the inhibitory effect of Pb toxicity by increasing antioxidant capacity (Ma et al. 2021). Thus, $H_2$ not only plays an important role in regulating plant growth and development as a signal modulator, but also participates in plant physiological and biochemical processes together with other gas molecules under stress conditions.

Both $H_2$ and SLs play key roles in regulating plant growth and development. To the best of our knowledge, there is no information about the relationships between $H_2$ and SLs in plants. Thus, the crosstalk between $H_2$ and SLs in root development in tomato seedlings under salt stress was investigated. It provides a basis for exploring our understanding of the signal transduction mechanisms of $H_2$ and SLs during plant root growth and development under abiotic stresses.

# Materials and methods

Plant materials and treatments

Tomato (*Solanum lycopersicum* L. 'Micro-Tom') seeds were surface sterilized with 3% (w/v) sodium hypochlorite for 20 min, and then soaked in distilled water for 4 h. Plant seedlings were germinated in petri dishes containing sterile with distilled water and grown under a cycle of 14 h light at 200 μmol $m^{-2}$ $s^{-1}$ photons irradiance at 25 ± 2°C and 7-h dark at 20 ± 2°C and 70% relative humidity. The germinated seedlings were transferred into erlenmeyer flasks containing 1/2 Hoagland solution for 4 d, and then transferred to 100% Hoagland solution for 7 d. After growing for approximately one month (four-leaf stage), seedlings with similar growth were collected and treated with different concentrations for 7 d. The seedlings root treated with the Hogland solution adding no extra compounds were served as control (CK). According to the previous study in our laboratory (Wang et al. 2021; Liu et al. 2022), the concentration of NaCl (150 mM), SLs synthesis mimic GR24 (15 μM), tricolactone synthesis inhibitor TIS108 (3 μM) and 75% HRW were selected. The treatments are as follows: CK (Hogland); NaCl (150 mM); NaCl (150mM) + HRW (75%); NaCl (150 mM) + GR24 (15 μM); NaCl (150 mM) + HRW (75%) + TIS108 (3 μM). Each treatment consisted of 3 replicates and each replicate consisted of 60 seedlings root, and plant seedlings in each replication were harvested separately and stored in an ultra-low temperature freezer at -80°C for the following experimental analysis.

The preparation of HRW

The preparation of hydrogen gas ($H_2$) refers to the method of Fang et al. (2020). Under our experimental conditions, $H_2$ (99.99%, v/v) was produced by a hydrogen generator (QL-300, Cyclos Hydrogen Energy Co., Ltd., Jinan, China) was bubbled into 1000 mL distilled water at a rate of 320 mL/$min^{-1}$ for 60 min continuously. The $H_2$ content of HRW could be kept at a relatively constant level for at least 12 h at 20°C. Then, the $H_2$ concentration in the freshly prepared HRW was analyzed using a dissolved hydrogen portable meter (Trustlex, Led, ENH-1000, Japan), and the $H_2$ concentration was 0.47 mM, which was

defined as 100% hydrogen-rich water (HRW). Finally, the corresponding HRW was rapidly diluted to the desired concentration (75%, [v/v]).

Determination of morphological indicators

After each tomato seedling was treated with the corresponding solution for 7 d. The aboveground parts of the tomato seedlings were removed, and the roots were carefully washed with distilled water, then the roots were photographed by root scanner (STD4800, Canada) to measure root length, surface area, and root volume. Roots from three plants of each treatment were analyzed with root scanning analysis system Win RHIZO (Pro 2.0 Version 2005; Regent Instruments, Quebec, QC, Canada). Then, the roots were fixed in an oven at 105°C for 30 min, and were baked dry at 80°C for 48 h to calculate the average dry weight.

Root/ shoot ratio = root dry weight / shoot dry weight

Determination of root activity

The root activity was determined according to Yan et al. (2022) with slight modifications. Firstly, the 0.5 g of fresh root samples was cut into 1 cm segments and placed for 1h with a mixture of 5 mL TTC solution (0.1% (w/v)) and 5 mL phosphate buffer (0.1 mol/L, pH 7.5) at 37°C. Next, the experiment was stopped by adding 2 mL of 1 mol/L $H_2SO_4$. Roots were dried with filter paper, and 5 mL ethyl acetate extraction and 5 mL quartz sand were performed, and ethyl acetate extraction and quartz sand was performed. Finally, the absorbance of the extract at 485 nm was recorded.

Anatomical observations of roots

The root internal structure was determined by conventional paraffin section method. Samples were obtained on 7 d after treatments. The roots were rinsed with sterilizing distilled water, and a section of about 5 mm in length was cut from the main roots. The samples were preserved and fixed in FAA (insisting of 90mL of 50% alcohol, 5mL of glacial acetic acid, and 5mL of formaldehyde). Then, the roots were dehydrated with gradient ethanol (30% alcohol for 2 h, 50% alcohol for 2 h, 70% alcohol for 2 h, 83% alcohol dehydration 1 h, 95% alcohol dehydration 2 h, and 100% alcohol dehydration 2 h), then diped with xylene gradient transparent (50% xylene: 50% absolute ethanol 1.5 h, pure xylene 1.5 h, 50% xylene) and impregnated with wax for 48 h (50% paraffin: 50% xylene), embedding (the pure paraffin was replaced 2–3 times, every 1 h), sectioning with a microtome (the thickness of the section was 8–14 um). After that, the sections were dewaxed with xylene, and double stained with safranin-Fast green. The sections were sealed with neutral gum after dehydration using ethanol gradient. Finally, a BX61 optical microscope (Olympus, Japan) was used to observe and photograph the internal tissues of the root system.

Measurement of strigolactone content

After 7 d of treatment, the content of SLs was measured basing on Vogel et al. (2010) with some modifications. Briefly, the roots of tomato seedlings (0.5 g) were quickly ground with liquid nitrogen and

then transferred to a test tube. The mixture was extracted in a refrigerator at 4°C overnight in the dark after adding 2 mL ethyl acetate. Then, the extractions were collected by centrifuging at 2500 *g* for 10 min at 4°C. The supernatant was transferred to a new 10 mL centrifuge tube, and the residue was suspended in 2 mL extraction solution again using the same condition, and then centrifuged. The extraction was repeated twice and the supernatant was merged. The supernatant was evaporated in a rotary suspension vacuum at 38°C for 3 h until dryness. The supernatant was dissolved with 1.5 mL of 50% acetonitrile. Then, the content of SLs was detected by high performance liquid Chromatography-mass Spectrometry (LC-MS) using Kromasil C18 (2.1 × 50 mm, 1.7 μM, Agilent). The mobile phase consisted of 0.15% formic acid (A) and 0.15% acetonitrile (B). 10 μL of extracts were injected at a chromatography temperature of 35°C and at a flow rate of 0.3 mL $min^{-1}$.

Extraction of RNA and Quantitative Real-Time PCR (qRT-PCR)

After 7 d of treatments, 0.5 g tomato seedlings root were ground into powder in liquid nitrogen. Extraction of total RNA was performed according to the TRIzol (Invitrogen Life Technologies) method described by Wang et al. (2022). RNA was reverse transcribed using the ABI Step One Plus System (Applied Biosystems, Carlsbad, CA, USA) and SYBR® Premix Ex Taq™ II (Takara, China) according to the manufacturer's recommendations. Reverse transcription conditions were as follows: 5 min at 37°C, 5 sec at 85°C, 4°C cycle. Quantitative real-time PCR (qRT-PCR) analysis was performed to assess the relative expression levels of each gene. PCR reaction conditions were as follows: 30 sec at 5°C, then 5 sec at 95°C, then 40 sec at 56°C, 90 sec at 72°C, 50 cycles, followed by separation. The tomato actin gene was used as an internal control gene. Primers used for PCR analysis were shown in Table S1.

Construction of VIGS vectors

Firstly, the specific fragments of *SlMAX1* and *SlD27* gene silencing were obtained by PCR amplification using cDNA as template. About 300 bp fragments of *SlMAX1* and *SlD27* genes were cloned gene-specific primers (*SlMAX1*-F, 5'-gtgagtaaggttaccgaattcAAGGAGAATAAAAGCGTCACTCCA-3'; *SlMAX1*-R, 5'-cgtgagctcggtaccggatccATGGGGTCTAGCCGGCCT-3'; *SlD27*-F, 5'-gtgagtaaggttaccgaattcATGGAGGCAAATCTTGTTCTATCTT-3'; *SlD27*-R, 5'-cgtgagctcggtaccggatccATTAAAGTTCACATACACAACCCTTGC-3') with EcoRI and BamHI restriction sites. After identifying the target fragment, the target fragment was ligated into the empty vector, and the successfully ligated TRV2 vector was transformed into competent *E. coli* DH5α and incubated at 37°C overnight to obtain positive colonies. Then, the sequenced and validated vectors pTRV1, pTRV2, pTRV2-*SlMAX1* and pTRV2-*SlD27* were transferred into *Agrobacterium* tumefaciens GV3101 as well. Meanwhile, the mixture solution of pTRV1 and pTRV2 (1:1, v/v, OD600 = 1.0-1.2) was used as empty pTRV vector. The mixture solution of Ptrv, pTRV2-*SlMAX1* and pTRV2-*SlD27* (1:1, v/v, OD600 = 1.0-1.2) was used to infect the cotyledon back of tomato seedlings with the same size (two cotyledons fully expanded). Finally, the overall area of the injected leaves is not less than 80% (the area of all injection is the best). After the injection, the plants were incubated in the dark for 24 h, and then placed in the plant growth room at 26°C and 70% relative humidity. Seedlings were treated and indicators were measured when they

grew to four leaves and one heart. Then, the silencing efficiency was detected by the expression levels of *SlMAX1* and *SlD27* genes. Our results demonstrate that the silencing efficiency of *MAX1* and *D27* was 86.8% and 59.1%, respectively (Fig. S1).

# Statistical Analysis

Statistical analysis of all experimental data was performed using Excel 2010 and SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). All experiments consisted of three independent replicates and results were expressed as mean ± standard error (SE). The analysis of variance (ANOVA) and Duncan's multi-range test were used to analyze the significant differences among different treatments ($P < 0.05$).

# Results

## Strigolactone was involved in HRW-improved root morphological characteristics of tomato seedlings under salt stress

HRW, GR24 (a SLs synthetic analog) and TIS (a SLs inhibitor) were applied to preliminarily explore the role and relationship between $H_2$ and SLs under salt stress. As shown in Fig. 1, NaCl treatment significantly inhibited root morphological parameters in comparison with the control. For example, root length, average diameter, root surface area, root volume, and root/shoot ratio were decreased by 65.1%, 38.4%, 76.9%, 24.5%, and 6.6%, respectively (Fig. 1a, c, e, f). However, compared to NaCl treatment, NaCl + HRW and NaCl + GR24 treatments significantly increased tomato seedling root length, fresh weight and dry weight, and root/shoot ratio (Fig. 1a, b, e, f). However, there was no difference in root fresh weight, dry weight, root volume, root surface area and radio between NaCl + HRW and NaCl + GR24 treatments (Fig. 1b, c, e). Compared with NaCl + HRW, NaCl + HRW + TIS108 treatment significantly decreased root surface area, root volume, root tip number, root fork number, and root /shoot radio by 44.4%, 35.2%, 40.6%, 43.8%, and 2.6%, respectively (Fig. 1c, d, e), indicating that the positive roles of HRW in root morphological parameters was weakened by TIS under salt stress. These results indicate that SLs and HRW could effectively stimulate the root morphological parameters in tomato seedlings under salt stress. In addition, SLs might be involved in HRW-alleviated salt stress in tomato seedlings.

## Strigolactone participated in HRW-enhanced root activity and root internal anatomical structure in tomato seedlings under salt stress

As shown in Fig. 2a, NaCl treatment significantly reduced root activity in comparison with the control. However, compared to NaCl treatment, NaCl + HRW and NaCl + GR24 significantly increased tomato seedling root avtivity by 53.2% and 42.2%, respectively. There was no difference in root activity between NaCl + HRW and NaCl + GR24 treatments (Fig. 2a). In addition, the root activity was significantly decreased by NaCl + HRW + TIS treatment in comparison with NaCl + HRW treatment.

Compared to the control, NaCl treatment serious damaged root internal anatomical structure (Fig. 2b). For instance, epidermal cells were dense and thin, with reduced cavity structure. The internal anatomical

structure of NaCl + HRW and NaCl + GR24 treatments was more complete than that of NaCl treatment. Compared with NaCl + HRW, NaCl + HRW + TIS108 treatment had incomplete internal anatomical structure (Fig. 2b). These results suggest that SLs and HRW could effectively alleviate salt stress in tomato seedlings by improving the root activity and internal anatomical structure.

HRW increased strigolactone content and strigolactone biosynthesis-related gene expression in tomato seedling root under salt stress

To further analyze the relationship between $H_2$ and SLs in salt stress response, the endogenous SLs content and its biosynthesis-related gene expression were detected in tomato seedling root (Fig. 3). NaCl treatment significantly increased the endogenous SLs content in comparison with the control (Fig. 3a). Compared to NaCl treatment, NaCl + HRW significantly increased the endogenous SLs content in tomato seedling root by 42.2%. In addition, HRW treatment alone significantly increased the endogenous SLs content. Similarly, compared to the control, NaCl treatment significantly improved the expression level of SLs biosynthesis-related gene, including *SlCCD7*, *SlCCD8*, *SlD27* and *SlMAX1*. In comparison with NaCl treatment, the expression level of *SlCCD7*, *SlCCD8*, *SlD27* and *SlMAX1* significantly was up-regulated by NaCl + HRW treatment. Additionally, HRW treatment alone significantly up-regulated the expression level of *SlCCD7*, *SlCCD8*, *SlD27*and *SlMAX1* (Fig. 3b). Thus, HRW might induce endogenous SLs generation under salt stress by increasing SLs biosynthesis-related gene expression.

HRW up-regulated the expression of signal transduction genes in tomato seedling root under salt stress

NaCl treatment significantly improved the expression level of signal transduction genes of *SlCD14* and *SlMAX2* in comparison with the control (Fig. 4). Compared with NaCl treatment, the expression level of *SlCD14* and *SlMAX2* significantly was up-regulated by NaCl + HRW treatment. HRW treatment alone significantly up-regulated the expression level of *SlCD14* and *SlMAX2* (Fig. 4). The above findings further demonstrate a crucial role for SLs in $H_2$-induced salt resistance at the transcriptional level.

HRW couldn’t alleviate salt stress in TRV-*SlMAX1* and TRV-*SlD27* silenced plants

As shown in Fig. 5, compared to CK, NaCl treatment significantly decreased root length, root fresh weight, dry weight in wild type (WT), TRV, TRV-*SlMAX1* and TRV-*SlD27* groups. Moreover, compared with NaCl treatment, NaCl + HRW treatment increased these indicators in WT and TRV groups. However, the fresh weight, dry weight and root length of TRV-*SlD27* and TRV-*MAX1* groups had no significant difference between NaCl and NaCl + HRW treatments. Therefore, HRW did not alleviate salt stress in TRV-*SlMAX1* and TRV-*SlD27* silenced plant lines. Compared with CK, NaCl treatment decreased the root/shoot ratio and root activity in WT, TRV, TRV-*SlMAX1* and TRV-*SlD27* plants (Fig. 5). Moreover, under salt stress, HRW treatment significantly increased the root/shoot ratio and root activity in WT and TRV empty vector-infected plants. However, interestingly, in TRV-*SlMAX1* and TRV-*SlD27* silenced plants, NaCl + HRW treatment did not improve the root/shoot ratio and root activity, in comparison with NaCl treatment.

HRW couldn’t increase endogenous SLs content after *SlMAX1* and *SlD27* gene silencing under salt stress

As shown in Fig. 6, compared with CK, NaCl treatment significantly increased the endogenous SL content of WT and TRV groups. In WT and TRV groups, endogenous SL content in NaCl + HRW treatment was significantly higher than that in NaCl treatment. However, there was no significant difference in SL content between NaCl and NaCl + HRW treatment in TRV-*SlD27* and TRV-*MAX1* groups, suggesting that after silencing *SlD27* and *MAX1*, the promote role of HRW in endogenous SLs content was inhibited under salt stress.

# Discussion

Salt stress seriously impairs various physiological, biochemical processes and molecular functions of plants, thereby affecting plant growth and development (Zörb et al. 2019). There is increasing evidence that $H_2$ and SLs are involved in plant root growth as important signaling molecules under abiotic stresses (Ma et al. 2021; Al-Babili et al. 2015). HRW treatment could increase adventitious root number and root length in marigold explants (Zhu et al. 2017). In addition, HRW treatment significantly increased root length and root shoot ratio of cucumber seedlings under salt stress (Yu et al. 2021). Similarly, in our study, HRW treatment increased root length and root fresh weight in tomato seedlings under salt stress (Fig. 1). In addition, it has also been reported that exogenous HRW treatment significantly increased the root length, dry weight, surface area, root volume, root/shoot ratio and average diameter of maize roots under salt stress (Yang et al. 2023). Our results indicate that HRW treatment significantly enhanced root length, dry weight, surface area, volume, root/shoot ratio, and average root diameter of tomato seedlings under salt stress (Fig. 1), indicating that $H_2$ might play a positive role in plant root growth under salt stress. In the present study, we found that SLs also increased the salt tolerance of tomato seedling roots. The root fresh weight, dry weight and root length were increased by GR24 treatment in tomato seedling under salt stress (Fig. 1). Santoro et al. (2021) recently reported that exogenous SLs increased the fresh weight, dry weight, primary root length and root number of tomato seedlings under low phosphorus conditions compared with high phosphorus seedlings. Meanwhile, 10 μM GR24 treatment increased root length, root number and average diameter of cherry rootstocks, thereby promoting adventitious root development in cherry rootstocks (Jiu et al. 2022). However, the positive roles of HRW in root morphological characteristics were blocked by TIS108 (a SLs inhibitor, Fig. 1), indicating that SLs might play a crucial role in $H_2$-alleviated salt stress in tomato seedlings. According to Liu et al. (2022), SLs could be a vital signaling molecule and function in the downstream of NO, thereby enhancing the resistance to salt stress in tomato seedling. Kolbert et al. (2019) found that MG132 (an inhibitor of SLs biosynthesis) prevented NO-induced root elongation in rice, indicating that SLs might be involved in NO signaling as a downstream element. Similarly, we also found that TIS108 (an inhibitor of SLs biosynthesis) prevented $H_2$-induced root elongation in tomato seedlings. Therefore, SLs might be involved in $H_2$-alleviated salt stress.

Roots absorb water through root epidermis, cortex, and endodermis to the xylem vessel of the stele, which is then transported upward to the aboveground part (Prince et al. 2017). Root anatomical structure is closely related to root activity, which can reflect plant growth status. Wu et al. (2015) observed that under

cadmium stress, exogenous application of $H_2$ improves the root activity in cabbage seedlings. In addition, HRW treatment induced aluminum tolerance in maize seedlings by increasing root activity (Zhao et al. 2017). Similarly, our study showed that HRW treatment increased root activity in tomato seedlings under salt stress (Fig. 2), implying that $H_2$ might alleviate the inhibition of root growth under abiotic stress. In the study, we also found that GR24 (a SLs synthetic analog) also improved the salt tolerance of tomato seedlings by increasing root activity. Ha et al. (2014) discovered that as a positive regulator, SLs could increase root hair length and density and reduce the number of lateral roots in Arabidopsis. Meanwhile, exogenous GR24 increased root relative water content, thereby alleviating the inhibition of root growth in switchgrass seedlings under Cd stress (Tai et al. 2017). Furthermore, salt toxicity also seriously damaged the anatomical structure of roots (Silva et al. 2021). Arafa et al. (2009) reported that sorghum treated with NaCl had a decreased root stele and cortical regions. Salt stress caused by NaCl decreased the thickness of the cross-sectional area of the xylem and cortex of the roots in wild conditions (Chen et al. 2021). Maia et al. (2022) also found that the exogenous application of EBR (24-Epibrassinolide) induced protection in tomato root anatomy under Pb stress. Besides, spermidine (Spd) and brassinosteroid (BRs) increased the number of root cortex in maize seedlings under waterlogging stress (Salah et al. 2022). Similarly, in this study, both SLs and HRW effectively alleviated salt stress in the roots of tomato seedlings, as evidenced by the internal anatomical structure (Fig. 2). However, TIS108 (a SLs inhibitor) could reverse the promotive effects of HRW on the internal anatomical structure of roots under salt stress (Fig. 2), indicating that SLs might be involved in HRW-mediated alleviation of salt stress in tomato seedlings. Therefore, our study suggests that SLs might play an important role in $H_2$-alleviated root growth and development under salt stress in tomato seedlings.

Previous studies have shown that SLs are defined as a new group of carotenoid-derived lactones isolated from root exudates that respond to abiotic stresses (Rasmussen et al. 2012; Hoffmann et al. 2014). Al-Babili et al. (2015) unraveled the involvement of *CCD7* and *CCD8* in SLs biosynthesis gene. Ren et al. (2018) found that drought increased the expression levels of the SLs-biosynthetic genes *SlCCD7* and *SlCCD8* in tomato shoots. Similarly, our study also found that NaCl treatment increased the endogenous SLs content and up-regulated the expression level of *SlCCD7* and *SlCCD8*. HRW also significantly improved endogenous SLs content and up-regulated the expression level of *SlCCD7*, *SlCCD8* in tomato seedlings root under salt stress, indicating that HRW might induce endogenous SLs generation under salt stress. Meanwhile, we also analyzed the expression level of *SlD27* and *SlMAX1*, and signal transduction-related gene *SlD14* and *SlMAX2* in tomato seedlings root. Banerjee and Roychoudhury (2018) reported that MAX1 was a cytochrome P450 monooxygenase, and production of 9-cis-β-carotene was catalysed by carotenoid isomerase, D27, which finally catalyzed the conversion of the compound to SLs. Furthermore, correlation analysis showed that the expression level of *VvD27* and *VvCCD8* was significantly up-regulated and positively correlated with the SLs content in grapevine roots (Yu et al. 2022). Ha et al. (2014) found that the expression level of the SLs-biosynthetic genes *D27* and *MAX1* was up-regulated by drought stress in rice shoots. In our study, NaCl up-regulated expression level of *SlD27* and *SlMAX1.* The expression level of *SlD27* and *SlMAX1* were significantly up-regulated by HRW under salt stress, indicating that $H_2$ might induce SLs biosynthesis (Fig. 3). Besides, Ruyter-Spira et al. (2013)

suggested that *MAX2* and *D14* could be involved in SLs-transduction gene perception. Exogenous 6-BA improved the relative expression of SLs transcription genes *FaD14* and *FaMAX2* in tall fescue leaves under drought stress (Ghaleh et al. 2020). A recent study proved that NO significantly increased the endogenous SLs content and up-regulated the expression level of *SlD14*, *SlMAX1*, *SlD27* and *SlMAX2* in tomato leaves under salt stress (Liu et al. 2022). Similarly, we found that NaCl treatment up-regulated SLs transduction gene *SlMAX2* and *SlD14*. The HRW further significantly up-regulated the expression levels of *SlMAX2* and *SlD14* in tomato seedlings root under salt stress (Fig. 4). Thus, our results further demonstrated that HRW might induce endogenous SLs generation under salt stress by up-regulating the expression of SLs biosynthesis-related gene and signal transduction gene. Therefore, SLs-related genes might play a crucial role in $H_2$-induced salt resistance.

It has been demonstrated that the biosynthesis of SLs is mainly responsible for four proteins (AtD27, MAX1, MAX3, and MAX4). Among them, β-carotene isomerase D27, as a key enzyme in the synthesis of SLs is also the first enzyme in the biosynthesis pathway of SLs (Chen et al. 2021). As a downstream gene in the synthesis of SLs, MAX1 plays an important role in the synthesis of SLs (Santoro et al., 2021). MAX1 encodes a cytochrome P450 family member that acts downstream of MAX3/4 to produce a carotenoid-derived branch-inhibiting hormone. In *Arabidopsi*s and rice, mutation of D27 resulted in reduced SLs production and increased branching (Lin et al. 2009; Waters et al. 2012). Meanwhile, decreasing *TaD27s* expression also increased branching in wheat. The ability of root exudates to stimulate germination in TaD27-RNAi plants was severely reduced compared to that in WT plants (Zhao et al. 2020). In our study, to further show the effect of *SlMAX1* and *SlD27* on salt tolerance of tomato seedlings, we successfully silenced *SlMAX1* and *SlD27* using VIGS (Supplemental Fig. S1). The SLs content in *SlMAX1* and *SlD27* silencing plants was significantly decreased compared with WT plants. Additionally, Wen et al. (2023) discovered that knockdown of *GhD27*, *GhMAX3*, and *GhMAX4* genes in cotton resulted in an increased number of axillary buds and leaves, decreased fiber length, and significantly reduced fiber thickness. In our study, *SlMAX1* and *SlD27* gene silencing decreased root length, fresh weight and dry weight in tomato seedlings (Fig. 5). Guillotin et al. (2017) also found that compared in control plants, the expression levels of *SlD27* and *SlMAX1* were down-regulated in the three RNAi *SlIAA27* tomato lines. Similarly, exogenous application of synthetic GR24 rescued the increased branching phenotype of *Atd27* mutant in rice (Waters et al. 2012). Meanwhile, under salt stress, exogenous addition of HRW couldn't enhance the root/shoot ratio and root activity in *SlD27* and *SlMAX1* silencing plants (Fig. 5). The endogenous SLs content in *SlD27*- and *SlMAX1*-silenced plants was not increased by HRW treatment (Fig. 6). Meanwhile, Yang et al. (2024) recently found that after silencing *SlD27*, the alleviation effect of $H_2S$ on salt stress in tomato seedlings was basically eliminated, and the endogenous SL content was not increased by $H_2S$, which was similar to our results. Thus, our results showed that SLs participating in HRW to alleviate salt stress of tomato seedlings by regulating SLs biosynthesis genes *SlD27* and *SlMAX1*.

# Conclusion

Taken together, both SLs and HRW alleviated the damage of salt stress and promoted root growth in tomato seedlings. In addition, SLs and HRW alleviated the adverse effects of salt stress on root morphological characteristics, root activity and internal anatomical structure. However, TIS108 (an inhibitor of strigolactone synthesis) inhibited the positive roles of HRW in roots under salt stress, suggesting that SLs might be responsible for $H_2$-regulated root growth under salt stress. Moreover, HRW might enhance SLs biosynthesis by increasing the expression of SLs synthesis genes in the roots of tomato seedlings under salt stress. Furthermore, further silencing of SL biosynthesis gene *SlMAX1* and *SlD27* significantly reduced dry weight, fresh weight, root length, root/shoot ratio, root activity, and SLs content in roots under salt stress (Fig. 7). It suggested that *SlMAX1* and *SlD27* may positively regulate tomato seedlings. Under salt stress, HRW couldn't alleviate salt stress and enhance endogenous SLs content when *SlMAX1* and *SlD27* were silenced, suggesting the participation of SLs biosynthesis genes *SlMAX1* and *SlD27* in $H_2$-enhanced the salt resistance in tomato seedlings (Fig. 7). The present study provides new insights into the roles and interactions of $H_2$ and SLs during salt stress. However, future work will need to be done to investigate the mechanisms of crosstalk $H_2$ with SLs in response to salt stress, including post translational modification (*S*-nitrosylation, tyrosine nitration and metal nitrosylation) and key gene mutant analyses.

# Declarations

### Author contributons

Weibiao Liao designed the research; Fujin Ye: methodology, formal analysis, Investigation, writing – original draft, writing – review & editing. Hua Fang: methodology, formal analysis, Investigation, writing – review & editing. Li Feng and Meimei Shi: methodology, investigation, formal analysis. methodology, investigation. Ruirui Yang: writing – review & editing. All authors have read and approved the final version of the paper.


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### Data availability

Data will be made available on request.

### Competing interests

The authors have no relevant fnancial or non-fnancial interests to disclose. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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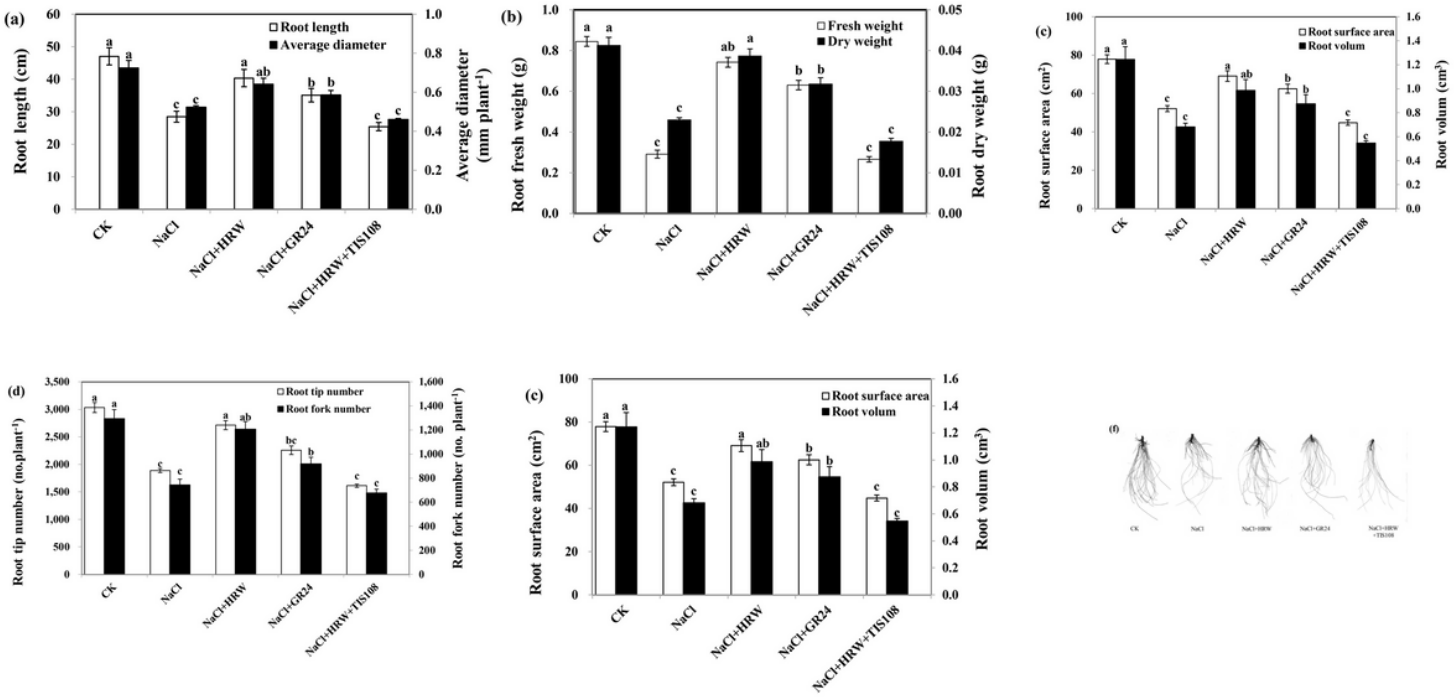
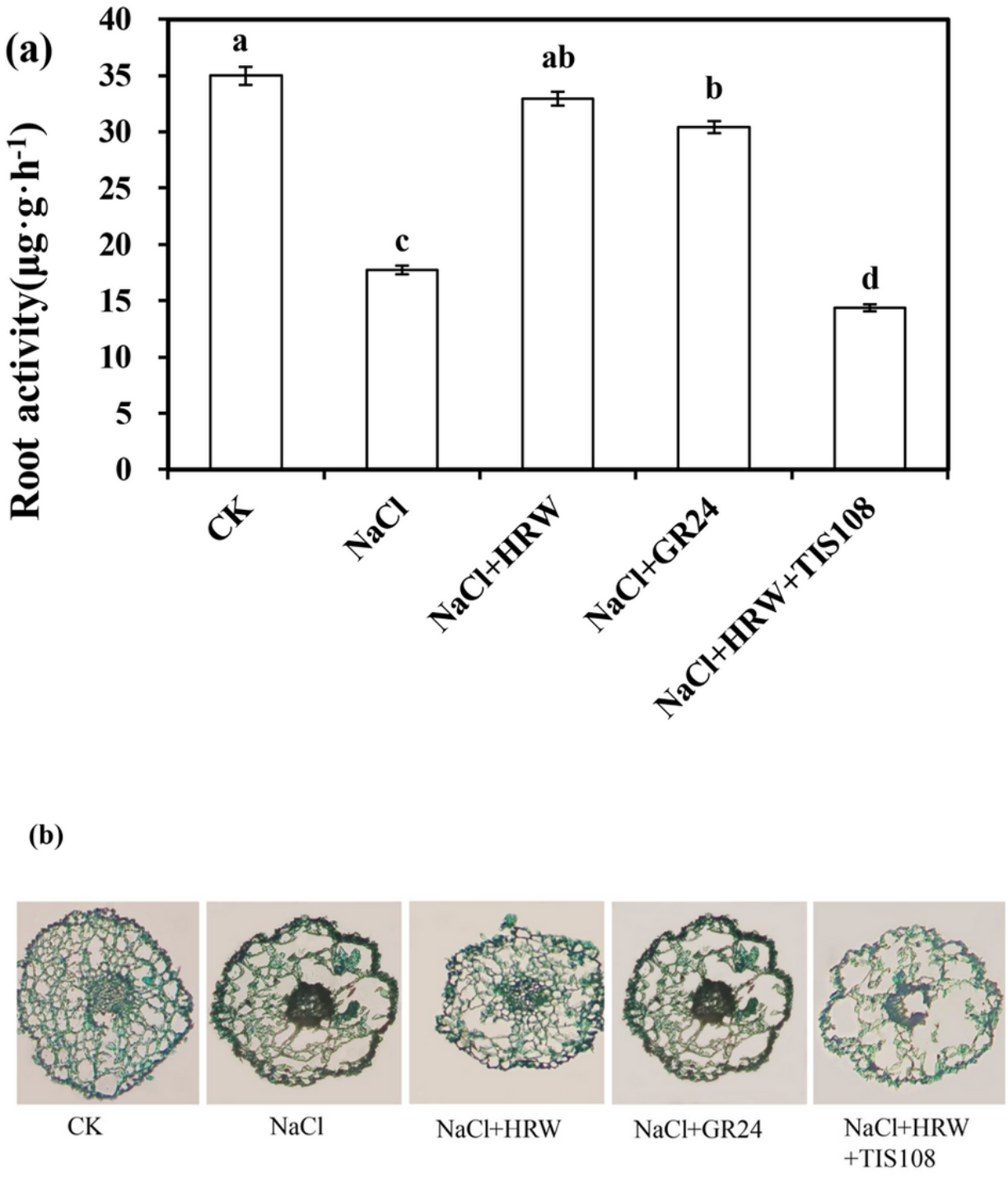
# Figures



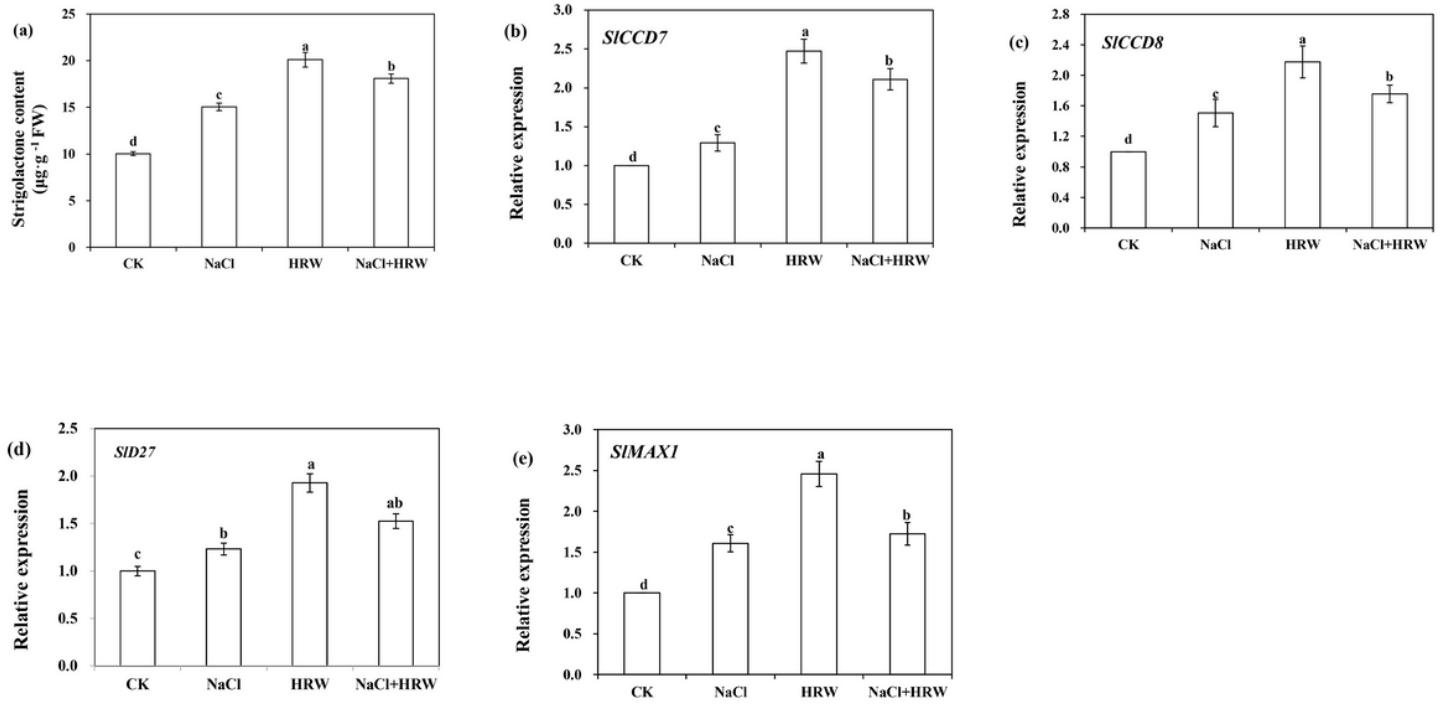

## Figure 1

Effects of GR24 and HRW on root parameters in tomato seedling under salt stress. Root length and average diameter (**a**); Fresh weight and dry weight (**b**); Surface area and root Volume (**c**); Root tip number and root fork number (**d**); Root/shoot ratio (**e**); Phenotype (**f**). Each value is the mean ± standard error (SE) of three independent means. Different letters indicate significant differences ($P$<0.05).



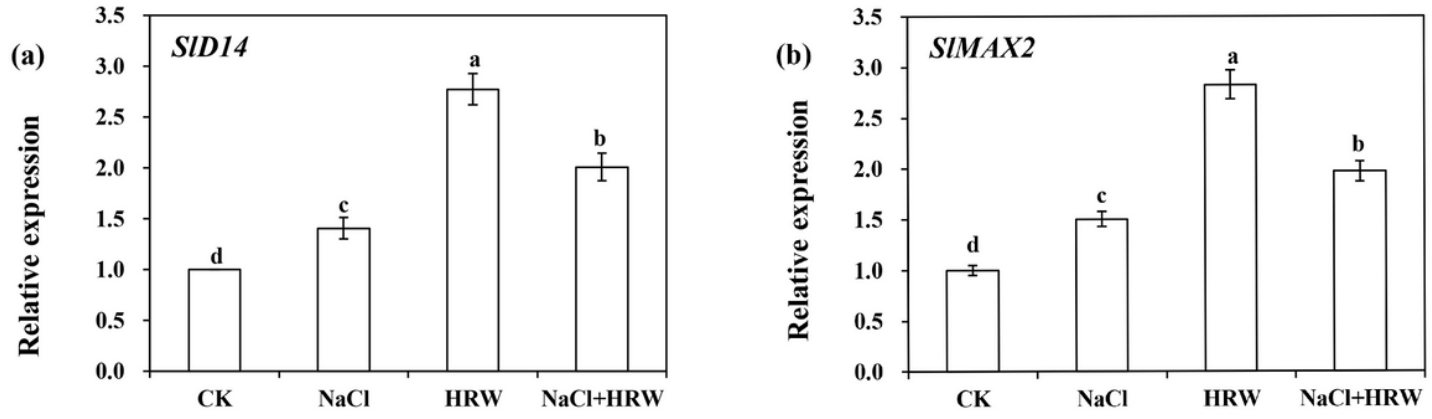

## Figure 2

Effects of GR24 and HRW on root activity and root internal anatomical structure in tomato seedling under salt stress. Root activity (**a**); Internal anatomical structure (**b**). Each value is the mean ± standard error (SE) of three independent means. Different letters indicate significant differences ($P$<0.05).



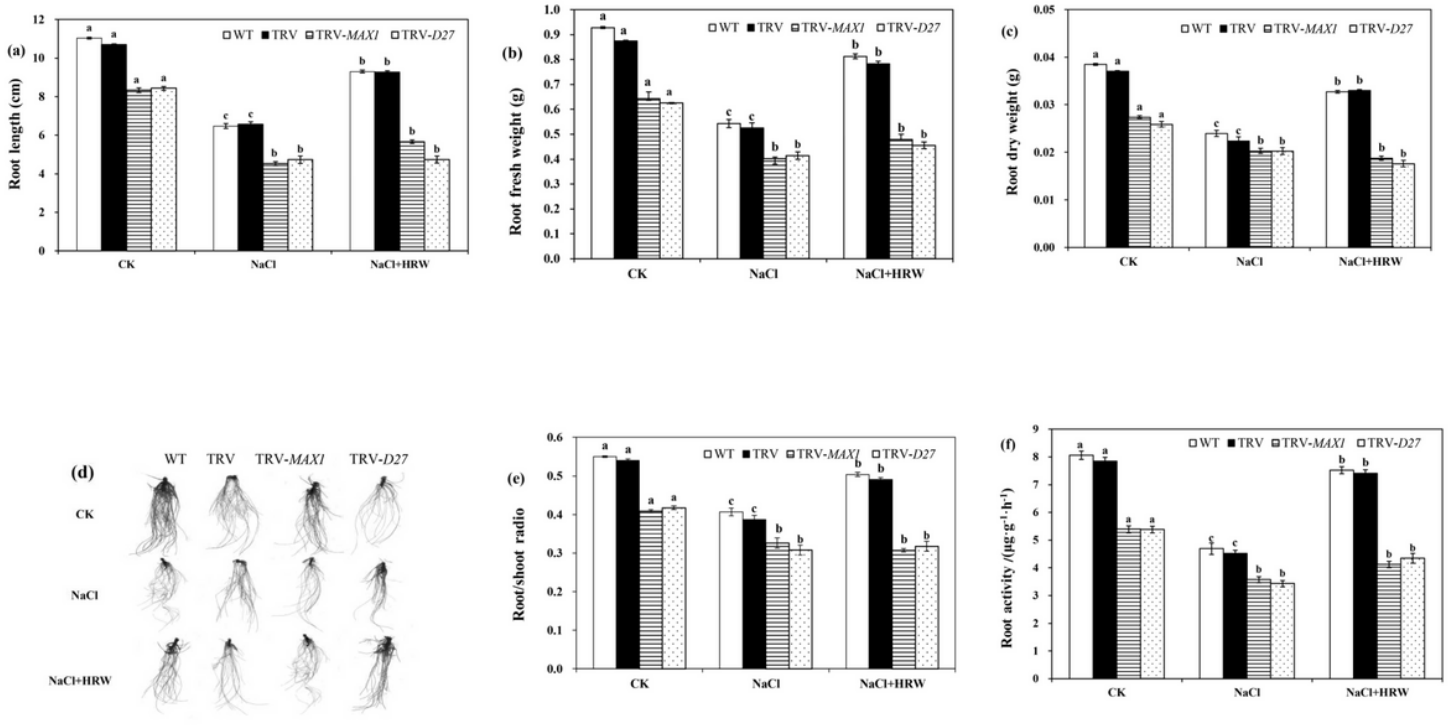

## Figure 3

Effects of HRW on endogenous SLs content and biosynthesis-related gene expression in tomato seedling root under salt stress. SLs content (**a**); *SlCCD7* (**b**); *SlCCD8* (**c**); *SlD27* (**d**); *SlMAX1* (**e**); Each value is the mean ± standard error (SE) of three independent means. Different letters indicate significant differences ($P$<0.05).



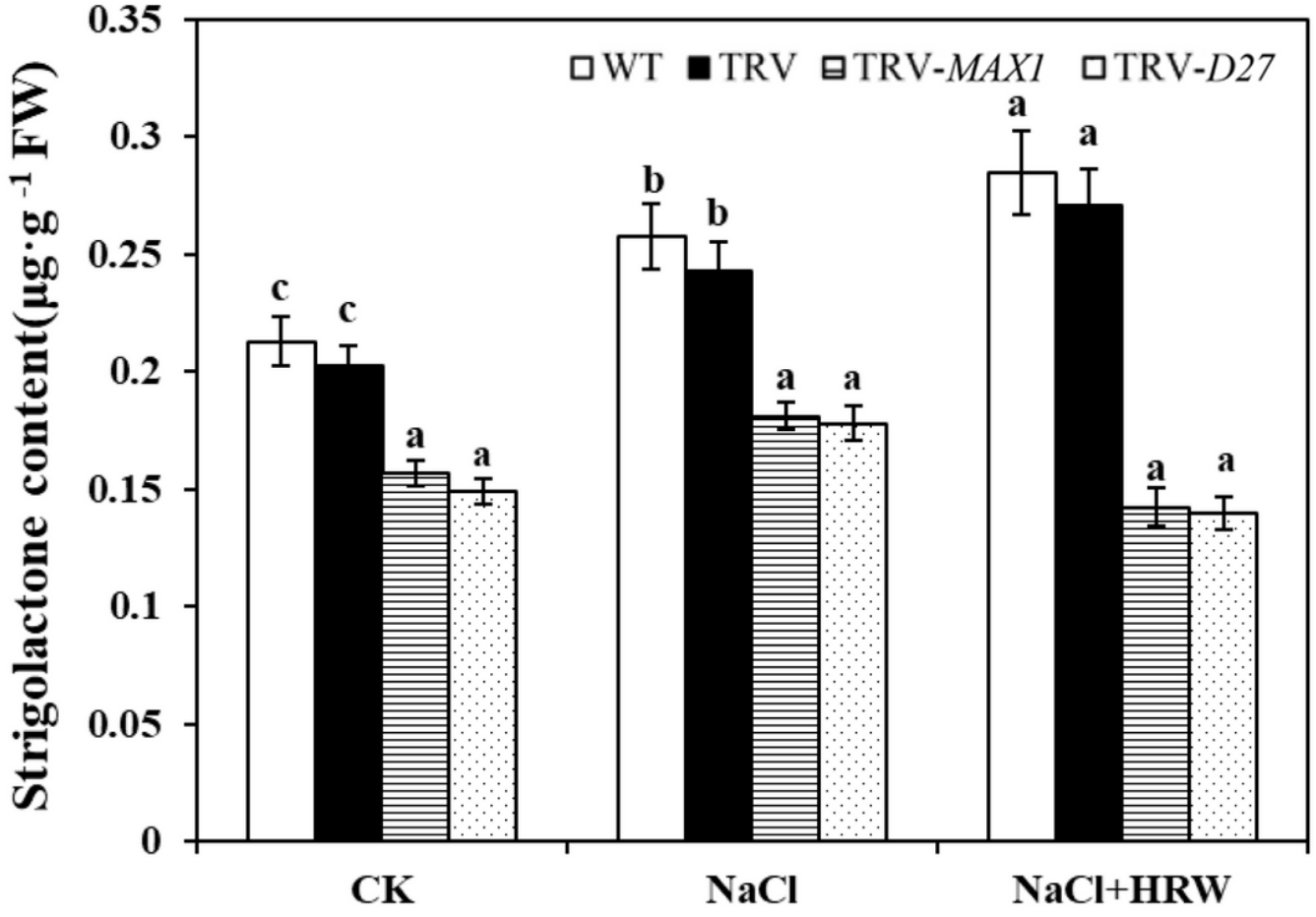

## Figure 4

Effects of HRW on the expression level of signal transduction gene *SlD14* and *SlMAX2* in tomato seedling root under salt stress. *SlD14* (a); *SlMAX2* (b). Each value is the mean ± standard error (SE) of three independent means. Different letters indicate significant differences ($P$<0.05).




## Figure 5

Effects of HRW, *SlMAX1* and *SlD27* gene silencing on root growth and development of tomato seedlings under salt stress. Root length (**a**); Root fresh weight (**b**); Root dry weight (**c**); Phenotype (**d**); Root/shoot radio (**e**); and Root activity (**f**). Different letters above the bars indicate significant differences among different treatments in the same line ($P$<0.05).




## Figure 6

Effects of HRW, *SlMAX1* and *SlD27* gene silencing on SLs content in tomato seedlings under salt stress. Different letters above the bars indicate significant differences among different treatments in the same line ($P$<0.05).

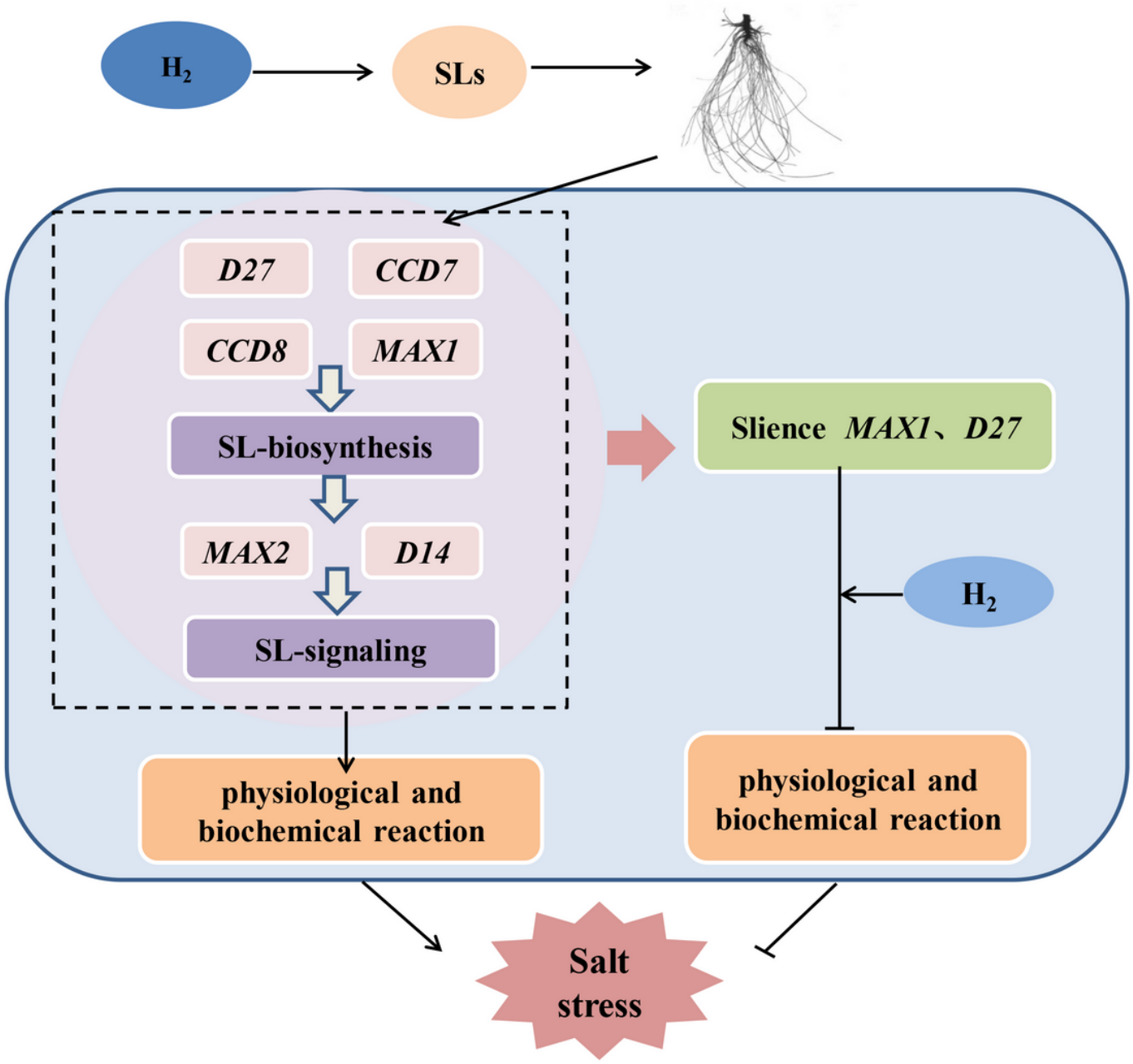



**Figure 7**

Mode of actin of strigolactones participated in $H_2$-improved salt stress in tomato seedlings.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterials.docx