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# Synthetic CsCEP3 Peptide Attenuates Salinity Stress via ROS and ABA Signaling in Cucumber Primary Root

**Zeping Shen †, Tingting Zuo †, Hailin Xia †, Shichang Ai, Qi Tao, Cheng Zeng, Xueping Guo and Huibin Han ***

College of Bioscience and Bioengineering, Jiangxi Agricultural University, Nanchang 330045, China; 15879060214@139.com (X.G.)

* Correspondence: huibinhan@jxau.edu.cn

† These authors contributed equally to this work.

**Abstract:** Salinity stress prominently limits cucumber growth and productivity. However, the mechanism underlying salinity response mediated by the C-TERMINALLY ENCODED PEPTIDE (CEP) peptide in cucumber primary root remains largely unclear. In this study, we show that salinity prominently inhibits cucumber primary root growth, and *CsCEP* gene expression is differentially induced by salinity. We further demonstrate that the exogenous application of synthetic CsCEP3 peptide partially suppresses salinity-triggered growth inhibition in cucumber primary root, although CsCEP3 peptide itself shows no obvious effect on cucumber primary root growth under normal conditions. Our transcriptomic and qRT-PCR data further reveal that CsCEP3 peptide may modulate gene expression related to abscisic acid (ABA) signaling pathway, reactive oxygen species (ROS) production, and slat-responsive transcription factors to attenuate the inhibitory effect of salinity on cucumber primary root growth. Taken together, our work provides a fundamental insight into CEP peptide-mediated cucumber salinity adaptation.

**Keywords:** *Cucumis sativus* L.; CEP peptide; root growth; salinity; transcriptome



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## 1. Introduction

Salinity is recognized as a world-wide problem, and high salt content in the soil remarkably suppresses crop growth, development, and yield [1–3]. Salinity stress elicits regulations at multiple levels, including salinity sensing, signal transduction, transcriptional/post-transcriptional regulations, and translation/post-translational regulations [4,5]. During the last decade, numerous gene loci and natural variations that are critical for salt stress response have been identified in various plant species [4,5]. Among them, plant phytohormones play vital roles in plant adaptations to salinity. Phytohormones are molecules produced in the plant body at very low concentrations, and they show extensive participation in plant development and growth regulations as well as salinity stress [6,7]. The exogenous application of phytohormones such as abscisic acid (ABA) is an effective strategy for crop improvements by reducing the negative impacts of salinity on plant development and growth [7]. Other signaling components, such as reactive oxygen species (ROS), are also induced by salt stress, and the *Arabidopsis* ROS production gene, *RbohD* and *RbohF*, play positive roles in salt stress [5]. In addition, many salt-responsive transcription factors (TFs) have been identified, and they show complex regulation mechanisms with ROS and phytohormones [3–7]. Nonetheless, the mechanisms underlying salt stress response are still largely unclear.

Besides the well-known phytohormones in abiotic stress response, the small signaling peptides also play crucial roles in plant adaptions to ever-changing environments [8–10]. Among the identified small peptide families, the C-TERMINALLY ENCODED PEPTIDE (CEP) signaling peptides play diverse roles in various plant developmental and environmental adaption processes [11]. The *CEP* gene encodes proteins with a secretory peptide at the N-terminal, a variable central region, and one or multiple CEP motifs at the

C-terminus [12–14]. CEP peptides belong to the post-translationally modified peptide family, the bioactive form of CEP is 15 amino acids in length, and application of synthetic CEP peptide can trigger plant physiological outcomes [12,15,16]. In *Arabidopsis*, *CEP* genes exhibit a distinct expression pattern in various tissues, and several environmental stressors can regulate *CEP* genes expression, indicating that *CEP*s may play diverse roles in *Arabidopsis* development and adaptive processes [12,13]. *Arabidopsis CEP3* gene expression is regulated by salinity, and the *cep3* mutant displays a large root system under salt stress conditions [13]. In addition, the CEP5 peptide has been reported to promote tolerance to drought and osmotic stress by interfering with auxin signaling [17]. In other species, *CEP*s expression is also regulated by salinity and drought stress [18]. Nevertheless, the undefined role of CEP peptide in the regulation of cucumber salt stress response remains largely untapped.

Cucumber (*Cucumis sativus* L.) is a world-wide cultivated vegetable with great economic value [19]. However, environmental fluctuations such as high temperature, chilling, salinity, and drought stress severely limit cucumber growth and yield. It has been reported that high salinity stress inhibits cucumber root growth [20,21], but the molecular mechanisms are still largely unknown. Previously, we have identified 6 *CEP* members in cucumber, and ROS is important for CsCEP4 peptide-mediated root growth regulation [22], but their role in salinity response is unknown. In this study, we aim to elucidate the potential role of CsCEP3 peptide in cucumber salinity stress response. As mentioned herein, ROS, salt-responsive TFs, and ABA paly crucial roles in salinity adaptions [3–7], so we also examine the involvement of ROS, salt-responsive TFs, and ABA in CsCEP3-mediated salt stress response in cucumber primary root. Our results show that salinity prominently suppresses cucumber primary root growth, and salinity also differentially induces *CsCEP* genes expression. Additionally, exogenous application of synthetic CsCEP3 peptide partially attenuates the negative effect of salinity on cucumber primary root growth, although CsCEP3 peptide itself shows no obvious effect on cucumber primary root growth under normal conditions. We perform a transcriptome assay to further identify the possible players that may be responsible for CsCEP3 peptide-mediated salinity adaption in cucumber primary root. Our results demonstrate that genes related to reactive oxygen species (ROS) production are regulated by CsCEP3 peptide under salt conditions. Our pharmacological and qRT-PCR results further indicate that CsCEP3 peptide may potentially modulate ROS level to promote cucumber primary root growth under salt stress. Additionally, ABA signaling and salt-responsive transcription factors are differentially regulated by salinity and CsCEP3 peptide. Taken together, our study demonstrates the potential relationship between CsCEP3 peptide, ROS, ABA, and salt-responsive TFs in cucumber salinity adaption, and our work provides an alternative strategy to remold cultivar cucumber with enhanced tolerance to salinity.

## 2. Materials and Methods

### *2.1. Plant Material and Growth Conditions*

The cultivar cucumber seeds (Jinchun si hao) were used in this study. The cucumber variety was developed by Tianjin Cucumber Research Institute with strong resistance to downy mildew disease, and the fruit length was 30–35 cm. The seed sterilization and germination procedure were described previously [22]. After germination, seedlings were sown on half-strength Murashige and Skoog medium and were kept growing in a plant growth chamber under 16 h:8 h (light–dark photoperiod) at 21 °C with a light intensity 112 μmol $m^{-2}$ $sec^{-1}$. All the experiments were performed (from the year 2022 to 2023) in the lab of Research Center of Plant Functional Genes and Tissue Culture Technology, Jiangxi Agricultural University, Nanchang, China.

### *2.2. Root Length Quantification*

After germination, cucumber seedlings with a similar primary root length were transferred to the new medium supplied with various chemicals including CsCEP3, CsCEP1a,

CsCEP4 peptides, DPI, CAT, $H_2O_2$, *L*-NAME, $Na_2WO_4$, SNP at indicated concentrations, and the seedlings were cultured for another 4 days, the EPSON 370 scanner was then used to capture the cucumber primary root, and root length was quantified using ImageJ software (Version 1.53t).

### *2.3. RNA Sequencing and Transcriptomic Data Processing*

After germination, the cucumber seedlings with a similar primary root length were transferred to the new half MS medium containing control, NaCl (100 mM), CsCEP3 peptide (1 μM), and CsCEP3 plus NaCl, and the seedlings were cultured for another 4 days. Then, the primary roots were collected for transcriptome analysis. Additionally, 1 g roots for each treatment were collected for RNA-seq analysis, and three independent biological replicates were performed. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA purity and quantification were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then, the libraries were constructed using VAHTS Universal V6 RNA-seq Library Prep Kit according to the manufacturer's instructions. The transcriptome sequencing was conducted with illumina NovaseqTM 6000 platform by OE Biotech Co., Ltd. (Shanghai, China). Raw reads of fastq format were firstly processed using fastpi, and the low-quality reads were removed to obtain the clean reads. The clean reads were mapped to the cucumber genome (Chinese Long V3). Differential expression analysis was performed using the DESeq2 software with fold change > 2, and $p$-value < 0.05 was set as the threshold for significantly differential expression genes (DEGs) [23].

### *2.4. Chemical Treatment*

The 15 amino acids motifs of the CsCEP1a, CsCEP3, and CsCEP4 peptides were previously synthesized [22] by DGpeptide company (http://www.dgpeptides.com/, accessed on 20 April 2020, Wuhan, China) with a purity higher than 90%. All synthetic peptides were dissolved with $ddH_2O$ and were stored at −20 °C. CsCEP1a peptide (1 μM), CsCEP3 peptide (1 μM), and CsCEP4 peptide (1 μM), catalase (CAT, a $H_2O_2$ scavenger enzyme, 100 units), diphenylene iodonium (DPI, an inhibitor of NADPH oxidase, 10 μM), $H_2O_2$ (1 mM), NG-nitro-L-Argmethyl ester hydrochloride (*L*-NAME, an NO synthase-like enzyme inhibitor, 25 μM), $Na_2WO_4$ (a nitrate reductase inhibitor, 5 μM), and sodium nitroprusside (SNP, NO donor, 50 μM) were obtained from Macklin company (http://www.macklin.cn/, accessed on18 March 2020, Shanghai, China) and were used at indicated concentration. Three independent biological replicates were performed, and 15 seedlings were transferred for each treatment.

### *2.5. Total RNA Extraction and Quantitative Real-Time PCR (RT-qPCR) Analysis*

For each treatment, 500 mg roots were harvested and ground in liquid nitrogen. Total RNA was extracted using the plant RNA extraction kit (DP432, TIANGEN Biotech, Beijing, China) according to manufacturer's instructions. Furthermore, 1 μg RNA was used for cDNA synthesis using StartScript II First-strand cDNA Synthesis Kit (GenStar A224, Beijing, China). The qPCRs were performed with 2 × RealStar Fast SYBR qPCR Mix (GenStar A304, Beijing, China) using LightCycler480 II (Roche). A previously reported cucumber Ubiquitin gene (*CsaV3_5G031430*) was used as an internal control, and the relative transcript level was calculated using the $2^{-\Delta\Delta CT}$ method [24]. Three independent biological replicates were performed, and for each independent biological replicate, the relative transcription level was calculated as the mean of three technical replicates. All qPCR primers used in this study were shown in Table S1.

### *2.6. DAB and NBT Staining*

The DAB and NBT staining were performed as previously described [22,25]. In brief, after germination, the cucumber seedlings with a similar primary root length were

transferred to the new half MS medium containing control, NaCl (100 mM), CsCEP3 peptide (1 μM), and CsCEP3 peptide plus NaCl for another 4 days, then DAB and NBT staining were performed in cucumber primary root. The relative DAB and NBT signal intensity was quantified via ImageJ software (Version 1.53t). Five roots were quantified for each treatment, and three independent biological replicates were performed.

### *2.7. Statistical Analysis*

All statistical analysis was performed using One-way ANOVA test with a significant difference via GraphPad Prism 8.0 (* $p < 0.05$; ** $p < 0.01$) developed by GraphPad Software company.

## 3. Results

### *3.1. Salinity Inhibits Cucumber Primary Root Growth and Regulates CsCEPs Gene Expression*

We first examined the salinity effect on cucumber primary root growth. After germination for 2 days, the cucumber seedlings with a similar primary root length were transferred to the new culture medium supplied with 75 mM, 100 mM, and 150 mM sodium chloride (NaCl), respectively. Compared to the control treatment, primary root growth was remarkably inhibited in cucumber seedlings treated with NaCl (Figure 1A). It has been suggested that salinity regulates *CEP* expression [13,18], so we investigated whether NaCl (100 mM) treatment can modulate the 6 *CsCEP* expression in cucumber primary root. Under our experimental conditions, our qRT-PCR result showed that *CsCEP*s were differentially regulated by salt stress. *CsCEP2*, *CsCEP3,* and *CsCEP5* were prominently up-regulated, while the expression of *CsCEP1*, *CsCEP4*, and *CsCEP6* was not obviously affected by NaCl treatment (Figure 1B). The differential expression patterns of *CsCEP*s under NaCl treatment further imply their potential roles in cucumber salinity response.

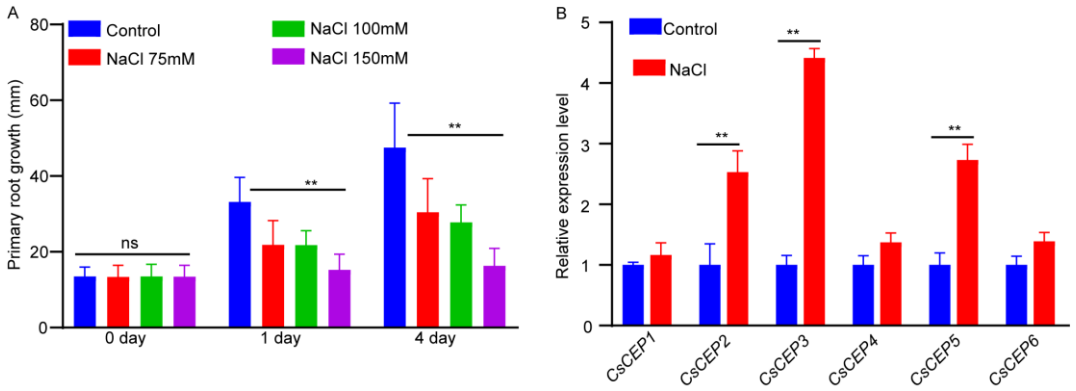


**Figure 1.** Salinity inhibits cucumber primary root growth. (**A**) Quantification of cucumber primary root length under 75 mM, 100 mM, and 150 mM of NaCl treatment. $n$ = 10–15, ** $p < 0.01$ was determined by Student's *t*-test. (**B**) qRT-PCR analysis of expression patterns of 6 *CsCEP* genes under 100 mM NaCl treatment. $n$ = 3, ** $p < 0.01$ was determined by One-way ANOVA test. ns: no significance.

### *3.2. CsCEP3 Peptide Supplement Partially Promotes Salinity Tolerance*

We next assessed whether exogenous application of the synthetic CsCEP1a, CsCEP3, and CsCEP4 peptides [22] can affect cucumber primary root growth under salinity stress. We found that the synthetic CsCEP1a and CsCEP4 peptides exhibited no visible impact on cucumber primary root growth under 100 mM NaCl treatment (Figure S1), which is in line with their unchanged expression under salt stress (Figure 1B). However, application of synthetic CsCEP3 peptide partially attenuated the negative effect of salinity on cucumber primary root growth, although CsCEP3 peptide itself displayed no significant effect on cucumber primary root growth (Figure 2) [22]. These results indicate that CsCEP peptides may play distinct but redundant roles in cucumber primary root growth under salinity, and CsCEP3 peptide can potentially promote cucumber salinity tolerance.

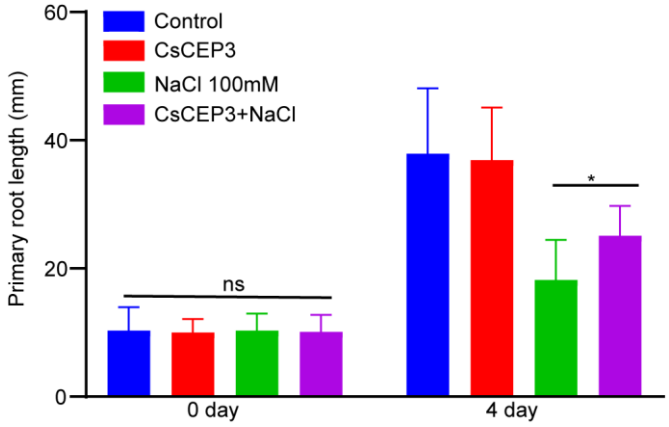


**Figure 2.** CsCEP3 peptide partially attenuates salinity effect on cucumber primary root growth. $n$ = 10–15, * $p < 0.05$ was determined by One-way ANOVA test. ns: no significance.

### *3.3. Synthetic CsCEP3 Peptide Modulates Multiple Pathways in Salt-Treated Cucumber Primary Root*

To further identify the possible downstream players that are responsible for CsCEP3 peptide-mediated salt tolerance in cucumber primary root, we performed a transcriptome analysis. We analyzed differential expressed genes (DEGs) in cucumber primary root under different conditions. As shown in Figure 3, 952 up-regulated genes and 578 downregulated genes were identified in the Control vs. NaCl group (Table S2); 27 up-regulated genes and 12 down-regulated genes were identified in the Control vs. CsCEP3 peptide group (Table S3); 318 up-regulated genes and 389 down-regulated genes were identified in the NaCl vs. CsCEP3 peptide group (Table S4); 344 up-regulated genes and 304 down-regulated genes were identified in the CsCEP3 + NaCl vs. CsCEP3 peptide group (Table S5); 156 up-regulated genes and 38 down-regulated genes were identified in the CsCEP3 + NaCl vs. NaCl group (Table S6); 695 up-regulated genes and 296 down-regulated genes were identified in the CsCEP3 + NaCl vs. Control group (Table S7). Our transcriptome data indicates that synthetic CsCEP3 peptide could regulate gene expression in diverse signaling pathways under salt stress.

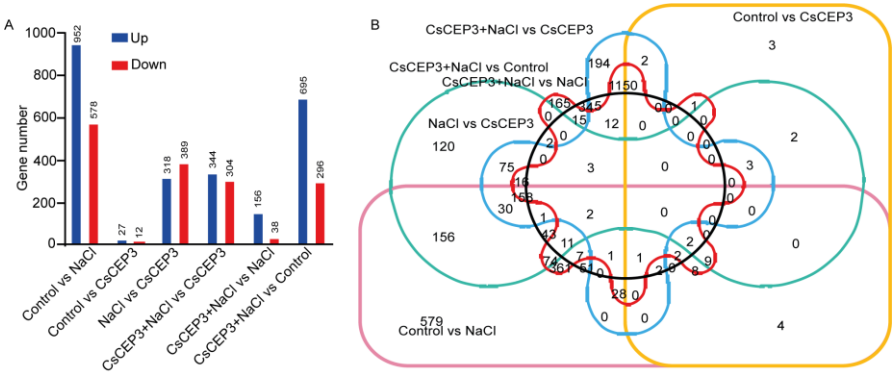


**Figure 3.** Identification of DEGs in cucumber primary roots upon salinity and CsCEP3 peptide treatment (**A**). The Venn graph (**B**) was built using TBtools software (accessed on 20 March 2023, Version 1.120).

### *3.4. CsCEP3 Regulates $H_2O_2$ and NO Signaling under Salt Stress*

Hydrogen peroxide ($H_2O_2$) and nitric oxide (NO), two main forms of reactive oxygen species (ROS), have been suggested to play a role in cucumber salt stress response [20,26–28]. The RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs) are considered as the main enzymes for $H_2O_2$ production [29]. Our transcriptomic data showed that cucumber genes encoding *RBOH*s including *CsaV3_1G002910*, *CsaV3_5G002350*, *CsaV3_5G027600*, *CsaV3_6G021970*, and *CsaV3_1G038860* were differentially regulated by salt stress and

CsCEP3 peptide (Figure 4A). Among them, *CsaV3_5G002350* and *CsaV3_1G038860* were up-regulated by salt stress, but they were down-regulated by CsCEP3 peptide upon salt stress treatment (Figure 4A). Our qRT-PCR data also showed a similar regulation of cucumber *RBOH*s gene expression by CsCEP3 peptide under salt conditions (Figure 4B). In addition to $H_2O_2$, we also checked the expression of NO biosynthesis gene, the *NITRATE RESDUCATE*s (*NR*s) [28]. Our qRT-PCR result revealed that *CsaV3_2G026760*, *CsaV3_4G028560*, and *CsaV3_5G023370* were up-regulated by salt stress. However, the salt-induced up-regulation of *CsaV3_2G026760*, *CsaV3_4G028560*, and *CsaV3_5G023370* was abolished by the CsCEP3 peptide (Figure 4C).

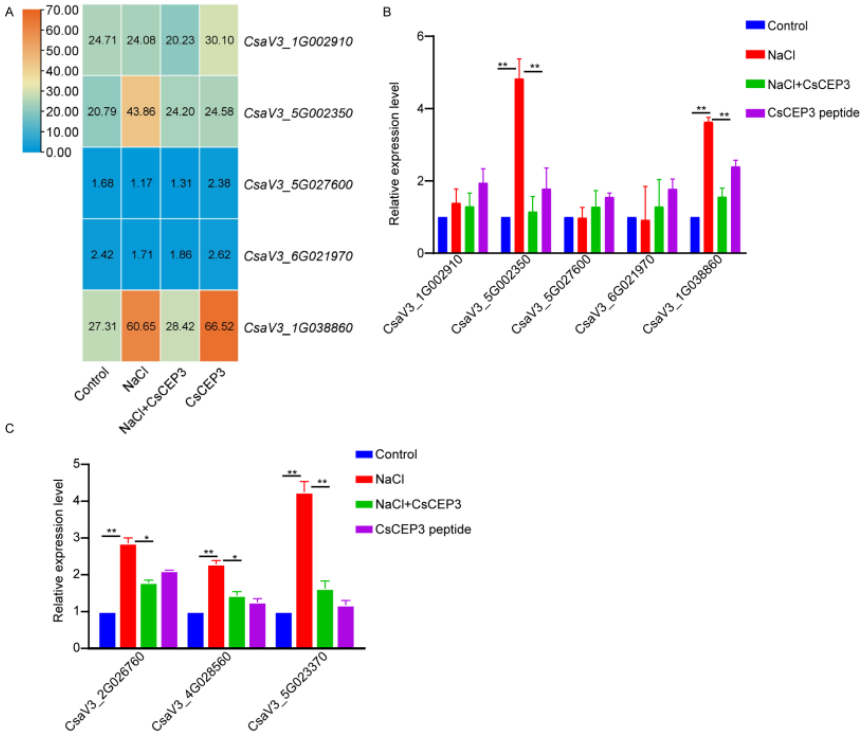


**Figure 4.** CsCEP3 peptide regulates $H_2O_2$ and NO biosynthesis gene expression under salt conditions. (**A**) Average of FPKM of $H_2O_2$ biosynthesis genes. qRT-PCR analysis of $H_2O_2$ biosynthesis genes (**B**) and NO biosynthesis genes (**C**) expression in cucumber primary roots, $n = 3$, ** $p < 0.01$ or * $p < 0.05$ was determined through One-way ANOVA test.

We next performed DAB and NBT staining to examine whether CsCEP3 peptide would regulate the accumulation of $H_2O_2$ and NO in cucumber primary root under salinity conditions. In line with previously reported results [20,28], salt stress prominently induced accumulation of $H_2O_2$ and NO in cucumber primary roots, and CsCEP3 peptide itself showed no significant effect on $H_2O_2$ and NO accumulation. However, CsCEP3 peptide partially abolished $H_2O_2$ and NO accumulation triggered by salinity (Figure 5).

To further corroborate our result, we investigated whether $H_2O_2$ and NO inhibitors can affect cucumber primary root growth under salinity conditions. Under our experimental conditions, we observed that $H_2O_2$ and NO inhibitors partially abolished the inhibitory effect of salt stress on cucumber primary root growth, and exogenous application of $H_2O_2$ and SNP could partially rescue the inhibitor impact. $H_2O_2$ and SNP enhanced the effect of salt stress on cucumber primary root growth. However, CsCEP3 peptide repressed $H_2O_2$ and SNP effect (Figure 6A,B).

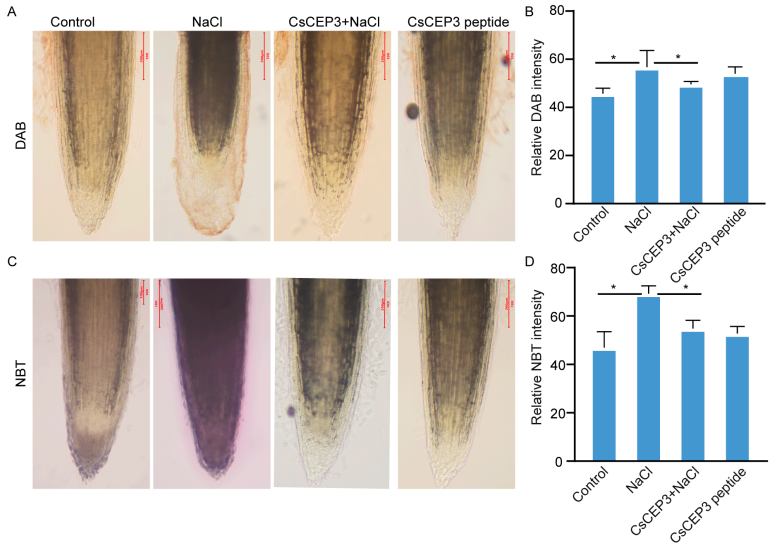


**Figure 5.** Synthetic CsCEP3 peptide regulates $H_2O_2$ and NO accumulation in cucumber primary roots under salt stress. Representative images showing DAB (**A**) and NBT (**C**) staining in cucumber primary roots. (**B**,**D**) quantification of DAB and NBT intensity in cucumber primary roots. $n = 5$, * $p < 0.05$ was determined by One-way ANOVA test.

Taken together, our data indicate that CsCEP3 peptide may eliminate the inhibitory effect of salinity on cucumber primary root growth by regulating $H_2O_2$ and NO production.

### *3.5. CsCEP3 Peptide Regulates Gene Expression of ABA Signaling Pathway*

It has been shown that salinity induces abscisic acid (ABA) content and ABA signaling gene expression in cucumber [30], indicating the role of ABA in salinity response. We also found that genes related ABA signaling pathway including *CsaV3_4G035560* (ABA receptor), *CsaV3_7G025250* (ABA receptor), *CsaV3_3G037220* (Abscisic acid insensitive protein), *CsaV3_4G007760* (9-cis-epoxycarotenoid dioxygenase, ABA biosynthesis enzyme), *CsaV3_4G028620* (ABSCISIC ACID-INSENSITIVE 5-LIKE PROTEIN), and *CsaV3_6G037310* (ABSCISIC ACID-INSENSITIVE 5-LIKE PROTEIN 4 isoform) were differentially regulated by salinity and CsCEP3 peptide (Figure 7A). Among these genes, *CsaV3_4G007760* and *CsaV3_7G025250* were up-regulated by salt stress, but their expression was down-regulated by CsCEP3 peptide under salt treatment. To confirm the transcriptome data, we hence performed a qRT-PCR analysis to assess the identified ABA signaling gene expression. Our data showed that *CsaV3_4G007760* and *CsaV3_7G025250* were up-regulated by salt stress, but CsCEP3 peptide reduced their expression under salinity conditions (Figure 7B). Our data indicate that salinity promotes ABA biosynthesis and activates ABA signaling pathway [30], but CsCEP3 peptide may play an antagonistic role in salt-stimulated ABA signaling.

### *3.6. CsCEP3 Peptide Regulates Gene Expression of Salt-Responsive Transcription Factors*

Transcription factors (TFs) such as WRKY and MYB family are essential regulators that directly bind to specific DNA regulatory elements of their target genes, thus regulating multiple stress responses including salt stress [31,32]. In *Arabidopsis*, overexpression of *WRKY8* [33], *WRKY33* [34], *WRKY53* [35], *MYB12* [36], *MYB30* [37], and *MYB44* [38] can promote root growth under salinity; whereas, over expression of *WRKY15* increases sensitivity to salt stress [39]. Our transcriptome data revealed that these slat-responsive TFs including *CsaV3_7G030110* (homolog of *AtWRKY8*), *CsaV3_6G043450* (homolog of *AtWRKY15*), *CsaV3_3G033350* (homolog of *AtWRKY33*), *CsaV3_7G025370* (homolog of *AtWRKY53*), *CsaV3_6G022540* (homolog of *AtMYB12*), *CsaV3_3G012100* (homolog of *AtMYB30*), and *CsaV3_4G034750* (homolog of *AtMYB44*) were differentially regulated by salinity and CsCEP3 peptide (Figure 8A). Our data implied that salt stress in-

duced *CsaV3_3G033350*, *CsaV3_6G022540*, and *CsaV3_4G034750* gene expression but down-regulated *CsaV3_6G043450*, *CsaV3_7G025370*, and *CsaV3_3G012100* expression. However, CsCEP3 peptide could facilitate *CsaV3_7G030110*, *CsaV3_7G025370*, *CsaV3_3G012100*, and *CsaV3_4G034750* expression level but repress *CsaV3_6G043450* expression level. Additionally, we also observed a similar expression pattern of these salt-responsive TFs by performing qRT-PCR analysis in cucumber primary roots (Figure 8B). These data indicate that CsCEP3 peptide could differentially regulate salt-responsive TFs expression to modulate cucumber primary root growth under salinity conditions.

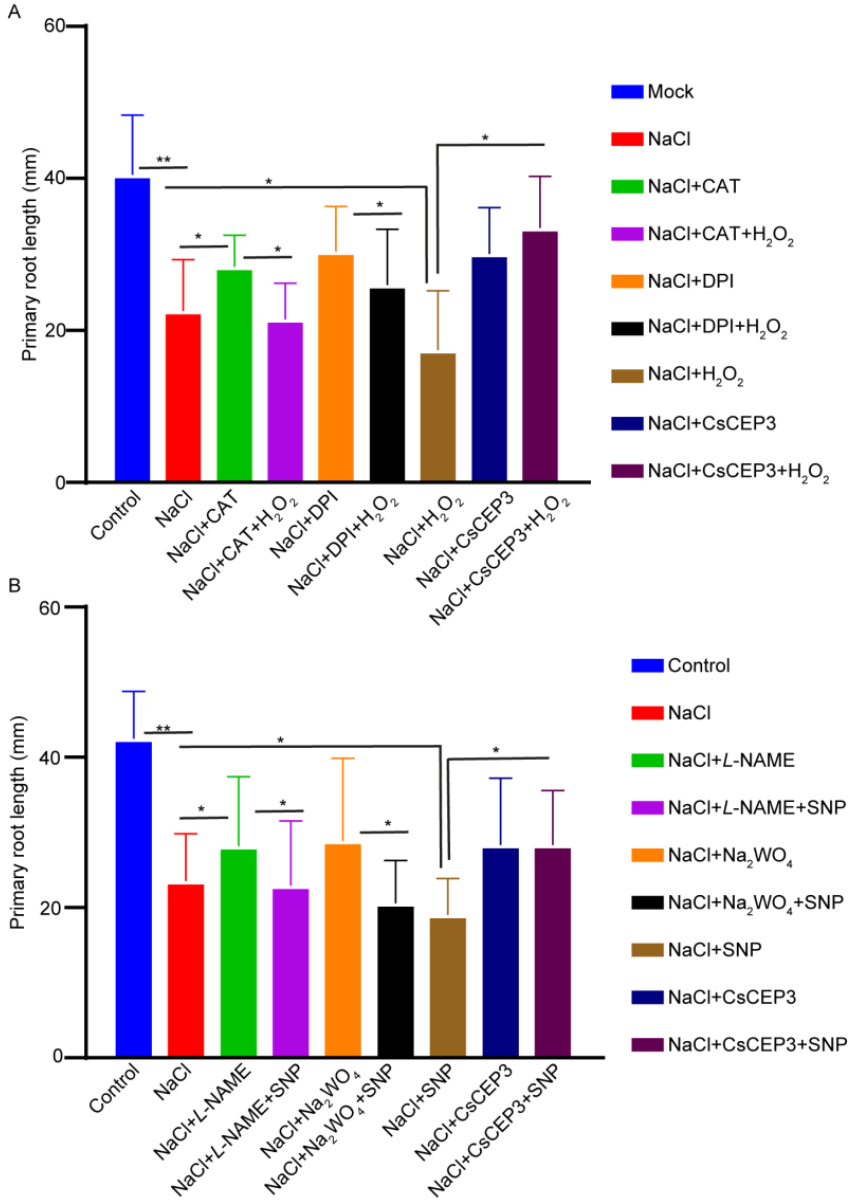


**Figure 6.** CsCEP3 peptide attenuates salinity effect on cucumber primary root growth via modulating $H_2O_2$ (**A**) and NO (**B**) signaling. Cucumber seedlings were treated with $H_2O_2$ (**A**) and NO M for 4 days. Then, the primary root length was quantified. $n$ = 10–15, ** $p < 0.01$ and * $p < 0.05$ was determined by One-way ANOVA test.

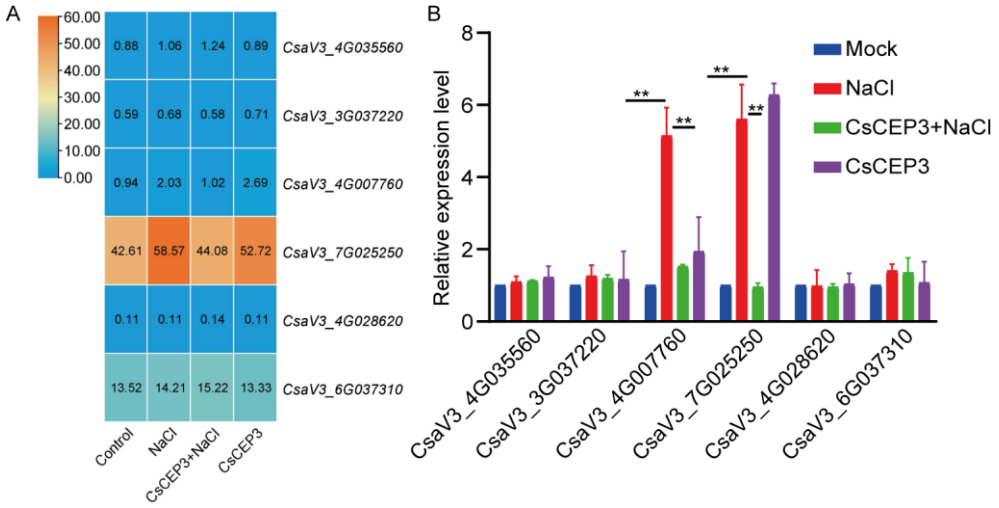


**Figure 7.** CsCEP3 peptide regulates ABA pathway gene expression under salinity. (**A**) Average of FPKM of ABA pathway genes. (**B**) qRT-PCR analysis of ABA pathway gene expression in cucumber primary roots, $n = 3$, ** $p < 0.01$ was determined by One-way ANOVA test.

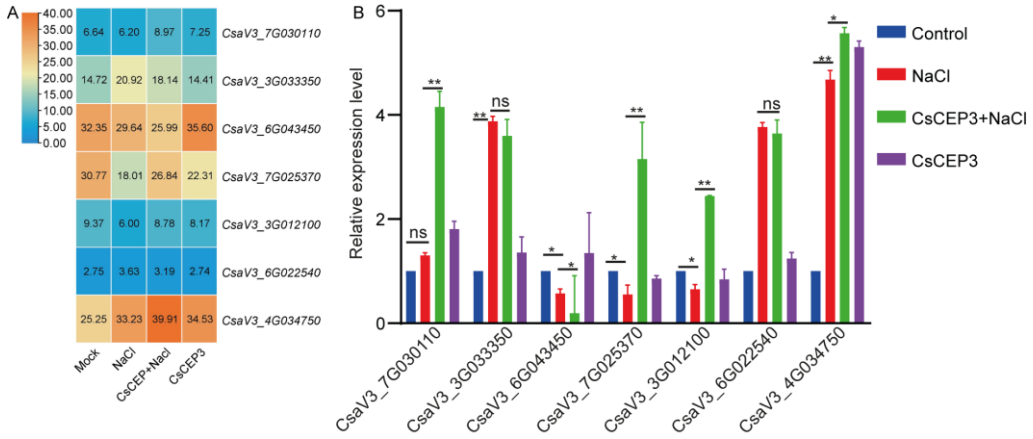


**Figure 8.** CsCEP3 peptide regulates salt-responsive TFs gene expression under salinity. (**A**) Average of FPKM of salt-responsive TFs. (**B**) qRT-PCR analysis of salt-responsive TFs expression in cucumber primary roots, $n = 3$, * $p < 0.05$ or ** $p < 0.01$ was determined by One-way ANOVA test. ns: no significance.

## 4. Discussion

The CEP peptides play critical roles in a wide range of plant developmental and adaptive processes [9–11]. Here, we showed that salt stress inhibited cucumber primary root growth, and *CsCEP3* expression was significantly induced by salt stress (Figure 1). In addition, exogenous application of synthetic CsCEP3 peptide can partially eliminate the inhibitory effect of salt stress on cucumber primary root growth probably via regulation of ABA signaling pathway, ROS production, and salt-responsive TFs gene expression. Our work hence provides a fundamental insight into CsCEP3 peptide in the regulation of cucumber primary root growth in response to salinity, which would suggest an alternative strategy to remold cultivar cucumber with enhanced tolerance to salinity.

The CsCEP peptides may play redundant roles in salt stress response, as CsCEP3 peptide only partially rescued the salinity effect on cucumber primary root growth (Figure 2). Therefore, we cannot exclude the undefined roles of CsCEP2 and CsCEP5 peptides in salt stress, as their expression is also prominently induced by salinity (Figure 1B). Importantly, it is very crucial to generate loss-of- and gain-of-function cucumber transgenic seedlings to further verify the positive role of *CsCEP3* in salt tolerance. In most cases, CEP peptide can be perceived by the plasma membrane localized receptor-like kinase CEP RECEPTOR1

(CEPR1) and CEPR2 to modulate downstream regulatory networks [40–43]. Receptor-like kinase (RLK) families have been identified from the cucumber genome [44–46], and the *CsaV3_3G048330* and *CsaV3_6G004690* genes encode RLK, which shows that 67.38% identify to CERP1, and 59.45% identify to CERP2, respectively (Figure S2). These two RLKs could be potential receptors for CsCEP3 peptide in salinity adaption, but we cannot exclude the role of other RLKs in CsCEP3-mediated salt response. Hence, it is appealing to identify putative receptors of CsCEP3 peptide using the loss-of-function RLK mutants, which will enable us to explore the downstream signaling pathways that are vital for CEP peptide function during cucumber salt adaption as well as other developmental processes.

$H_2O_2$ and NO are two main forms of reactive oxygen species (ROS), and they act as essential signaling molecules in plant stress response [47]. ROS signaling output is tightly regulated by ROS generation, scavenging, and transport [47]. Enzymes including RBOHs and CATALASE (CAT), GLUTATHIONE PEROXIDASE (GPX), and ASCORBATE PEROXIDASE (APX) are the main producers and scavengers of $H_2O_2$ in plants, respectively [47,48]. Moreover, AQAPORINs (AQPs) are considered to be $H_2O_2$ transporters [49]. The receptor kinase HYDROGEN-PEROXIDE-INDUCED CALCIUM 417 INCREASES 1 (HPCA1) has been identified as a sensor to perceive extracellular $H_2O_2$ [50]. NITRATE REDUCTASE (NR) has been proposed as a critical enzymatic NO source in cucumber salt stress response [28]. Our data demonstrated that RBOH-dependent and NR-dependent $H_2O_2$ and NO production (Figures 4–6) are crucial for CsCEP3 peptide-regulated cucumber root growth under salt stress. However, how CsCEP3 peptide regulates $H_2O_2$ and NO homeostasis through these sensors, metabolic enzymes, and transporters requires detailed investigations in the future.

Plants have developed various pathways to cope with diverse abiotic stresses [6], and the plant phytohormone ABA plays an indispensable role in regulating plants responses to abiotic stress [7]. In plants, ABA homeostasis is mostly controlled by its biosynthesis, transportation, catabolism, and conjugation with other metabolites [7]. Many genes related to ABA biosynthesis, transport, and metabolism have been identified [7], and our transcriptomic data suggested that salinity can induce ABA signaling and biosynthesis gene expression, but CsCEP3 peptide down-regulated these genes expression (Figure 7). However, it remains unclear whether other genes related to ABA signaling pathway are also regulated by salt stress and CsCEP3 peptide. In rice, ABA regulates the expression of *OsP5CR* and *OsGST4* to promote ROS clearance and enhances the salt tolerance of rice [51]. Our data showed salt stress significantly stimulated the accumulation of ROS in cucumber root, and CsCEP3 peptide eliminated ROS accumulation under salt stress conditions (Figure 5). This ROS clearance triggered by CsCEP3 peptide resulted in cucumber growth promotion (Figure 6). It is likely that CsCEP3 provokes ROS clearance via regulating ABA pathway (Figure 7) [51], but it needs more investigations in the future. On the other hand, we have to note that other phytohormones may also participate in CsCEP3 peptide-mediated salt adaptions, as ABA and ROS interact with other phytohormones in response to salinity [7,52].

Many plant TF families such as WRKY and MYB can orchestrate regulatory networks underlying salt stress [31,32]. WRKYs and MYBs have been suggested to regulate multicomponent signaling pathways including but not limited to ion transport, antioxidant response, and hormone signaling pathway to modulate plant tolerance to salinity [31,32]. The modulation of $Na^+$, chloride ($Cl^-$), and $K^+$ transport is a key trait to promote plant salinity tolerance [53]. The $K^+$ uptake transporter HKT [54], SALT OVERLY SENSITIVEs (SOSs) protein kinase [55], and NHX proteins [56] play an important role to maintain ion homeostasis under salt stress conditions. The MYB and WRKY have been reported to modulate ion homeostasis under salt stress [31,32]. For example, *Arabidopsis wrky8* mutant accumulated less $K^+$ and more $Na^+$ by regulating *SOS*s gene expression under salt stress conditions [33]. Our transcriptome and qRT-PCR data showed that *CsaV3_7G030110* (homolog of *AtWRKY8*) was induced by CsCEP3 peptide upon salt treatment, although its expression was not significantly stimulated by salt (Figure 8), indicating that CsCEP3

peptide may potentially reduce $Na^+$ accumulation in cucumber primary root by regulating SOSs pathway. In addition, the salt-responsive WRKYs and MYBs can also activate or suppress redox homeostasis and ABA signaling pathway [31,32]. Our data showed that these salt-responsive WRKYs and MYBs (Figure 8), ROS production genes (Figure 4), and ABA pathway genes (Figure 7) were differentially regulated by salt stress and CsCEP3 peptide, indicating that CsCEP3 peptide may integrate complex signaling pathways to optimize cucumber primary root growth under salt stress. However, the precise mechanisms require more detailed investigations in the future with cucumber genetic mutants.

## 5. Conclusions

In this study, we combined physiological and transcriptomic analysis to investigate how synthetic CsCEP3 peptide regulated cucumber primary root growth under salt stress. Our data indicated that CsCEP3 peptide positively promotes cucumber primary root growth under salt stress by regulating the gene expression of ROS production, ABA signaling pathway, and salt-responsive TFs. Taken together, our work would provide valuable information and novel strategies for future salt resistance cultivation and high cucumber yield.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9080921/s1, Figure S1: CsCEP1a and CsCEP4 peptide show no effect on cucumber primary root growth under 100 mM NaCl treatment; Figure S2: Protein alignment of *Arabidopsis* CEPR1 and CEPR2 receptors with their potential homologous in cucumber; Table S1: qRT-PCR primers used in this study; Table S2: DEGs identified in Control vs. NaCl group; Table S3: DEGs identified in Control vs. CsCEP3 peptide group; Table S4: DEGs identified in NaCl vs. CsCEP3 peptide group; Table S5: DEGs identified in CsCEP3 + NaCl vs. CsCEP3 peptide group; Table S6: DEGs identified in CsCEP3 + NaCl vs. NaCl group; Table S7: DEGs identified in CsCEP3 + NaCl vs. Control group.

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**Data Availability Statement:** The DEGs are shown in Tables S2–S7, and the RNA-seq raw data can be obtained from corresponding author upon request.

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