Application of GC7 to reduce hypusination via inhibiting deoxyhypusine synthase in *Arabidopsis thaliana* seedlings exposed salt stress

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A R T I C L E I N F O

A B S T R A C T

Salt stress is one of the most harmful stress types affecting our agriculture and food safety. Despite the extensive knowledge of plant responses induced by salt stress, the details of the regulation of metabolite-dependent posttranslational modifications occurring during salt stress have rarely been investigated. Hypusination is polyamine-dependent posttranslational modification of eukaryotic translation factor 5A (eIF5A), which is conserved in all living organisms. Some evidence suggests that this modification plays an important role in salt stress tolerance, but the significance of the enzymes participating in the synthesis of hypusine is unknown. GC7 (N1-guanyl-1,7-diaminoheptane) is a special compound which can inhibit the enzyme activity of deoxyhypusine synthase (DHS). In our study, we applied GC7 in order to examine the applicability and efficiency of this compound and to decipher the modulation of polyamine metabolism during salt stress in plants. Our results support the significance of DHS during salt stress and of GC7 as an efficient tool to study hypusination in plant species.

1. Introduction

Salt stress is one of the most powerful stress factors globally, which causes soil degradation threatening our food safety affecting 833 million hectares of land and 1.5 billion inhabitants (FAO, 2021; Negacz et al., 2022). Climate change conditions could be enhancing the significance of the alleviation of salt stress, therefore cultivation of salt-tolerant crops on salt-affected land could be one of the sustainable solutions. Despite the extensive knowledge of plant responses to salt stress, it is an urgent task to elucidate the details of protein translation in plants during salt stress.

Hypusine, a rare polyamine-derived amino acid is critical for eukaryotic translation (Park et al., 2018)and hypusination a so-called metabolic posttranslational modification (PTM) which needs spermidine, one of the main free polyamines, essential in all living organisms (Park, 2006; Park et al., 2010).. The eukaryotic translation factor eIF5A is the only protein known to be activated by the posttranslational modification of a specific lysine residue to hypusine through hypusination, a process consisting of two steps catalysed by deoxyhypusine synthase (DHS, EC 2.5.1.46.) and deoxyhypusine hydroxylase (DOHH, EC 1.14.99.29.) The first step is catalysed by DHS is reversible while the DOHH mediated second step is irreversible providing the hypusine

amino acid (Dever et al., 2014; Pálfi et al., 2021). Polyamines (PAs) are essential N-containing molecules with versatile functions not only in growth and development but also in stress responses (Alcázar et al., 2010; Chen et al., 2019). The main PAs are putrescine (Put), spermidine (Spd), and spermine (Spm). Spd has a special role in hypusination: its presence is crucial for the first step of the biosynthesis of hypusine, when DHS adds the aminobutyl moiety of Spd to the eIF5A precursor protein. The axis of Spd/eIF5A is involved in plant growth and development (Belda-Palazón et al., 2016) and scant evidence also suggests its role in stress responses. The level of Spd and the other PAs could affect the success of hypusination, however the precise mechanism is not well understood. There is evidence to suggest that eIF5A promotes the translation of some rare motifs, for example the polyproline motif by alleviating ribosome stalling (Gutierrez et al., 2013). The frequencies of PPP (Pro-Pro-Pro) and PPG (Pro-Pro-Gly) motifs are demonstrated to be higher in multicellular eukaryotes like plants than in unicellular organisms (Wolff et al., 2007; Mandal et al., 2014) indicating phylogenetically that hypusination could contribute to the better adaptation of plants to changing conditions. eIF5A was also shown to be involved in many processes from cell division to cell death (Thompson et al., 2004). eIF5A has some isoforms in plants; for example, in *Arabidopsis thaliana* there are three isoforms of eIF5A (Thompson et al., 2004). These

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isoforms are expressed under different conditions: eIF5A-1 and eIF5A-2 are induced during xylem formation and by pathogen infection (Hopkins et al., 2008), whereas eIF5A-3 is known to be expressed during osmotic and nutrient stress (reviewed by Pálfi et al., 2021). eIF5A-2 plays a crucial role in plant growth and development by regulating cell division, cell growth and cell death (Feng et al., 2007). RceIF5A from *Rosa chinensis* can enhance the thermotolerance and the oxidative and osmotic stress resistance of *A. thaliana* (Xu et al., 2011).

Some studies suggest that modulation of hypusination can be conducted by reducing DHS activity, the reversible step of hypusination. Suppressing DHS expression induced pleiotropic effects in *A. thaliana* (Wang et al., 2001, 2003). Besides vegetative growth, the reproductive system was also affected by antisense suppression of DHS in tomato resulting in delayed fruit softening and altered growth and development (Wang et al., 2005). There is some evidence on the salt stress-mediated responses of plant hypusination (Maršálová et al., 2016; Belda-Palazón et al., 2016); however, the role of hypusination in salt stress responses of plants is not well known.

To examine hypusination processes in plants, it is a widely used method to apply pharmacological inhibitors; however, it is very important to investigate the efficiency of these inhibitor compounds during stress conditions. We have selected GC7 (N1-guanyl-1,7-diaminoheptane), a specific inhibitor compound targeting the Spd-binding site of DHS, frequently used in animal organisms for inhibiting hypusination. In this study, some biochemical parameters are examined to describe a mechanism that supports the inhibition of hypusination by GC7 without any crucial drastic damage. We propose that GC7 could be effective to inhibit salt stress-induced hypusination, therefore these results could highlight an opportunity to examine the significance of hypusination in abiotic stress tolerance in plants.

2. Materials and methods

2.1. Plant material and experimental design

The *Arabidopsis thaliana* Columbia-0 ecotype was used for the experiments. Seeds were provided by Edit Horváth (Department of Plant Biology, University of Szeged). Seeds were surface sterilized with 70 % (v/v) ethanol and 20 % bleach (v/v) solution. After washing with distilled water, the seeds were stored at 4 °C for 1 day. Seedlings were grown in the greenhouse of the Department of Plant Biology, University of Szeged. Seeds were planted on 0.5 × Murashige and Skoog agar (0.8 %) medium with the addition of 0.5 % sucrose (w/v) (pH adjusted to 5.5 with NaOH) in plastic Petri dishes with ten seeds per Petri dish in a single line, positioned vertically (Marik et al., 2019). Plants were grown for 7 days in a controlled environment under 200 μmol m^{-2} s^{-1} photon flux density (F36W/GRO lamps, OSRAM SYLVANIA, Danvers, MA, USA) with a 12/12-h light/dark period, day/night temperatures of 24/22 °C and a relative humidity of 55–60 %. Treatments were conducted in the growing medium of the 1-week-old seedlings, GC7 was applied at 1 mM, and NaCl was added to the growing medium at a concentration of 100 mM.

2.2. Biomass

The biomass of plants grown under various treatments was measured by an analytical scale (Adam Equipment NBL2541, Milton Keynes, United Kingdom); for further biochemical analyses, samples were kept at −20 °C. Growth of the seedlings was monitored by image capture by Canon EOS 700D, Canon, United Kingdom. Images of the Petri dishes were saved in jpeg format, and root lengths were analysed by ImageJ software ver. 1.52a (National Institute of Mental Health, Bethesda, Maryland, USA) (http://imagej.nih.gov/ij).

2.3. Chlorophyll content

Photosynthetic pigments were determined by the method of Faragó et al. (2018). Fresh plant tissues were ground in ethanol and the homogenate was centrifuged (Eppendorf centrifuge 5424R, Eppendorf GMBH, Germany) at 12.000 rpm, 4 °C, for 10 min. The supernatants were analysed at wavelengths of 664, 648, and 470 nm (Synergy HTX plate reader, BioTek Instruments, Winooski, VT, USA). Pigment contents were normalized for 1 mg fresh weight (Faragó et al., 2018).

2.4. Soluble protein analysis

Homogenization of plant tissues and analysis of total soluble protein contents were performed by the method of Bradford (1976). Samples were ground in ice-cold phosphate buffer (KH$_2$PO$_4$ and Na$_2$HPO$_4$, 50 mM, pH 7.0) and centrifuged in an Eppendorf centrifuge (Eppendorf 5424R, Eppendorf GMBH, Germany) for 10 min at 4 °C. After adding Bradford reagent, the extinction of the supernatant was measured at 595 nm (Synergy HTX plate reader, BioTek Instruments, Winooski, VT, USA).

2.5. Hydrogen peroxide analysis

H$_2$O$_2$ levels of tomato leaf and root tissues were measured according to Horváth et al. (2015). A total of 100 mg of sample tissue was homogenized in 0.75 mL of ice-cold, 0.1 % trichloroacetic acid. After centrifugation, 0.5 mL of supernatant was used for the measurement. The reaction mixture contained 0.5 mL 50 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The reaction was started by the addition of the supernatant followed by incubation for 10 min at 25 °C. The absorbance values were recorded by spectrophotometer (KONTRON, Milano, Italy) at 390 nm. A standard curve was prepared using the H$_2$O$_2$ standard. The results were expressed as μmol H$_2$O$_2$ g^{-1} FW.

2.6. Microscopic analysis of reactive species

NO was detected with a specific fluorescent dye, 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM DA) (Sigma-Aldrich, St. Louis, MO, USA); the superoxide anion (O$_2^-$) was visualized using 10 μM dihydroethidium (DHE) solution (Szepesi et al., 2022b), and hydrogen peroxide (H$_2$O$_2$) was visualized using 50 μM Amplex Red solution (Lehotai et al., 2012). Fluorescence intensity was detected by a Zeiss Axiowert 200 M-type fluorescent microscope (Carl Zeiss Inc., Jena, Germany) equipped with an objective × 10. Digital photographs of the samples were taken with a high-resolution digital camera (Axiocam HR, HQ CCD camera; Carl Zeiss Inc., Jena, Germany) with a filter set 10 (excitation 450–495 nm, emission 515–565 nm). Fluorescence intensities (pixel intensity) of root tips were measured on digital images within circular areas of 100 μm radii using Axiovision Rel. 4.8 software.

2.7. Free polyamine analysis by HPLC

The levels of free Put, Spd, and Spm were determined by high-performance liquid chromatography (HPLC) separation of benzoyl-derivatized PAs, as described by Szepesi et al. (2022a). After homogenization in 5 % (v/v) perchloric acid, samples were centrifuged and the supernatant was neutralized with 2 M NaOH. Then, the PAs were derivatized with benzoyl chloride to produce benzoyl-polyamines. Diethyl ether was added to the aqueous phase to obtain organic phase for drying in a vacuum evaporator. Acetonitrile was pipetted onto the dry sample and injected into the JASCO HPLC system (JASCO, Tokyo, Japan). Separation was made in a reverse-phase C18 column (250 × 4.6 mm internal diameter, 5 μm particle size; Phenomenex, Torrance, CA, USA). Detection of benzoyl-PAs was made by UV/VIS detector (JASCO HPLC System, Japan) at a wavelength 254 nm. The mobile phase was

water/acetonitrile in 55:45 (v:v) ratio with a flow rate of 0.5 mL min^{-1}. The applied standards were Put, Spd, and Spm in the form of hydrochlorides from Sigma-Aldrich, Merck GMBH, Germany. The results are the means of three independent biological samples expressed in μmol g^{-1} fresh weight^{-1}.

2.8. Western blot detection of protein abundance of enzymes involved in hypusination

Arabidopsis seedlings were ground with double amount of extraction buffer (50 mM Tris-HCl, pH 7.6–7.8, containing 0.1 % (v/v) Triton X-100, 0.1 mM EDTA and 10 % (v/v) glycerol). The homogenised sample was centrifuged at 9300 rpm for 20 min on 4 °C. The supernatant was treated with 1 % (v/v) plant protease inhibitor cocktail (Sigma-Aldrich, cat. No. P9599), and used in further experiments.

The denatured sample containing 12.5 μg protein was subjected to SDS-PAGE on 12 % (w/v) acrylamide gels (Oláh et al., 2020). Separated proteins were transferred to PVDF membrane using wet blotting technique (25 mA for 16 h). Membranes were blocked in 5 % (w/v) non-fat milk blocking solution, prepared in TBS-T buffer (20 mM Tris-HCl, pH 7.8, 178 mM NaCl, 0.05 % (v/v) Tween 20). To detect each protein, the primary antibody was set accordingly (DHS-Abcam, cat. No. SAB4560654, DOHH- Sigma-Aldrich, cat. No. HAP041953) in a 1:2000 dilution. As loading control, anti-actin antibody (Agrisera, AS 13 2640) was used. Goat anti rabbit secondary antibody (Sigma-Aldrich, cat. No. A3687) was used in 1:10.000 dilution. Proteins were visualised using the NBT/BCIP reaction and by chemiluminescence method by Meng et al. (2013).

2.9. Statistical analysis

The experiments were repeated three times. Statistical analysis of two-way analysis of variance (ANOVA) was carried out using GraphPad Prism version 8.0.1.244 for Windows (GraphPad Software, La Jolla, CA, USA). Different letters on the bars denote significant differences ($p < 0.05$) based on Tukey's post hoc test for multiple comparisons.

3. Results

3.1. Morphological parameters

We first assessed GC7 impact on some morphological attributes of *Arabidopsis* plants subjected to salt stress induced by 100 mM NaCl. As expected, NaCl treatment caused severe stress to plants, as a significant decrease of biomass was observed compared to the control plants (Fig.1).

We checked that GC7 could affect biomass production during salt stress. We could conclude that GC7 significantly decreased the biomass in control plants but not in salt-treated seedlings (Fig. 2). Herein GC7 did not appear to alter biomass under stress conditions.

3.2. Root system

Investigating the root system, it can be seen that NaCl severely affected the growth of seedlings, causing a drastic growth reduction of rosettes and shorter roots. After application of GC7, the primary root (PR) length and lateral root (LR) numbers did not show any significant difference in NaCl-treated seedlings (Fig.3).

Collectively, it can be concluded that GC7 was not effective to mitigate the salt stress induced root growth reduction in Arabidopsis seedlings.

3.3. Biochemical parameters

In comparison to the control, there was a reduction in protein levels after NaCl treatment. GC7 application did not induce any alterations
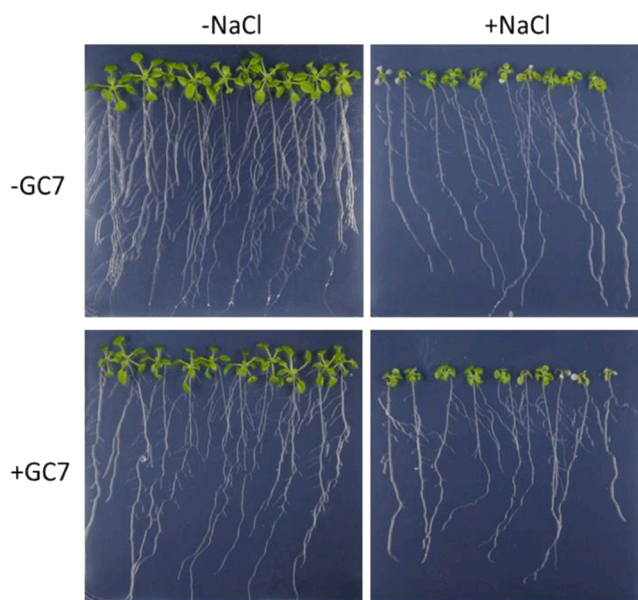

Fig. 1. Representative image of GC7- and NaCl-treated *Arabidopsis thaliana* seedlings after 1 week of treatment. Concentration of GC7 was 1 mM, and NaCl was applied at 100 mM for one week.
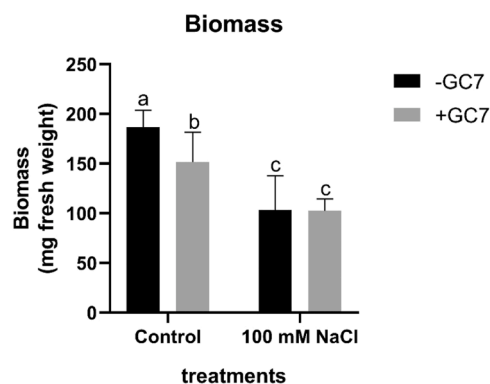

Fig. 2. Biomass production of NaCl- and GC7-treated *Arabidopsis thaliana* seedlings. Mean±SD, $n = 3$.

with or without NaCl treatment. These results demonstrated that GC7 application did not affect the protein levels in plants (Fig. 4).

We then evaluated the impact of GC7 treatment on chlorophyll contents, which are important pigments for photosynthesis in plants. GC7 treatment was effective to reduce pigment contents in all cases, more significantly in the case of NaCl-treated plants (Fig. 5).

Salt stress is known to induce oxidative stress, thus we evaluated H_2O_2 levels as a related stress marker, H_2O_2 being one of the most important reactive oxygen species in plants.

GC7 treatment could reduce H_2O_2 levels with or without NaCl stress treatment, indicating that reduction in hypusination could affect ROS production in plants under stress conditions (Fig. 6).

3.4. Microscopic analysis of reactive species

Salt stress induces imbalance of reactive oxygen and nitrogen species in plants. In order to identify the origin of reactive species, we conducted fluorescent microscopic analysis. First, superoxide anion content was observed in root tips. As expected, NaCl treatment induced enhancement of the superoxide level. In untreated root tips, GC7 caused a significant increase of superoxide levels, but in the salt-treated root tips GC7 did not
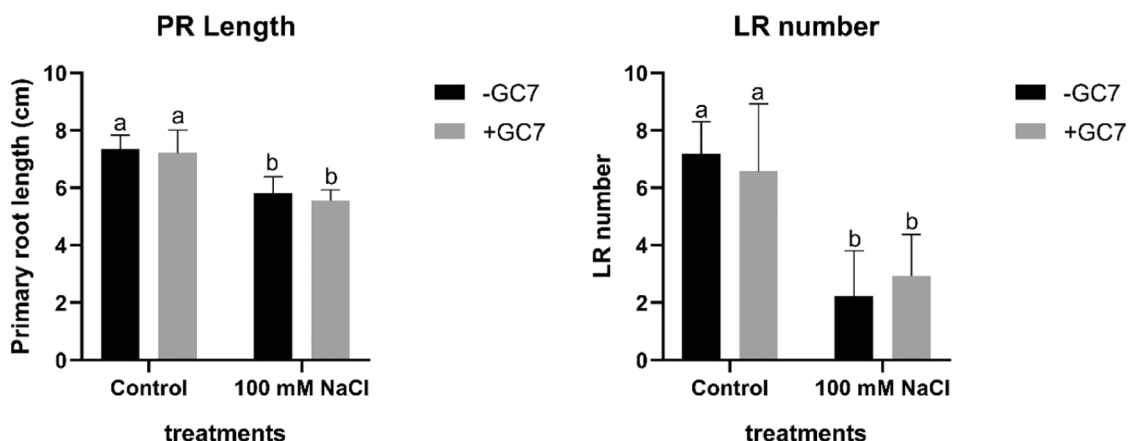
PR Length

LR number

Fig. 3. Effect of GC7 and NaCl on root system of *Arabidopsis thaliana* seedlings. Data of primary root length and number of lateral roots are the mean ± SD, at least three biological replicates, $n = 25$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$).
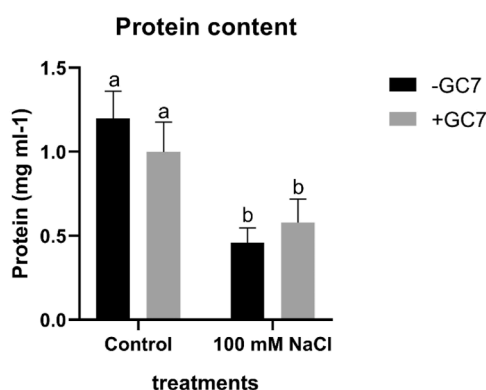
Protein content

Fig. 4. Effect of GC7 and NaCl on protein content of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 3$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$).

alleviate this increment (Fig. 7).

H_2O_2 is generated from superoxide anions in different ways. GC7 was effective to reduce H_2O_2 levels in root tips after salt stress, alleviating the salt stress induced increment (Fig. 8).

Because NO and polyamines share the same biosynthetic pathways, we evaluated the effect of GC7 on NO levels. GC7 alone was effective to increase NO levels in root tips; however, GC7 treatment did not have an impact on NO levels in the root tips of salt-stressed plants. Our results suggest that NO metabolism is not affected by reduction in hypusination in plants during salt stress (Fig. 9).

3.5. Free polyamine spectra

Hypusination is strongly connected to PA content, especially to Spd content. To evaluate the effect of GC7 on polyamine levels, we evaluated free polyamine contents after salt stress by HPLC. No difference was observed in Put and Spm levels in GC7-treated control plants, only Spd increased after GC7. In plants exposed to salt stress, GC7 treatment caused a significant increase in polyamine levels such as Spd and Spm, whereas Put levels diminished (Fig. 10).

3.6. Western blot analysis of enzymes involved in hypusination

We performed western blot evaluation of the protein levels of two enzymes involved in hypusination, namely DHS and DOHH. DHS catalyses the first step, when deoxyhypusinated intermedier is synthesized, whereas DOHH is responsible for the second, irreversible step to hypusine biosynthesis for activation of eIF5A. In NaCl-treated plants, we observed significant inhibition of the levels of both proteins with or without GC7 treatment, whereas in the case of GC7 alone, only the level of the DHS protein showed a slight decrease compared to the control (Fig. 11).

4. Discussion

Salt stress is one of the most crucial stress conditions, which could drastically affect our food safety and food production (FAO, 2021). To investigate the processes involved in protein synthesis during salt stress is of high importance for enhancing the stress tolerance of our crop plants. Hypusination, which is a PA-dependent posttranscriptional modification of eIF5A, can contribute to stress tolerance in plants (Pálfi
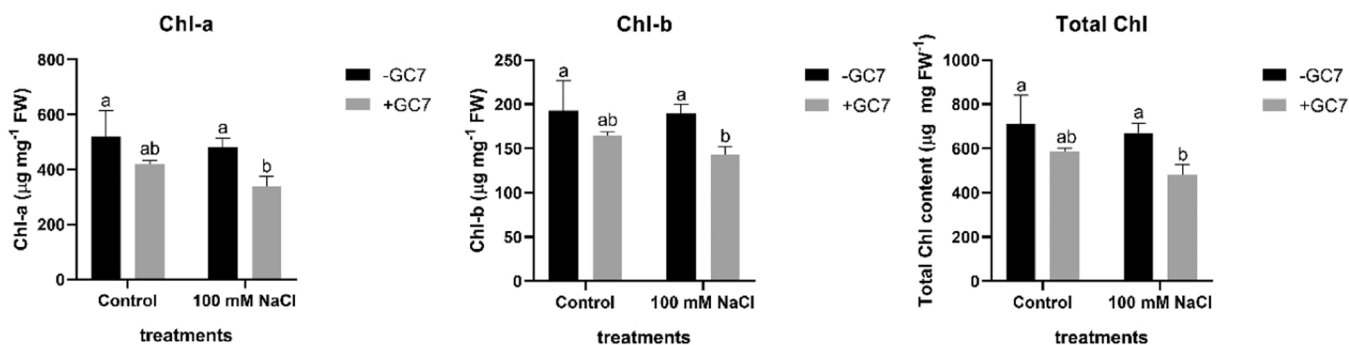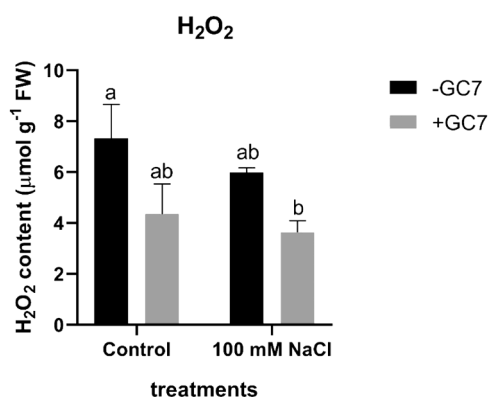
Chl-a

Chl-b

Total Chl

Fig. 5. Effect of GC7 and NaCl on chlorophyll content of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 4$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$).

Fig. 6. Effect of GC7 and NaCl on H_2O_2 content of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 3$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$).

et al., 2021); however, only scant results are available. Pharmacological inhibition of hypusination could be useful for examining the involvement of hypusination in salt stress in plants. Using the model plant *Arabidopsis thaliana*, we demonstrate that the using eIF5A hypusination inhibitor GC7 could be effective tool for inhibiting hypusination in plants.

GC7 application caused a decrease in biomass, but in NaCl-treated plants GC7 did not induce noticeable effects (Figs. 1, 2). As the suggested role for GC7 is to reduce DHS activity, our results suggest that GC7 could induce these effects in another way, because suppression of DHS in plants induced growth enhancement and bigger leaves (Wang et al., 2003; Duguay et al., 2007). It is important to note that this DHS suppression was examined during drought stress, suggesting that reducing hypusination by decreased DHS activity could be dependent on stress conditions.

Optimal growth of the root system during salt stress is crucial for surviving these harsh conditions. GC7 did not cause any reduction in PR length (Fig. 3). However, combined with NaCl stress, GC7 was not effective to improve root growth, remaining questionable that how insufficient hypusination during salt stress could affect the root system.

To further investigate the effect of GC7, we performed an analysis of protein contents. GC7 was also shown to not inhibit protein synthesis with or without NaCl treatment (Fig. 4). The data of protein contents suggests that the effect of GC7 was not toxic for *Arabidopsis* plants in this concentration.

There is some evidence demonstrating that hypusination is involved in photosynthetic mechanisms (Wang et al., 2012). Liu et al. (2019) showed that PhDHS is involved in chloroplast development in petunia, suggesting the strong involvement of hypusination in the efficiency of photosynthesis in plants. Suppression of DHS caused abnormal chloroplast development. We observed here that GC7 was effective to reduce chlorophyll content in control and NaCl-treated plants as well (Fig. 5). It
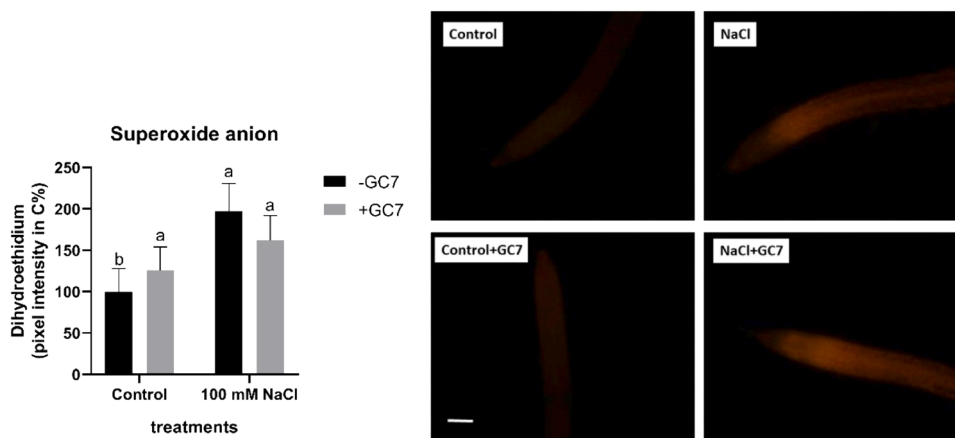
Fig. 7. Effect of GC7 and NaCl on H_2O_2 levels of root tips of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 10$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$). Scale bar=100 μm.
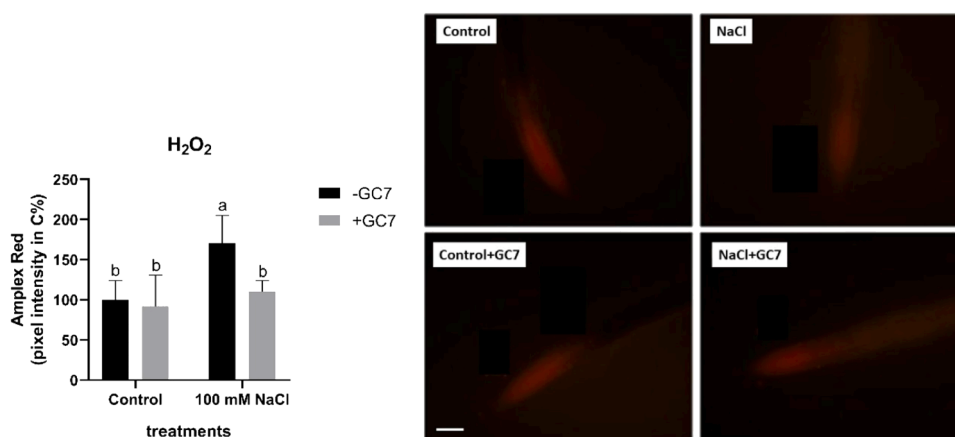
Fig. 8. Effect of GC7 and NaCl on H_2O_2 levels of root tips of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 10$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$). Scale bar=100 μm.
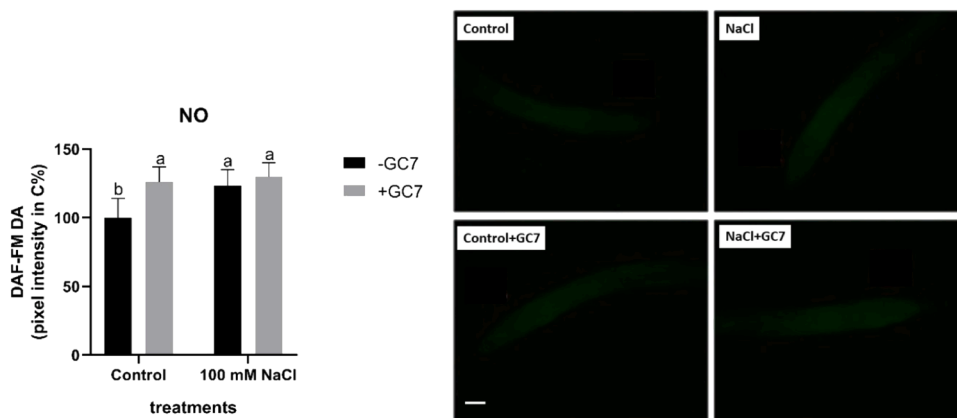
Fig. 9. Effect of GC7 and NaCl on NO levels of root tips of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 10$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$). Scale bar=100 μm.
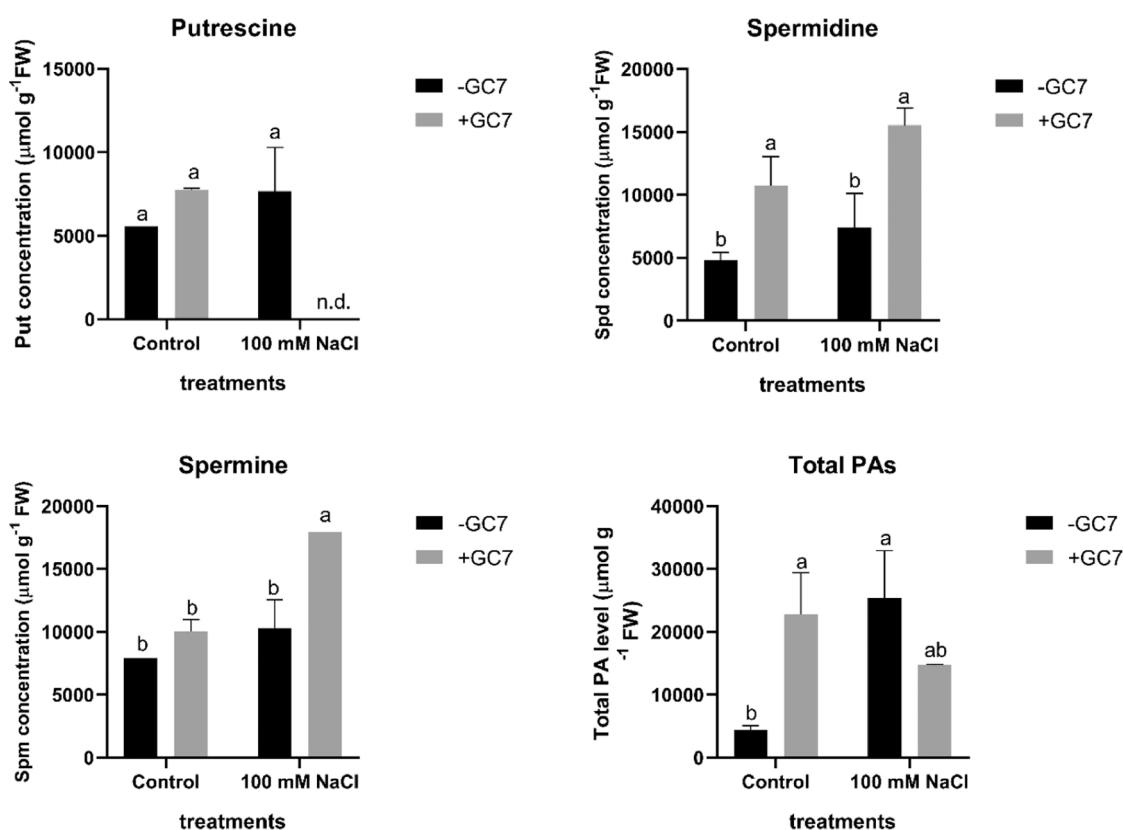

Fig. 10. Effect of GC7 and NaCl on free polyamine levels of *Arabidopsis thaliana* seedlings. Data are the mean ± SD, at least three biological replicates, $n = 3$. Different letters denote significant differences (two-way ANOVA, Tukey's post hoc test, $p < 0.05$).

is important to note that in the case of NaCl treatment, GC7 reduced chlorophyll content to a higher extent, suggesting that sufficient hypusination is important for efficient photosynthesis during salt stress (Wang et al., 2012).

It has been reported in animal organisms that GC7 has some antioxidant properties beside reducing DHS activity (Oliveiro et al., 2014). To elucidate its role in ROS scavenging, we tested its antioxidant properties in plants. As salt stress induces oxidative stress, we evaluated the level of H_2O_2. GC7 was able to reduce H_2O_2 levels in control plants, but after salt stress its effect was more pronounced (Fig. 6), indicating its antioxidant effect. In order to understand the origin of this ROS, we observed other reactive species by fluorescent microscopic analysis. An increase of superoxide anion level was found in GC7-treated control

plants; however, after salt stress there were no significant alterations (Fig. 7). This was accompanied by reduced H_2O_2 level (Fig. 8), suggesting that GC7 induced the antioxidant defense system in these plants; however, in order to fully explain these effects, the other antioxidant components should be identified.

The suppressive effect of ROS production might contribute to its ameliorating effect against salt stress, providing a plausible explanation for the protective effect of hypusination.

L-arginine amino acid could be a precursor of both PAs and intracellular messenger NO in plants. Our hypothesis was that by reducing DHS in hypusination, more Spd should be synthesized and therefore more NO could be produced. In control plants, the NO level increased after GC7 application, but during salt stress there was no difference
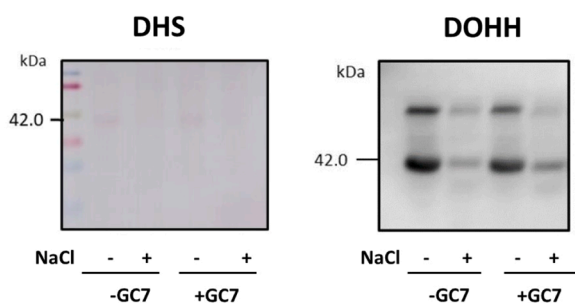
Fig. 11. Western blot analysis of protein levels of DHS and DOHH after GC7 and NaCl treatment of *Arabidopsis thaliana* seedlings.

Fig. 12. Schematic model of a proposed effect of GC7 on the interaction between polyamine metabolism and hypusination in plants. Spd is essential to synthesize the deoxyhypusine intermediate from precursor eIF5A by DHS. Effects of GC7 on DHS are indicated in red: inhibiting DHS, the first reversible step of hypusination results in an increased Spd level in plants. This increasing could affect positively the PA metabolism, while the level of hypusination could be alleviated by inhibiting DHS activity. The influence of increased PA levels on hypusination process remains to be studied further in plants. Abbreviations used in the figure are as follows: Put, putrescine; Spd, spermidine; Spm, spermine; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase.

between GC7- and NaCl-treated plants (Fig. 9). The results of this study are the first evidence that imbalance of hypusination could induce alterations of NO levels in plants. More experiments are needed to investigate how GC7 treatment may improve the superoxide/NO ratio during salt stress.

Polyamines are known to have a crucial role in growth and development and also in abiotic stress responses (Alcázar et al., 2020). Spd is involved in hypusination as a substrate for deoxyhypusine synthesis by DHS. To examine whether GC7 could affect the level of this substrate for hypusination, we conducted HPLC analysis of free PAs. Our hypothesis was that reducing DHS activity will increase the level of Spd. Under control conditions, only Spd showed higher levels compared to the control after GC7 treatment, but in the case of NaCl-treated plants, Spd and Spm increased, whereas Put decreased (Fig. 10). GC7 treatment could be effective to synthesize higher PAs, such as Spd and Spm presumably from Put during salt stress. Our results suggest that pharmacological interference with the polyamine-eIF5A-hypusine axis by GC7 might have a novel role in investigating hypusination in plants. While our results on free PAs' biosynthesis reveal the potential of GC7 to shift PA synthesis to more effective higher PAs, it would be interesting to measure other types of PAs such as conjugated or bound PAs under salt stress conditions.

In order to provide evidence about the relevance of impaired hypusination, we analysed by western blotting the protein levels of both enzymes involved in hypusination. The inhibition of eIF5A hypusination by GC7 demonstrated that not only DHS was affected but also the other enzyme, DOHH. In the presence of NaCl, DOHH had lower protein levels, suggesting the effect of stress (Fig. 11). All observed changes suggest that reduction of hypusination by GC7 did not alleviate the effect of salt stress. Also, future studies should clarify how GC7 could affect not only DHS but also DOHH in plants.
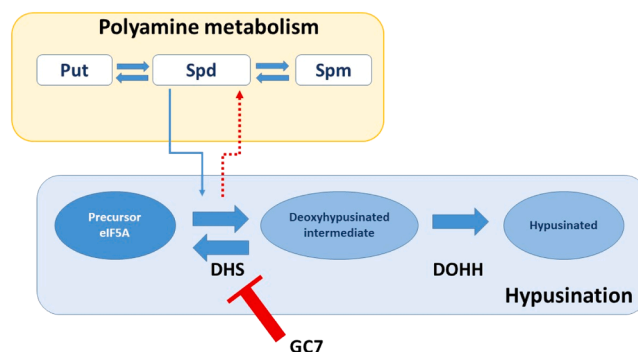
In the future, targeting eIF5A should be under investigation for treating salt stress in plants in order to clarify whether reducing hypusination could alleviate salt stress (Fig.12).

For example, to investigate the hormonal aspects of hypusination could enhance our knowledge about protein synthesis during salt stress. An interesting connection is the ABA-dependent regulation of eIF5A hypusination in *Arabidopsis thaliana* (Belda-Palazón et al., 2014). Also, designing and testing new inhibitor compounds in plants could identify some specific effects of plant hypusination. New theories of binding mechanisms of GC7 to DHS in other model organisms could contribute to designing new inhibitors of hypusination (D'Agostino et al., 2020).

The effect of GC7 should be tested in plant cultivars with different salt sensitivities. The different isoforms in *Hordeum* species were examined by Maršálová et al. (2016), who compared the proteomic responses of *Hordeum vulgare* species with different salt tolerances.

5. Conclusion

Hypusination is a Spd-dependent posttranslational modification of eIF5A translation factor, which has significant role in growth and

development as well as in stress responses, however its precise role during salt stress remains to be explored. GC7 pharmacological treatment seems to be a promising alleviating approach to reduce hypusination by inhibiting DHS, the enzyme responsible for the first step of hypusination in plants to limit salt stress induced damages. Our study provides new insight into GC7-induced effects in plants during salt stress, providing evidence that GC7 treatment enhanced the antioxidant defense mechanism before and after salt stress. As GC7 decreased not only the level of DHS protein but also that of DOHH, we can speculate that DOHH could be affected by an unknown manner in plants, but this assumption needs further studies. These findings related to hypusination could be potentially applicable for salt tolerance strategies. Therefore, further studies are needed to be conducted to get knowledge about the benefits of GC7 in cultivars with different salt sensitivity.

CRediT authorship contribution statement

Ágnes Szepesi: Conceptualization, Methodology, Formal analysis, Writing – original draft, Funding acquisition. **Edina Kakas:** Methodology. **Réka Szőllősi:** Investigation, Software, Writing-original draft, Writing-review & editing. **Árpád Molnár:** Methodology. **Péter Pálfi:** Writing – review, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Sípos Master student from the University of Szeged.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2023.100257.

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