Comparative study of salt stress-induced physiological and molecular responses in tomato (*Solanum lycopersicum* L.)

Ph.D. Dissertation

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Introduction

Plants perceive and respond rapidly to environmental changes. They have evolved complex physiological, biochemical and molecular mechanisms to adapt to a variety of stresses. Plant stress can be divided into two groups depending on the origin. Abiotic stress is a physical (e.g. light, temperature and water) or chemical (e.g. salinity, heavy metals) insult in contrast to biotic stress, which is caused by living organisms (e.g. bacteria, fungus and insects).

High salinity is one of the major environmental stress factors globally that reduces growth and significantly limits crop productivity. The cultivated tomato, *S. lycopersicum*, is a widely-grown crop plant and its production is concentrated in warm and dry areas where high level of salinity appeared in the soil or in irrigation water causes serious damages in tomato production. Therefore more attention to salinity is required in the production of tomato.

Response to salt stress is a complex phenomenon and involves morphological and physiological changes. Salinity has two major effects: an early osmotic stress caused by the high salt concentration in soil solution and ionic stress caused by high salt concentration within the cytoplasm of plants cells which is expressed after a longer period. As a result of salt stress, K^+ content can dramatically and irreversibly fall in the roots. This reaction is accompanied ROS generation and protease activation. Interestingly, Ca^{2+} elevation, ROS generation, reduction of cytoplasmic K^+/Na^+ ratio and activation of proteases together establish a clear system resulting in PCD.

Plant hormones are central integrators of the growth and development and they are able to control the interaction between plants and environments, including plant responses to salt stress. The phytohormone, ABA plays vital role in alleviating salt stress in different ways. ABA is produced under water-deficit conditions and it regulates plant water balance and osmotic stress tolerance via stomatal regulation. Moreover little is known about the relation between ABA and cysteine proteases, however, molecular and biochemical data confirmed that ABA suppressed GA-induced PCD inhibition via suppression of cysteine protease, SlCysEP.

PCD is a well-defined, genetically controlled process and it plays a critical role in response to stress. PCD often includes protein degradation, mediated by protease- or proteasome-based degradation pathways. There are two major systems for intracellular protein degradation: (a) protease pathway or (b) proteasome-based degradation pathway.

Proteases are key regulators controlling different developmental processes and response to stress by maintaining strict protein quality control and degrading specific sets of proteins. However, they are involved in most cellular processes, still very little is known about their substrate specificity, physiological roles or cellular location of many of putative proteases. We investigated cysteine (Cys) protease family which is the largest protease family besides serine hydrolases. Some types of PCD are regulated by vacuolar processing enzymes (VPEs), and by papain-like Cys proteases (PLCPs).

The 26S proteasome is a highly conserved protein complex consists of a catalytic 20S core particle and a 19S regulatory particle which has a crucial role in selective protein degradation during cell death and development. The active sites are in the central chamber of 20S core particle, residing in three catalytic subunits: β1, β2 and β5.

Protein degradation was intensively studied in the past decades and nowadays powerful tools are available to broaden our knowledge, especially under high salinity since salt stress-induced protein degradation is still less explored.

Objectives

Our aim is to investigate the main differences between the effects of sublethal (100 mM NaCl) and lethal (250 mM NaCl) salt stress on tomato plants. We are interested in the physiological and molecular changes depending on whether the plants survive or salt stress induces PCD. In addition, contribution of ABA deficiency to salt stress sensitivity was studied in *sitiens* mutants deficient in ABA biosynthesis and thus in ABA content focusing on the early events of PCD.

We focused on the following questions:

1. How do different tissues react to salt stress? What are the differences between responses of roots and leaves upon early stages of low- or high salt stress?

2. Are there any effects of salt stress on the two main protein degradation pathways: Cys protease and proteasome activity in tomato? How does protein turnover and protease activity change upon the different salt treatments?

3. How does ABA deficiency modify the sensitivity of tomato roots to salt stress? Which physiological or molecular responses will change in *sitiens* roots under control and different salt stress conditions?

4. Does ABA deficiency affect protease activity?

Materials and methods

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum* L. cv. "*Rio Fuego*") and *sitiens* mutant (*Solanum lycopersicum* cv. "*Rheinland*") plants were germinated at 26 ^{o}C for 3 days in the dark, and the seedlings were subsequently transferred to perlite for 2 weeks. Plants were grown hydroponically in a controlled environment in a greenhouse (300 µmol m^{-2} s^{-1} photon flux density with 12/12 light/dark photoperiod, 25^{o}C, and 55-60% relative humidity) for 3 weeks. Tomato plants were treated with 0-, 100- and 250 mM NaCl in the nutrient solution (2 mM $Ca(NO_3)_2$, 1mM $MgSO_4$, 0.5 mM KCl, 0.5 mM, KH_2PO_4, 0.5 mM Na_2HPO_4, 0.001 mM $MnSO_4$, 0.005 mM $ZnSO_4$, 0.0001 mM $(NH_4)_6Mo_7O_{24}$, 0.01 mM H_3BO_4, 0.02 mM Fe(III)-EDTA). Treatments were performed at 9 a.m. and samples were taken in triplicate at 1, 6 and 24 hours after salt exposure.

FDA Staining

Fluorescein diacetate (FDA) was used to determine cell viability according to Gémes et al (2011). Fluorescence intensity was detected with Zeiss Axiowert 200M type fluorescent microscope equipped with an 5X objective. Digital photographs were taken from the samples with a high-resolution digital camera using a filter set 10 (excitation 450-495 nm, emission 515-565 nm) or filter set 20HE (excitation: 535–585 nm, emission: 600–655 nm).

Electrolyte leakage and elemental analysis

Cell death was determined with the measurement of electrolyte leakage (EL) by the method of Poór et al. (2014).

Detection of ROS and NO

ROS was visualized by using 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 20 min in 10 mM MES-TRIS/KCl buffer (pH 5.8) in the dark at 37 °C and rinsed once with 10 mM MES-TRIS/KCl buffer (pH 5.8). NO production was visualized using 10 µM 4,5 diaminofluorescein-diacetate (DAF-2 DA) for 20 min in 10 mM MES-TRIS/KCl buffer (pH 5.8) in the dark at room temperature and rinsed once with 10 mM MES-TRIS/KCl buffer (pH 5.8). Fluorescence intensity was detected with Zeiss Axiowert 200 M-type fluorescent microscope equipped with an objective ×10. Digital photographs were taken from the samples with a high-resolution digital camera with a filter set 10 (excitation 450–495 nm, emission 515–565 nm). Fluorescence intensities (pixel intensity) were measured on digital images within circular areas of 45 lm radii using Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

Determination of the H_2O_2 level

The H_2O_2 level was measured spectrophotometrically as described by Horváth *et al*. (2015) with some modifications. After homogenisation of 200 mg of tissues with ice-cold 1 mL of 0.1% trichloroacetic acid (TCA), the samples were centrifuged at 10.000 g for 20 min at 4 ^{o}C. The reaction mixture contained 0.25 mL of a 10-mM phosphate buffer (pH 7.0), 0.5 mL of 1-M potassium iodide (KI) and 0.25 mL of the supernatant. The absorbance of the samples was measured after 10 min at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared from the dilution of H_2O_2 stock solution.

Determination of protein concentration

For the analysis of the soluble protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard.

Determination of proteolytic activity

Azocasein assay

Azocasein was applied as a nonspecific substrate to measure the total proteolytic activity. 50 µl of tissue extract, 300 µl 1% azocasein (w/v) and 650 µl potassium phosphate buffer (pH 5.5) were incubated at 37 ^{o}C for 2 h. The reaction was stopped by the addition of 300 µl 10% (w/v) trichloroacetic acid (TCA) at 4 ^{o}C for 20 min. After 20 min on ice, the samples were centrifuged (10 min at 4 ^{o}C, 11300 g) and the yellow color of the supernatant was measured at 440 nm. One unit of total proteolytic activity (U) was defined as the amount of enzyme yielding 0.01 unit of absorbance per min under the assay conditions.

Gelatin-SDS PAGE

20 µg/ml protein extract was loaded in each lane on 12,5% SDS PAGE containing 0,1% gelatin substrate with a 5% stacking gel overlaid. The proteins were run under denaturing conditions at 90 V for 20 min, then at 70 V for 120 min. The gels were then renatured in 2.5% Triton X-100 twice for 40 min (40 rpm, 25 ^{o}C) and developed in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$, pH 5,5) overnight at 37 ^{o}C. Gels were stained in 250 ml of 0,1% Coomassie R-250 containing 40% methanol and 10% acetic acid for 30 min and destained in 40% methanol and 10% acetic acid solution twice for 30 min. Gels were stored in distilled water till development. Areas of protease activity were revealed as cleared bands on a blue background. The images were quantified using ImageJ 1.48V.

Small-Scale Labelling Reaction

Sample preparation

Root tissue was homogenized in 50 mM Tris buffer at pH 7.5 containing 5 mM DTT for labelling of the proteasome. The extract was mixed and centrifuged at 10000 g for 10 min at 4 °C to remove cell debris and the supernatant was collected and used for labelling.

Labelling of PLCPs

Protein extract of 100 µg/ml was incubated with 0.3 µM MV201 for 4 hours at room temperature in the dark in 200 µl of total volume. Equal volumes of dimethyl sulfoxide (DMSO) were added for the no-probe-control. For inhibition assays, protein extracts were pre-incubated with 50 µM E64 or DMSO for 30 min at room temperature and these extracts were labelled with 0.3 µM MV201. The labelling reaction was stopped by precipitation using the chloroform/methanol precipitation protocol (Friedman 2007). 1 X SDS–PAGE loading buffer containing β-mercaptoethanol was added to the pellet and it was heated for 10 min at 95^{o}C. The reaction mixture was separated on 12% SDS gel at 200 V for 1 h. Labelled proteins were visualized by in-gel fluorescence scanning using a Typhoon 9400 Imager.

Labelling of proteasome subunits

Protein extract of 100 µg/ml was labelled with 2 µM MV151 for 3 h or 0.2 µM MVB072 or co-labelled with 0.8 µM LW124/MVB127 for 2 h at room temperature in the dark in 60 µl total volume. Equal volumes of DMSO were added for the no-

probe-control. For inhibition assays, extracts were pre-incubated with 50 or 100 µM epoxomicin or with one of 50 µM N3β1, N3β5 or DMSO and these extracts were labelled with the suitable probe. The labelling reactions were stopped by adding gel loading buffer containing β-mercaptoethanol and they were heated at 95 °C for 10 min. The reaction mixture was separated on 15% SDS gel at 200 V for 75 min. Labelled proteins were visualized by in-gel fluorescence scanning using a Typhoon 9400 Imager.

Large-Scale Labeling and Affinity Purification

Sample preparation for PLCPs

Root tissue was homogenized in 50 mM NaOAc buffer at pH 6.0 containing 5 mM DTT. The extract was mixed and centrifuged at 10000 g for 10 min at 4 °C to remove cell debris. Supernatant was collected and preincubated with 50 µl of high-capacity streptavidin agarose resin for 30 min at 4 °C. The mixture was centrifuged twice at 1400 g for 10 min at 4 °C. Protein extract was cleaned by passing through a 0.22 µm filter attached to a syringe.

Affinity purification

Protein extracts were separated to 500 µl aliquots containing 0.5 mg/ml protein. Labelling reaction was performed by incubating 5µM DCG-04 at room temperature for 6 h. Aliquots were collected and precipitated via the chloroform/methanol precipitation method. The pellet was dissolved in 1.2% SDS-PBS by sonication, then it was heated at 95^{o}C for 10 min and diluted to 0.2% SDS-PBS by adding 1 X PBS. The resulting solution was incubated with 100 µl of pre-equilibrated high-capacity streptavidin beads for 2 h at room temperature. The beads were collected by centrifuging at 1400 g for 5 min. Beads were washed successively three times with 5 ml of 1.2% SDS/PBS solution, three times with 5 ml of 1.0% SDS/PBS solution, and three times with 10 ml of 1 X PBS buffer. The final wash occurred with 10 ml of distilled water. The captured proteins were eluted by boiling the beads at 95 °C in 20 µl of 6 X gel loading buffer. The eluted proteins were separated on 12% protein gels at 200 V for 1 h, and the protein gels were stained overnight with SYPRO Ruby. To detect the proteins, we scanned the gels with an LPG filter using a Typhoon 9400 Imager. Specific bands were excised from the gel and subjected to in-gel tryptic digestion and subsequent MS analysis.

RNA extraction and expression analyses with qRT-PCR

The expression rates of the selected genes from tomato leaves were determined by quantitative real-time reverse transcription-PCR using SYBR green dye after the extraction of RNA from 100 mg of plant material according to Chomczynsky and Sacchi (1987) as described in Horváth et al. (2015).

IEF 2D SDS PAGE

Labelled and precipitated proteins were resuspended in UTC buffer (8M urea, 2M thiourea, 4% (w/v) 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1 g AG 501-X8 Resin) containing 1% (v/v) ampholyte and 65 mM DTT. Samples were isoelectrically focused on 7 cm immobilized pH gradient (IPG) 3–10 pH strips using BioRad PROTEAN i12 IEF system and the second dimension electrophoresis was run on a 15 % SDS gel. Gels were imaged using a Typhoon 9400 Imager using excitation and emission wavelengths of 532/580 nm. Images were quantified using ImageJ 1.48V.

In-gel digestion and MS

Bands were excised by hand and treated with trypsin as described elsewhere. Tryptic digests were desalted on home-made C18 StageTips as described by Rappsilber et al. (2007). After elution from the StageTips samples were dried using a vacuum concentrator and the peptides were taken up in 10 µL of 0.1 % formic acid solution.

LC-MS/MS experiments were performed on an Orbitrap Elite instrument that was coupled to an EASY-nLC 1000 liquid chromatography (LC) system.

PNGaseF Treatment of Labeled Proteins

9 µl of MVB072-labelled tomato root extract and Bovine Fetuin (Promega) were treated with 1 µl of 10X glycoprotein denaturing buffer and heated at 95°C for 5 minutes. The denatured proteins were chilled on ice. 2 µl 10X GlycoBuffer, 2 µl 10% NP40 and 6 µl H_2O was added to the reaction. The mixture was treated with 1 µl PNGase F or with 1 µl H_2O and incubated at 37 ^{o}C for 1 h. Samples were analyzed on 16% SDS-PAGE.

Protein Deglycosylation of Labeled Proteins

18 µl of MVB072-labelled sample and Bovine Fetuin (Promega) were treated with 2 µl of 10X denaturing solution and heated at 95°C for 10 minutes. The denatured proteins were chilled on ice for 5 min. To the denatured samples 5µl of 10X Deglycosylation Reaction Buffer, 5µl of 10% NP40 and 15µl of water were added. Samples were treated with 5µl of Protein Deglycosylation Mix and incubated at 37 ^{o}C for 8 h. Samples were analyzed on 16% SDS-PAGE.

Protein Phosphatase treatment

60 µl of MVB072-labelled sample containing 1M NaCl, 25 mM $MgCl_2$ 10 mM DTT and 12.5X protease inhibitor cocktail were treated with 1 or 5 µl of alkaline phosphatase and incubated at 37 ^{o}C for 1 h. Samples were analyzed on 16% SDS-PAGE, whereas the remainder of the sample were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane for detection of phosphorylated MAPK using primary antibody, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody and secondary Goat anti-Rabbit IgG and visualized using chemiluminescent substrates.

Bioinformatics

Genes encoding the β subunits of tomato were identified by BLASTp searches of the predicted proteome (ITAG release 2.40) for homologs of the seven Arabidopsis β subunits at the SolGenomics website. The β2a protein sequence was modeled onto polypeptide H of the structure of the yeast proteasome using Swiss Model. This β2a model was used in PyMol to replace the β2 in the structure of the yeast proteasome.

Statistics

Results are expressed as mean ± SE. Statistical analysis was performed with GraphPad using Student's t-test (*P B 0.05, **P B 0.01, ***P B 0.001).

Results and discussion

In this study the effects of sublethal- (100 mM NaCl) and lethal (250 mM NaCl) salt stress were compared. A robust and quick PCD was detected upon high salinity where robust alteration of membrane integrity, quick ROS- and NO burst and increased proteolysis were revealed in tomato. The changes were specific for different plant organs, therefore, leaf- and root tissues of tomato were studied separately.

PCD events

Salt stress has rapid and stronger effect on the viability of the root tips compared to the leaf tissue. We observed suppression of the viability of the roots also after one hour, which develops further after six hours upon high salt treatment and delayed, slight decrease of viable cells was detected upon sublethal salt treatment after 24 hours. Tomato roots appears to be more susceptible to salinity regarding both K^+ efflux and Na^+ influx even if it was treated by sublethal salt treatment after 24 hours. 250 mM salt treatment dramatically alters the Na^+ content of leaf tissue probably by transportation of excess Na^+ ions to the shoot.

Electrolyte leakage showed extreme increments already after one hour in tomato root tips exposed to 250 mM NaCl. Overall, leaves showed slightly delayed and milder response in the aspect of membrane integrity, ROS and NO accumulation, total proteolytic activity and consequently decreased protein content. Only H_2O_2 level was significantly higher and increased in leaves compared to the decreased H_2O_2 content of roots supporting the hypothesis that H_2O_2 can have a systemic signalling role in response to high salinity and contributes to the acclimation of leaf tissues. Moreover, the simultaneous alteration of ROS and NO content as a function of time can also be a crucial point in the context of tissue-specified survive or salinity-induced PCD.

Cys poteases

PLCPs

We detected four PLCPs in the roots and five PLCPs in the leaves that might be activated during salt stress-induced PCD. They belong to RD21A-like, RD19A-like, SAG12-like, aleurain-like

and XBCP3-like protease subfamily. Interestingely, some of the detected PLCPs show tissue-specificity. There are several studies which suggest the roles of aleurains and granulin-containing proteases in early processes of PCD. Expression level of the identified proteases increased after 24 hours. At the early stages of PCD probably those proteases are activated which are basically stored as proproteases and they quickly reach their active form due to the salt stress. It is important to note that ABPP gives information only about the activated form of proteases not about their exact protein level. It might be that synthesis of more subsets of Cys proteases is needed to accomplish proteolysis during PCD.

VPEs

We confirmed with several tests that VPEs are exclusively targeted by JOPD in tomato. VPEs are responsible for maturation and activation of vacuolar enzymes and are involved in PCD during plant immune response. Several abiotic stresses, such as NaCl- or H_2O_2 stress, induce expression of certain VPEs. Activity of VPEs was revealed as an opposite response in different tissues. VPEs are activated in leaves while their activity decreased in roots upon lethal salt stress.

Proteasome modification during salt stress

We discovered robust alteration in the activity profile of proteasome catalytic subunits. Both catalytic β2 and β5 subunits shifted to a higher MW and β5 also has a negative pI shift upon salt stress. The recurrence of a normal proteasome profile at 24 hours upon treatment with 250 mM NaCl indicates that this change is reversible and occurs concomitantly with PCD.

The reason for proteasome modification could be an altered preference for substrates. Salt stress induces protein oxidation and an altered proteasome might be required to degrade these oxidized proteins. The three catalytic subunits have different peptidase activity and their modification may cause changes in their activity and specificity.

There are several molecular mechanisms that might underpin MW/pI shifts of catalytic subunits: existance of an alternative proteasome in plants; alternative splicing of transcripts resulting different isoforms; or post-translational modification.

Investigation of the role of ABA in response to high salinity

Detection of cell viability

ABA is a key endogenous messenger in plants participating in responses to salinity. Salt stress causes massive transient increments in ABA level resulting in physiological changes in plants. Tomato mutant with reduced ABA level (*sitiens*) was investigated upon low (100 mM NaCl)- and high (250 mM NaCl) salt stress at six hours. It is well known that *sitiens* is not able to withstand salt stress even if it is a sublethal NaCl concentration for tomato due to their defective ABA production. Importantly, significantly decreased cell viability was observed in *sitiens* roots upon low salt treatment, too.

ABA controls several mechanism in response to salt stress. Besides regulation of stomatal closure it regulates water flow, specifically by having effects on aquaporins, induces accumulation of osmoprotectants or activates detoxifying mechanisms. Indeed, ABA deficiency provokes many cellular damages already at this early stage of salt stress in roots leading to PCD such as oxidative burst or disruption of membrane integrity.

Detection of oxidative burst

First, we observed highly increased ROS production without salt treatment and ROS level massively increased in *sitiens* roots while it slightly decreased in wild type roots upon 100 mM salt treatment, however, it significantly decreased upon lethal salt treatment in both genotypes at 6 hours. It can be suggested that ABA has a crucial role in control of ROS scavenging mechanisms by regulating non-enzymatic and enzymatic antioxidant mechanisms. Reduced ABA level can ruin this balance and a low salt stress can induce oxidative damages.

Second, both of H_2O_2 content and NO production decreased in the root tips of *sitiens* mutants compared to the wild type. Overall, their signalling pathways are complex and it is likely that ABA has a crucial role in regulation of H_2O_2 and NO level in this early stage of PCD in *sitiens* roots.

Investigation of protein degradation in sitiens

We hypotesized that ABA deficiency could result in increased proteolytic activity in the early stage of PCD. Little is known about the link between ABA deficiency and proteolysis, although there are some previous results that can reinforce our assumption. For instance, ABA delayed the accumulation and maturation of SlCysEP, a cysteine endopeptidase, which is an indicator of PCD in tomato endosperm. In agreement with previous reports we revealed increased total protease activity in the absence of ABA. Importantly, the difference between wild type and *sitiens* was detected only upon 100 mM NaCl treatment. Low salt stress reduced protein content and simultanously, increased proteolytic activity in *sitiens* roots whereas proteolytic activity was increased in wild type and in the mutants at the same degree under lethal salt condition.

Gelatin-SDS PAGE zymography revealed very remarkable differences in the activity of high MW proteases between wild type and mutant plants. ABPP revealed substantial differences between Cys protease activities of the roots at 34 and 28 kDa in the wild type and mutant plants. These proteases were much more active in *sitiens* mutants under salt stress which suggests that ABA accumulating under salt stress may suppress the activity of specific Cys proteases in the roots of tomato.

We could demonstrate that there are differences not only in the activity of protease proteins but also in the gene expression level.

Summary

In this study we compared sublethal and lethal salt stress-induced events during the first 24 hours of the salinity stress. To summarize our results we listed our findings:

1. Overall, the leaves showed delayed response to salt stress in contrast to the roots. Decreased viability at six hours upon lethal salt treatment indicates a massive and quick cell death, irreversible loss of membrane integrity, high electrolyte leakage, low K^+/Na^+ ratio and DNA degradation that is completed within 24 hours compared to sublethal salt treatment that caused a slower loss of viability of root cells. Lethal salt stress induced parallel ROS and NO peaks at the beginning of salt exposure in roots and only after six hours in leaves.

2. High salinity-induced reduction in total protein content appeared only after 24 hours both in leaves and roots. In parallel with protein degradation increased activity of proteases appears. We discovered increased PLCPs activity from three different subclasses. Interestingly, we detected PLCPs which were tissue-specific and were identified only either in the roots or in the leaves and

which were presented in both organs. Furthermore, we revealed tissue-specific overexpression of C14, AALP-like, RD19-like proteases that were overexpressed specifically in the leaves. The root-specific RD21A-like protease was overexpressed during salt stress-induced PCD. However, the overexpression of PLCPs occurred typically after 24 hours which can indicate that further protease synthesis is required for PCD execution.

5. We also investigated VPEs, which one of the protease subfamilies carrying caspase-like activity. We revealed that activity of VPEs is tissue-specific in tomato. Here, we demostrated that their activity increased only upon lethal salt stress, thus it can be assumed that they might have a role in salt stress-induced bulk protein degradation and they can also support the maturation of other vacuolar proteases.

6. High salinity altered the proteasome catalytic subunits in the roots. β2 and β5 subunits were activated upon salt stress-induced PCD which caused a shifted band in the labeling profile. As the changes of activity profile of PLCPs, VPEs and proteasome catalytic subunits were not detected at sublethal (100 mM) salt concentration, the identified PLCPs, VPEs and the altered β2, β5 catalytic subunits might be involved in PCD at the early stages of high salt stress in a tissue specific manner. Reoccurence of normal proteasome profile at 24 hours upon lethal salt treatment indicates that this change is reversible and confirms that it occurs concomitantly with PCD.

7. Most prominent alteration between wild type and ABA deficient, *sitiens* mutant under salt stress is that sublethal salt treatment seems to induce PCD in *sitiens*. Basic profile of almost all of the physiological responses was changed including cell viability, ROS content, NO production and protein degradation. Cell viability of the root cells in the mutants significantly declined not only upon 250 mM NaCl treatment but also upon 100 mM NaCl treatment compared to the wild type. Surprisingly, membrane integrity still remained stable upon sublethal treatment in sitiens.

8. Importantly, under normal condition total ROS content was already increased and it became higher upon sublethal salt stress while H_2O_2 and NO content decreased in sitiens compared to wild type. There is a clear correlation between ROS burst, disruption of membrane integrity and protease activation. Interestingly, increased electrolyte leakage is not needed to increase activity of proteases and simultaneously for degradation of proteins induced upon sublethal salt treatment in sitiens roots.

9. It is suggested that while a number of proteases are differentially activated in wild type and *sitiens* roots, the activities of certain Cys proteases (at ~34 and 28 kDa) increases to much higher extent in the mutant under salt stress indicating that ABA may suppress specific proteolytic activities under high salinity. Similar tendencies can be found in the case of certain proteases of higher MW, which suggests that ABA plays a crucial role in the inhibition of proteolitic activity under salt stress, especially under low salt concentrations.

List of publications

Papers related to the thesis

Kovács J, Poór P, Kaschani F, Chandrasekar B, Hong T, Misas-Villamil J, Xin BT, Kaiser M, Overkleeft H, Tari I and van der Hoorn RAL (2017) Proteasome activity profiling uncovers alteration of catalytic β2 and β5 subunits of the stress-induced proteasome during salinity stress in tomato roots. Frontiers in Plant Science 8:107. (IF: 4.495)

Kovács J and van der Hoorn RAL (2016) Twelve ways to confirm targets of activity-based probes in plants. Bioorganic & Medicinal Chemistry 24:3304-3311. (IF: 2.923)

Poór P, Borbély P, Kovács J, Papp, A, Szepesi Á, Takács Z and Tari I (2014) Opposite extremes in ethylene/nitric oxide ratio induce cell death in cell suspension culture and in root apices of tomato exposed to salt stress. Acta Biologica Hungarica 65: 428-438, DOI: 10.1556/ABiol.65.2014.4. (IF: 0.97)

Additional papers

Misas-Villamil JC, Van der Burgh AM, Grosse-Holz F, Pages M, Kovács J, Kaschani F, Schilasky S, Emron Khan Emon A, Ruben M, Kaiser M, Overkleeft HS and van der Hoorn RAL (2017) Subunit-selective proteasome activity profiling uncovers uncoupled proteasome subunit activities during bacterial infections. The Plant Journal TPJ-00974-2016. (IF: 5.468)

Shindo T, Kaschani F, Yang F, Kovács J, Tian F, Kourelis J, Hong TN, Colby T, Shabab M, Chawla R, Kumari S, Ilyas M, Hörger AC, Alfano JR and van der Hoorn RAL (2016) Screen of non-annotated small secreted proteins of Pseudomonas syringae reveals a virulence factor that inhibits tomato immune proteases. PLoS Pathogens. 12: e1005874. (IF: 7.64)

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Poór P, Kovács J, Borbély P, Takács Z, Szepesi Á and Tari I (2015) Salt stressinduced production of reactive oxygen- and nitrogen species and cell death in the ethylene receptor mutant Never ripe and wild type tomato roots. Plant Physiology and Biochemistry, 97: 313-322. (IF: 2.93)

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Tari I, Guóth A, Benyó J, Kovács J, Poór P and Wodala B (2010) The roles of ABA, reactive oxygen species and nitric oxide in root growth during osmotic stress in wheat: comparison of a tolerant and a sensitive variety. Acta Biologica Hungarica 209-216. (IF: 0.793)

Declaration

I the undersigned Professor Renier A. L. van der Hoorn, certify that Judit Kovács contributed to the implementation of the following publications "Proteasome activity profiling uncovers alteration of catalytic β2 and β5 subunits of the stress-induced proteasome during salinity stress in tomato roots" and "Twelve ways to confirm targets of activity-based probes in planta".

The above mentioned scientific evidences have not been published elsewhere, so far, and it is not going to be used by other PhD candidates

Oxford, ...10... 3... 2017

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Professor Renier A. L. van der Hoorn (Supervisor, Corresponding author)

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Judit Kovács (doctoral (Ph.D.) candidate

Declaration

I, the undersigned Dr. Miklósné Görgényi Dr. Irma Tari, certify that Judit Kovács contributed to the implementation of the following publication "Opposite extremes in ethylene/nitric oxide ratio induce cell death in cell suspension culture and in root apices of tomato exposed to salt stress".

The above mentioned scientific evidences have not been published elsewhere, so far, and it is not going to be used by other PhD candidates.

Szeged, ...

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Dr. Miklósné Görgényi Dr. Irma Tari (Supervisor, Corresponding author)

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