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The Expressions of Small Ubiquitin-like Modifier (SUMO) Related Genes Under Metal (Cu, Zn and Fe) Toxicity in *Arabidopsis thaliana*

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Abstract

Aim of this work was to investigate effects of toxic levels of Cu, Zn and Fe treatments on small ubiquitin-like modifier (SUMO) machinery of *Arabidopsis thaliana*. SUMO is a 100-115 amino acid post-translational modifier that can regulate stability, activity or sub-cellular localization of target proteins. *A. thaliana* plants were treated with 50 µM Cu, 700 µM Zn and 400 µM Fe for 7 d and then expressions of genes related to SUMOylation and deSUMOylation of target proteins were measured with qRT-PCR. Only Cu treatment was able to induce genes related to SUMOylation (*SUM3, SAE2, SIZ1*) of target proteins, while all of the three metals used in this study was effective in inducing a deSUMOylation related gene. Results of this study indicate that deSUMOylation of proteins might be a part of plant response to metal toxicity.

Keywords: *Arabidopsis thaliana*, deSUMOylation, metal toxicity, small ubiquitin-like modifier (SUMO), SUMOylation

1. Introduction

As sessile organisms, plants should rapidly respond to changes in environmental conditions to survive. These responses can be at different levels such as transcriptional, post-transcriptional, translational or post-translational [1]. Among these, post-translational modifications are rapid since there is no need for transcription of a gene and synthesis of a new protein and are involved in stress perception, signaling and acclimation [2]. Post-translational modifications are defined as addition or removal of small molecules to target proteins [3]. In eukaryotic cells activity, stability or sub-cellular localization of proteins can be controlled via modifications such as phosphorylation, acetylation, methylation, glycosylation, disulfide bond formation or ubiquitination [4]. Another lesser known post-translation modification that is involved in regulation of cellular functions is small ubiquitin-like modifier (SUMO) [5]. SUMO is a polypeptide that contains 100-115 amino acids and is encoded by four genes in model plant *Arabidopsis thaliana* (*SUM1, 2, 3* and *5*) [6]. During SUMOylation SUMO covalently binds to a lysine residue of a protein and this is reversible. In *A. thaliana* SUMOylation occurs with involvement of E1, E2 and E3 enzymes, which are SUMO activation, conjugation and ligation enzymes, respectively [7].

SUMO is activated by E1 SUMO activation enzyme, which is comprised of two small (SAE1a and SAE1b) and one large sub-unit (SAE2) [8]. Following activation, SUMO polypeptide is transferred to E3 ligases by the E2 SUMO conjugation enzyme (SCE1). E3 ligases that transfer SUMO to target proteins are encoded by *HIGHPLOIDY2* (*HPY2*) and *SAP & MIZ1* (*SIZ1*) in Arabidopsis [9]. On the other hand, removal of SUMO modification from target proteins is done by deSUMOylation enzymes such as *OVERLY TOLERANT TO SALT 1* (*OTS1*), *OTS2, EARLY IN SHORT DAYS 4* (*ESD4*), *ESD4 like SUMO PROTEASE* (*ELS1*), *ULP1b*, *ULP2a* and *ULP2b* [10].

SUMOylation and deSUMOylation can affect protein activity and cell metabolism in two different ways. The first of these is the regulation of protein-protein interactions and therefore signaling pathways and circuits in the cell. The second effect of SUMOylation on protein activity is that it protects proteins against ubiquitin-mediated protein breakdown [11]. Since ubiquitin and SUMO bind to the same lysine residues on target proteins, binding of SUMO to a protein prevents ubiquitin from binding to the protein of interest. It is well documented that ubiquitination of a protein creates a signal for the degradation of that protein by 26S proteasome [12]. The accumulation of

SUMOylated proteins in response to environmental stresses such as heat shock, cold, drought, salinity is vital for the plant. With the increase in amount of SUMOylated proteins, the levels of the free SUMO levels decrease. Following this with the relief of stress amount of free SUMO levels increases rapidly [13]. Moreover, mutants missing the SIZ1 allele were susceptible to abiotic stresses. For example, SUMOylation of ICE1 mediated by SIZ1 in Arabidopsis controls CBF3/DREB1A expression and affects freezing tolerance [14].

Metals such as Cu, Zn, Mn, Fe, Ni, and Co are essential for plant growth and development; however, accumulation of excess levels of these metal ions in plant cells can cause various detrimental toxic effects [15]. For example, direct or indirect production of reactive oxygen species and oxidative stress is a common effect caused by most of the metals, especially that of redox active metals such as Fe and Cu. On the other hand, some metals such as As, Cd, Cr, Pb, Hg might affect protein function by replacing other metals in active site of the proteins or by interfering with function of functional groups [16]. Metal toxicity is a wide-spread phenomenon observed in arable lands that can be caused by natural or anthropogenic activities such as mining, excessive use of fertilizers and irrigation with groundwater [17]. Therefore, it is vital to understand plant response to metal toxicity for sustainable plant productivity in metal contaminated soils. There are numerous studies that investigate uptake, translocation, sequestration of metals in plants and plant response to heavy metals at biochemical and molecular level such as antioxidant response, regulation of metalloenzymes and accumulation of phytochelatins [18, 19]. However, transcriptional response of SUMO metabolism to essential metals such as Cu, Zn and Fe has not been elucidated before.

Therefore, aim of this work was to elucidate how metal toxicity affects SUMO machinery of *A. thaliana* at transcriptional level. For this *A. thaliana* plants were treated with toxic concentrations of Cu, Zn and Fe and expressions of genes related to SUMOylation and deSUMOylation were measured with qRT-PCR.

2. Materials and Methods

2.1. Materials

Arabidopsis thaliana Col-0 ecotype was used in this work as plant material.

2.2. Methods

2.2.1. Growth Conditions and Treatments

Sterilized seeds were sown on ½ Murashige-Skoog (MS) medium with Gambrog's vitamins [20] and 1% sucrose in petri dishes and then were grown in a growth chamber in 22/20 °C, 12/12 dark/light and 60% relative humidity conditions. After 2 days of germination period, 5 days old seedlings were treated with 50 µM Cu, 700 µM Zn and 400 µM Fe for 7 days. Metal concentrations were selected according to previous studies [21-23]. At the end of experiments whole seedlings were harvested, then flash frozen in liquid nitrogen and were stored at -80 °C until further analysis.

2.2.2. Quantitative Real-Time PCR Analysis

Measurement of gene expressions was done according to Ozgur *et al.* [24]. 0.1 g fresh samples were used for RNA isolation which was determined with NucleoSpin RNA Plant Kit (Macherey-Nagel) according to directions. DNAse I was used for DNA digestion to prevent any genomic DNA contamination. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to perform reverse transcription (1µg of RNA). SYBR green Master mix (Applied Biosystems) were used to perform qRT-PCR. PCR amplification protocol was as follows, 95 °C for 5 minutes, 95 °C (15 s), 60 °C (15 s) and at 72 °C (30 s) for 40 cycles. StepOne Plus software was used to analyze the resulting data. Control group was designated as the reference and the value of the control groups was set to 1 when calculating the expression levels of the genes. *ACTIN8* gene was used as house-keeping gene and expressions of other genes were normalized according to it. Primers used in this study can be found in Table 1.

Table 1. List of qRT-PCR primers used in the study.

SUMO	*SUM1*	F	GACCGGCAATCTGTGGACAT
		R	CCATGTCAAGCTCATCGGGA
	SUM2	F	AGGGACAGGCATTTTTCGTTG
		R	TCCACAGACTGACGGTCACA
	SUM3	F	CAAGAGCCAGGATGGAGACG
		R	TCTCCAGGCCACCTATACGA
	SUM5	F	TGGTGAGTTCCACAGACACAA
		R	ATCCTCTGCTCCCTGTTGGT
E1	*SAE1a*	F	TCCTCGGAGAACAGCAAAGC
		R	TCTGGCAAGATCGAGTAGCG
	SAE1b	F	CACAAAGAAAAAGCTTGATGAAACA
		R	TCCACGGTACTGAAACTGCC
	SAE2	F	ACGGAAGCATTCTCACAGTCG
		R	GAGTTTAGGGAAAGTCGATGGT
E2	*SCE1*	F	GATGGAGACCAGCCATCACC
		R	AACCATCTGTCTGTGCAGGG
E3	*HPY2*	F	TGTCTCCGATAACAGTTCCACG
		R	TCAAGGTCCTTAACCTTGTCCG
	SIZ1	F	TTTTGGGTTACAGTGGCACA
		R	ACACTCTGCATTGTGCTTGC
deSUMOylation	*ELS1*	F	TTGGAGACAAGATGAAGAACCA
		R	TTGAGATGGTAGCCCAACCT
	OTS1	F	TGCGAGCGAGTACAGCCTCA
		R	AATCTTGGCAGCGACCGCCA
	OTS2	F	GGGAAAGCTGAGCACAGTGCA
		R	TCCCAAGACCACTCCCTAGGAGT
	ACT8	F	TCAGCACTTTCCAGCAGATG
		R	ATGCCTGGACCTGCTTCAT

2.2.6. Statistical Analysis

Experiments were repeated twice with 3 replicates (n = 6). The results were expressed as mean and error bars were used to indicate the standard error of the mean. (± SEM). Treatment groups were compared to controls using students-t test. Significant differences ($p < 0.05$) were marked with an asterisk (*).

3. Results and Discussion

3.1. The Expressions of SUMO Encoding *SUM1*, *SUM2*, *SUM3* and *SUM5* Genes

Cu, Zn and Fe treatments did not have statistically significant effects on the expressions of *SUM1*, *SUM2* and *SUM5* genes (Figure 1). However, the expression of *SUM3* changed with Cu treatment. Cu toxicity enhanced the expression of *SUM3* gene by 3.2 folds as compared to controls. SUMO3 is responsible for mono-SUMOylation of proteins while SUMO1 and 2 can form poly-SUMO chains on proteins. These results indicate that mono-SUMOylation might be required for plant response to Cu as it was shown in drought stress previously [25]. Although there is no information in literature related to transcriptional response of different *SUM* genes to metal stress, it has been demonstrated that oxidative stress and heat shock can induce accumulation of SUMO conjugates in Arabidopsis [26].

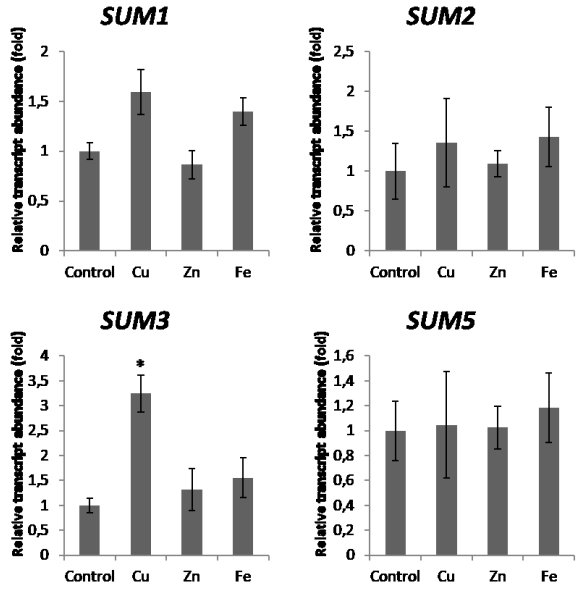

Figure 1. qRT-PCR analysis of *SUM1*, *SUM2*, *SUM3* and *SUM5* genes under Cu,Zn and Fe toxicity.

3.2. The Expressions of Genes Encoding SUMO E1 Activating Enzymes (*SAE1* and *SAE2*) and SUMO E2 Conjugating Enzyme (*SCE1*)

The expressions of *SAE1A* and *SAE1B* did not change under toxic levels of Cu, Zn and Fe treatments (Figure 2). However, Cu treatment enhanced *SAE2* expressions 2.3 folds as compared to controls while Zn treatment decreased it by 2 folds as compared to controls. On the other hand, metal treatments did not have any effect on the expression of *SCE1* expression. Previous studies demonstrated that *SCE1* expression was induced with high temperature stress in rice [27]. Moreover, *SCE1* was also induced in tomato species that is tolerant to bacterial pathogens [28]. E1 complex is comprised of SAE1A, SAE1B and SAE2, but results of this study indicates that SAE1 subunits does not respond to metal toxicity, while SAE2 can be induced by Cu toxicity. How this changes stoichiometry and activity of this protein deserves further scrutiny.

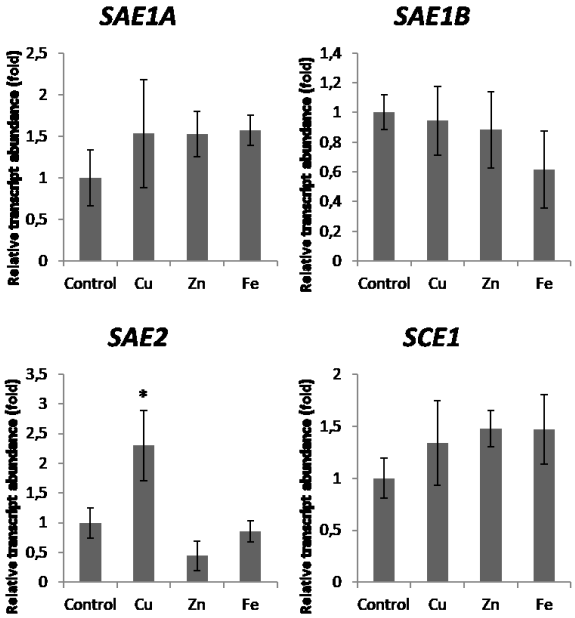

Figure 2. qRT-PCR analysis of *SAE1A*, *SAE1B*, *SAE2* and *SCE1* genes under Cu,Zn and Fe toxicity.

3.3. The Expressions of SUMO E3 Ligase Encoding Genes *HPY2* and *SIZ1*

The expression of *HPY2* gene decreased by 2 folds as compared to control under Cu toxicity, while Zn and Fe treatments did not change the expressions of *HPY2* (Figure 3). Moreover, Cu toxicity increased the expression of *SIZ1* by 2.3 folds as compared to controls, whereas Zn and Fe treatments did not have any effects on the *SIZ1* expressions. SIZ1 and HPY2 are thought to have different roles in plant stress response since heat-stress induced SUMOylated protein patterns in *siz1* and *hpy2* mutant plants are different [29]. It has been demonstrated that *siz1* mutation causes Cu stress sensitivity [30]. Chen et al. [30] demonstrated that SUMOylation controls mRNA levels of YSL1 and YSL3 (*Yellow Stripe-Like1* and *Yellow Stripe-Like3*), which encode Cu transporters, either through regulation of transcription or affecting mRNA stability under excess Cu conditions. Moreover, they showed that that SIZ1 activity is required for maintenance of basal transcription levels of YSL1 and YSL3 under non-stress conditions Another role of SIZ1 is related to low

temperatures, where SIZ1 mediated SUMOylation of ICE1 is required for freezing stress tolerance in *Arabidopsis thaliana* [14]. Moreover, overexpression of SIZ1 leads to increased tolerance to cold and salt stresses and attenuates response to abscisic acid in *Arabidopsis thaliana* [13]. Results of current study supports the idea that HPY2 and SIZ1 have different functions in plants, which is evident from induced expression of *SIZ1* under Cu toxicity, while *HPY2* did not change.

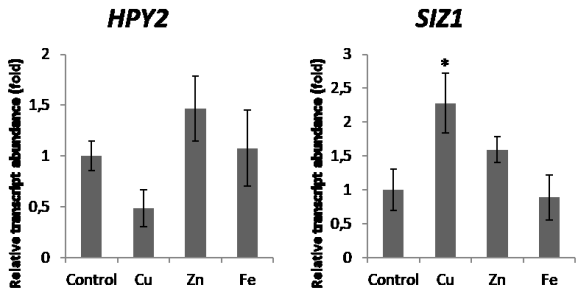

Figure 3. qRT-PCR analysis of *HPY2* and *SIZ1* genes under Cu, Zn and Fe toxicity.

3.4. The Expressions of Genes Encoding DeSUMOylation Enzymes ELS1, OTS1 and OTS2

Cu and Zn treatments enhanced the expressions of *ELS1* by 3.7 and 8 folds respectively as compared to controls while Fe toxicity showed no significant effect on the expression of *ELS1* (Figure 4). The expression of *OTS1* under Cu and Fe treatments also showed no differences, but Zn treatment enhanced the expression of *OTS1* by 2 folds as compared to control.

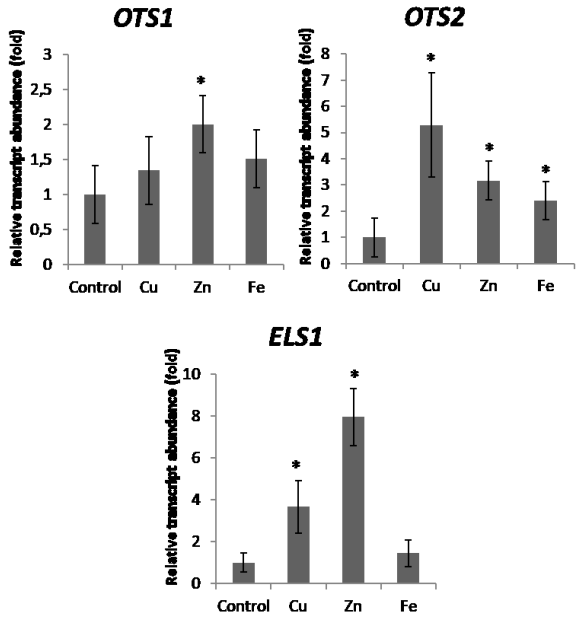

Figure 4. qRT-PCR analysis of *ELS1, OTS1* and *OTS2* genes under Cu, Zn and Fe toxicity.

The expression of *OTS2* was enhanced with all three metal treatments. Cu treatments enhanced it by 5 folds, while Zn treatments induced it 3 folds as compared to control. Moreover, Fe toxicity also enhanced the expression of *OTS2* by 2 folds as compared to controls. deSUMOylation enzymes are vital to control accumulation of SUMO substrate and can provide specificity since they are encoded by a higher number of genes [11]. Moreover, deSUMOylation enzymes are important components of plant stress tolerance. For example, *ots1 ots2* double mutant is sensitive to salt stress and accumulates higher levels of SUMO1/2 conjugated proteins [10]. On the other hand, over-expression of *OTS1* can confer plants tolerant to salt stress by reducing SUMO1/2 conjugates [10]. Results obtained in this study are consistent with the literature since all of the three deSUMOylation genes investigated are induced with metal treatments, indicating that protein deSUMOylation might be more critical than SUMOylation during metal toxicity.

4. Conclusion

Overall, this study demonstrates that among the metals tested only Cu induces genes related to SUMO polypeptides or SUMOylation enzymes (*SUM3, SAE2, SIZ1*) and Zn and Fe toxicity does not have such effect. On the other hand, all three metals, Cu, Zn and Fe, induced expressions of deSUMOylation related genes, especially that of *ELS1,* indicating that deSUMOylation of proteins might be a part of plant response to metal toxicity. Since *ELS1* can also play a role in maturation of SUMO peptides [11] further roles of ELS1 in metal toxicity tolerance should be evaluated by investigating SUMO conjugate levels in wild-type and ELS1 mutants under metal toxicity.

Author's Contributions

Baris UZILDAY: Designed and performed the experiments and evaluated the results. He prepared figures, drafted and wrote the manuscript.

Ethics

There are no ethical issues after the publication of this manuscript.

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