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Functional analysis of the SUMO conjugation/deconjugation system during the development and stress response of *Arabidopsis thaliana*

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Functional analysis of the SUMO conjugation/deconjugation system during the development and stress response of *Arabidopsis thaliana*

ABSTRACT

Living organisms are subjected to constantly changing environmental conditions that compromise survival. Perception of these changes precedes the triggering of signaling events that ultimately activate an adequate response. Since plants are sessile organisms they are considerably more exposed to an instable environment, having adopted a wide range of strategies to adapt, avoid or tolerate stress. Knowledge on the molecular basis of these strategies will be key for future crop improvement, particularly with a rapidly changing environment. Several studies have singled out how fast and reversible responses to stress correlate with post-translational modification (PTM) of key proteins. An increasingly important mechanism involves protein modification by small peptides such as ubiquitin and other ubiquitin-like modifiers (UBLs). Focus on small ubiquitin-related modifier (SUMO), a UBL family member, has increased massively in the last years. SUMO attachment, or sumoylation, may exert different effects depending on the target protein, controlling its conformation, or even creating or blocking interacting interfaces. To be attached to a target, pre-SUMO peptides are first processed by SUMO proteases (ULP/SENP family), and then conjugated to a target's lysine via SUMO E1 activases and SUMO E2 conjugases, aided by SUMO E3 ligases. Deconjugation of the SUMO peptide can be carried out by SUMO proteases. In plants, SUMO homeostasis is critical because mutations in pathway components result in embryonic lethality or pleiotropic phenotypes. One interesting feature of SUMO is that SUMO-conjugates accumulate rapidly upon stress imposition, placing SUMO in the forefront of the plant response to stress, most likely associated to transcriptional re-programming. Studies of SUMO function in plants have been based mostly on reverse genetics approaches in the plant model *Arabidopsis thaliana*. Similarly, we employed T-DNA insertion mutants to characterize the role of the major SUMO E3 ligase SIZ1 and SUMO proteases ULP1c, ULP1d, ULP2a and ULP2b in the plant stress response.

Reactive oxygen species (ROS) are known internal signals caused by stress that can induce SUMO-conjugate accumulation. In the present work we addressed SUMO-ROS interplay, and report that SIZ1 is essential for SUMO-conjugate induction in response to ROS, and is involved in oxidative stress tolerance. Additionally, *siz1* mutants displayed altered ROS homeostasis, constitutively accumulating hydrogen peroxide, superoxide ion and singlet oxygen. The *siz1* phenotype was partially dependent of salicylic acid (SA) levels since several *siz1* defects were greatly recovery by the expression of the transgenic salicylate hydroxylase *NahG*. The *siz1* mutant displays constitutive autoimmune phenotypes, including SA-accumulation. Analysis of oxidative stress-responsive targets of sumoylation suggests a major role for transcription remodeling proteins in the SUMO-dependent response to oxidative stress.

Bioinformatic analysis revealed that the transcriptome profile of adult *siz1* is similar to that of mitogen-activated protein kinase (MAPK) pathway mutants. These also share common features of autoimmune responses, such as the accumulation of ROS. Results suggest a crosstalk between the SUMO and MAPK pathways, but a direct interaction between MKK2-MPK4 and SUMOs or in vitro sumoylation of MKK2-MPK4 was not observed. Nonetheless, we introgressed *mkk1/2* and *mpk4*

mutants into *siz1*, and sumoylation profiles suggest that MPK4 acts as a negative regulator while MKK1/2 is seemingly epistatic to SIZ1.

In contrast to the low number of components involved in SUMO conjugation, there are several SUMO proteases in plants. SUMO proteases are sources of selectivity, since they can discriminate different SUMO isoforms and targets to be de-sumoylated. They also display different expression patterns and subcellular localizations. We report how phylogenetic reconstruction of the Arabidopsis ULP family outlines the existence of two major branches, and within these, four phylogenetic subgroups. We characterized two of these subgroups, one composed by ULP1c/ULP1d and another by ULP2a/ULP2b. We show how ULP1c/d function redundantly to regulate several developmental traits. Using GUS report assays we also established their spatial expression pattern. A microarray analysis of differentially expressed genes in a double T-DNA insertion mutant was carried out, suggesting a deregulation of drought and abscisic acid-related genes. Taking this into consideration, a characterization of related phenotypes was performed in the double mutant.

We additionally demonstrated how ULP1c/d are negative regulators of the plant response against the hemibiotroph pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. The response is however complex, since infection triggered down-regulation of *ULP1c* and *ULP1d* transcripts, which may correlate with the observed increase in SUMO-conjugates that follows infection. Analysis of the transcriptome of *ulp1c/d* after *Pst* DC3000 challenging revealed the existence of many de-regulated genes involved in both biotic stress responses and hormonal signaling, including auxin-responsive genes. We subsequently observed *ulp1c/d* sensitivity to exogenous auxins. Overall results suggest a ULP1c/d-dependent modulation of auxin in response to *Pst* DC3000.

ULP2a and ULP2b were structural and phylogenetically characterized, revealing a set of common features with ULP2-type proteases of other biological models. T-DNA insertion mutants for this ULP pair were characterized, showing diverse phenotypic defects and constitutive accumulation of SUMO-conjugates. ULP2a and ULP2b displayed unequal redundancy, placing ULP2b as the most preponderant. Microarray analysis evidenced a specific transcriptional signature that suggests the involvement of ULP2s in secondary metabolism, cell wall remodelling and nitrogen assimilation. The *ulp2a/b* mutant also displayed an antagonistic morphological phenotype to that of the well characterized SUMO E3 ligase mutant *siz1*. Most significantly, the triple mutant *ulp2a/b siz1* was phenotypically *siz1*-like, which places ULP2a/b as epistatic and downstream of SIZ1.

The current work highlights the importance of both SIZ1 and various SUMO proteases in development and the plant response to external challenges. Subsequent functional studies will help unravel the fundamental role that SUMO dynamics seems to play at a molecular level, in the control of plant development, hormone regulation, stress response and transcription programming.

Análise funcional do sistema de conjugação/desconjugação do SUMO no desenvolvimento e na resposta ao stresse em *Arabidopsis thaliana*

RESUMO

Os organismos vivos estão constantemente sujeitos a alterações ambientais que comprometem a sua sobrevivência. A percepção destas alterações precede eventos de sinalização que culminam na activação de uma resposta adequada. Dado as plantas serem organismos sésseis, elas estão consideravelmente mais expostas a ambientes instáveis, tendo desenvolvido um vasto leque de estratégias para adaptar, evitar ou tolerar o stresse. O conhecimento das bases moleculares subjacentes a estas estratégias é chave para o melhoramento de cultivares de interesse, especialmente estando estes sujeitos a ambientes instáveis. Diversos estudos têm evidenciado como respostas rápidas e reversíveis se correlacionam com mecanismos de modificação pós-tradução (PTM) de proteínas-chave. Mecanismos de reconhecida importância envolvem modificações por pequenos péptidos como a ubiquitina e modificadores semelhantes à ubiquitina (UBLs). O estudo do pequeno modificador relacionado com ubiquitina (*small ubiquitin-related modifier*, SUMO), um membro da família UBL, tem sido crescente nos últimos anos. A ligação do SUMO a uma proteína (sumoilação) poderá exercer diferentes efeitos consoante a proteína-alvo, controlando a sua conformação, e criando, ou bloqueando, interfaces de interacção. Para ser ligado a um alvo, o péptido pre-SUMO terá que ser inicialmente processado por SUMO proteases (família ULP/SENP), e então conjugado ao resíduo de lisina do alvo através de SUMO E1 activases e SUMO E2 conjugases, auxiliadas por SUMO E3 ligases. A desconjugação do SUMO poderá ser levada a cabo por SUMO proteases. Em plantas, a homeostasia do SUMO é fundamental uma vez que mutações em componentes da via resultam em letalidade embrionária ou em fenótipos pleiotrópicos. Um aspecto interessante do SUMO é o facto dos seus conjugados serem acumulados rapidamente em resposta ao stresse, posicionando o SUMO nos primeiros passos da resposta da planta ao stresse, muito possivelmente associada à reprogramação transcripcional. Estudos da função do SUMO em plantas assentam maioritariamente em estratégias de genética inversa onde é utilizada a espécie modelo *Arabidopsis thaliana* (Arabidopsis). De forma semelhante, o presente estudo utilizou mutantes de Arabidopsis por inserção de T-DNA para caracterizar o papel da SUMO E3 ligase SIZ1 e das SUMO proteases ULP1c, ULP1d, ULP2a e ULP2b na resposta da planta ao stresse.

As espécies reactivas de oxigénio (ROS) estão descritas como sendo sinalizadores internos originados pelo stresse que podem induzir a acumulação de conjugados de SUMO. No presente trabalho foi abordada a interacção SUMO-ROS, tendo-se obtido evidências de que SIZ1 é essencial para a indução de conjugados de SUMO em resposta a ROS, estando SIZ1 envolvida na tolerância ao stresse oxidativo. Adicionalmente, os mutantes *siz1* revelam alterações na homeostasia das ROS, acumulando constitutivamente peróxido de hidrogénio, ião superóxido e oxigénio singleto. O fenótipo de *siz1* é parcialmente dependente dos níveis de ácido salicílico (SA) uma vez que os defeitos fenotípicos de *siz1* são grandemente recuperados pela expressão do transgene salicilato hidrolase *NahG*. O mutante *siz1* apresenta um fenótipo de auto-imunidade constitutiva, incluindo a acumulação de SA. A análise dos alvos de sumoilação que respondem a stresse oxidativo sugere um papel preponderante de proteínas reguladoras da transcrição na resposta ao stresse oxidativo dependente do SUMO.

A análise bioinformática revelou que o padrão do transcriptoma de *siz1* é semelhante ao de mutantes da via das MAP cinases (MAPK). Estes apresentam também características de resposta auto-imune, como a acumulação de ROS. Os resultados sugerem uma intercepção entre as vias do SUMO e MAPK, porém não foram observadas interacções entre MPK4 e péptidos SUMO, ou sumoilação in vitro de MKK2-MPK4. Contudo, as plantas resultantes da introgressão de mutantes *mkk1/2* e *mpk4* com *siz1* apresentam padrões de sumoilados alterados, permitindo sugerir a MPK4 como sendo um regulador negativo da sumoilação, enquanto as MKK1/2 poderão ser epistáticas de SIZ1.

Em contraste com o número limitado de componentes da via de conjugação do SUMO, em plantas existem várias SUMO proteases. As SUMO proteases são uma fonte de selectividade, uma vez que são capazes de discriminar isoformas de SUMOs e descriminar alvos a serem dessumoilados, reconhecem diferencialmente isoformas do SUMO, possuem diferentes padrões de expressão e diferente localização subcelular. A reconstrução filogenética da família ULP de Arabidopsis evidenciou a existência de dois grandes ramos, divididos em quatro subgrupos filogenéticos. Procedemos à caracterização de dois destes subgrupos, um composto por ULP1c/ULP1d e outro por ULP2a/ULP2b. Foi demonstrado que as ULP1c/d funcionam redundantemente na regulação de diversos aspectos do desenvolvimento. Recorrendo a ensaios pelo gene repórter GUS, foi possível estabelecer o padrão de expressão espacial destes genes. Analise por *microarray* de genes diferencialmente expressos no duplo mutante de inserção por T-DNA para ambos genes identificou uma desregulação de genes envolvidos na secura e ácido abscísico. Tendo este resultado em consideração, uma caracterização de fenótipos relacionados com estes processos foi realizada no duplo mutante.

Demonstrou-se igualmente que as ULP1c/d são reguladores negativos na defesa da planta ao microorganismo patogénico hemibiotrófico *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. No entanto esta resposta é complexa já que a infecção diminui os níveis de transcritos de *ULP1c* e *ULP1d*, o que poderá estar correlacionado com o aumento de conjugados de SUMO que se seguem à infecção. Uma análise do transcriptoma do *ulp1c/d* após eliciação com *Pst* DC3000 revelou a existência de muitos genes desregulados envolvidos nas respostas ao stresse biótico e sinalização hormonal, incluindo genes de resposta a auxinas. Em suma, os resultados apontam para um papel das ULP1c/d na modelização das auxinas em resposta a *Pst* DC3000.

ULP2a e ULP2b foram caracterizadas estrutural e filogeneticamente, revelando um conjunto de características comuns às proteases do tipo ULP2 de outros modelos biológicos. Mutantes por inserção de T-DNA para este par de ULP mostraram diversos defeitos fenotípicos e uma acumulação constitutiva de conjugados de SUMO. ULP2a e ULP2b apresentam redundância desigual, sendo ULP2b a mais preponderante. A análise por *microarray* evidenciou uma assinatura transcripcional especifica que sugere o envolvimento das ULP2s no metabolismo secundário, remodelação da parede celular e assimilação de azoto. O mutante *ulp2a/b* apresenta também um fenótipo morfológico antagónico ao fenótipo bem caracterizado do mutante da SUMO E3 ligase SIZ1. Foi interessante constatar que o triplo mutante *ulp2a/b siz1* é fenotipicamente semelhante a *siz1*, colocando as ULP2a/b numa posição epistática e a jusante de SIZ1.

O presente trabalho salienta a importância tanto de SIZ1 como de várias SUMO proteases na resposta da planta a desafios externos. Estudos funcionais subsequentes permitirão revelar, ao nível molecular, papéis fundamentais da dinâmica do SUMO no controlo do desenvolvimento, da regulação hormonal, da resposta ao stresse e da programação transcripcional.

TABLE OF CONTENTS

CHAPTER 3

Crosstalk between SUMO and MAPK signalling cascades

CHAPTER 4

SUMO proteases ULP1c and ULP1d are required for development and drought stress responses in *Arabidopsis thaliana*

CHAPTER 5

The ULP1c/ULP1d SUMO protease pair negatively regulates *Arabidopsis thaliana* defence against *Pseudomonas syringae* pv. *tomato* DC3000

CHAPTER 6

Arabidopsis thaliana ULP2a and ULP2b are SUMO proteases involved in plant development

CHAPTER 7

Concluding remarks and future perspectives

APPENDIXES

Abbreviations and Symbols

$^1O_2$	singlet oxygen
2D	two dimensional
3D	three dimensional
°C	degrees Celsius
Ψw	water potential
ABA	abscisic acid
ABRE	abscisic acid responsive elements
AD	activation domain
amiR	artificial microRNA
APX	ascorbate peroxidase
ATG	autophagy peptide
AUX	auxin
BD	binding domain
BL	brassinolide
bp	base pair
BR	brassinosteroid
CAT	catalase
cDNA	complementary DNA
CFU	colony forming unit
CoIP	co-immunoprecipitation
Col	Colombia-0
Da	Dalton
DAB	3,3-diaminobenzidine
DEG	differentially expressed gene
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DPI	days post-inoculation
E	Einstein
EDTA	ethylenediaminetetraacetic acid
EFR	EF-Tu receptor
ERF	ethylene response factor
ET	ethylene
ETI	effector-triggered immunity
FLG	flagellin
FW	fresh weight
g	gram
g	relative centrifuge force
GFP	green fluorescent protein
GO	gene ontology
GUS	beta-glucuronidase
h	hour
H_2O_2	hydrogen peroxide
HL	high-light
HMWC	high molecular weight SUMO conjugate
$HO^·$	hydroxyl radical
HS	heat shock
HSF	heat shock factor
HSP	heat shock protein
IgG	immunoglobulin G
IAA	indole-3-acetic acid
JA	jasmonate
KIU	Kaonashi ULP-like
L	Litre
LB	lysogeny broth medium
M	molar
m	meter
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
MEKK	MAPK kinase kinase
MES	2-(N-morpholino)ethaesulfonic acid sodium salt
min	minute
MJ	methyl jasmonate
MKK	MAPK kinase
mol	mole
mRNA	messenger RNA
MS	Murashige and Skoog culture medium
MV	methyl viologen
MW	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NahG	salicylate hydrolase transgene
NBT	nitroblue tetrazolium
NDSM	negatively charged amino acid-dependent SUMO motif
$O_2^{·-}$	superoxide ion
OE	over-expression
ORF	open reading frame
Pa	Pascal
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PCA	principal component analysis
PCR	polymerase chain reaction
PDSM	phosphorylation dependent SUMO motif
PEG	polyethylene glycol
Pi	inorganic phosphate
PPI	protein-protein interaction
PPOD	pyrogallol peroxidase
PR	pathogen related gene
PRX	peroxidase
PS	photosystem
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
PTM	post-translation modification
qPCR	quantitative real-time PCR
R-protein	resistance protein
RBOH	respiratory burst oxidase homologue
RGN	ROS gene network
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RT	room temperature
RUB	related to ubiquitin
s	second

SA	salicylic acid
SAE	SUMO activating enzyme
SAUR	small auxin up-regulated
SAR	systemic acquired resistance
SCE	SUMO conjugating enzyme
SDS	sodium dodecyl sulphate
SEM	standard error of the means
SENP	sentrin-specific protease
SIM	SUMO interacting motif
SOD	superoxide dismutase
SOSG	singlet oxygen sensor green
STUbL	SUMO targeted ubiquitin ligase
SUMO	small ubiquitin-like modifier
T3SS	type III secretion system
T-DNA	transfer DNA
Taq	*Thermus aquaticus* polymerase
TBA	2-thiobarbituric acid
TF	transcription factor
Tm	melting temperature
Ub	ubiquitin
UBL	ubiquitin-like modifier
ULP	ubiquitin-like protease
UPS	ubiquitin proteasome system
UTR	untranslated region
V	volt
v/v	volume per volume
w/v	weight per volume
Wt	wild-type
XTH	xyloglucan endotransglucosylase/ hydrolase
Y2H	yeast two-hybrid

Amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartate
E	Glu	Glutamate
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophane
Y	Tyr	Tyrosine
X		unspecific amino acid
ψ		large hydrophobic residue

Nucleotides

A	Adenine	
C	Cytosine	
G	Guanine	
T	Thymine	
U	Uracil	
R	A or G	Purine
Y	C or T	Pyrimidine
W	A or T	
S	C or G	
M	A or C	
K	G or T	
B	C, G or T not A	
D	A, G or T not C	
H	A, C or T not G	
V	A, C or G not T	
N	A, C, G or T	

AMP	Adenosine monophosphate
ATP	Adenosine-5'-triphosphate
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycitidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	2'-deoxynucleotide-5'-triphosphater
dTTP	2'-deoxythymidine-5'-triphosphate
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate