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An Abscisic Acid-Inducible Gene *NICOTINAMIDASE 3* Is Regulated by REPRESSOR OF SILENCING 1 in *Arabidopsis*

애기장대의 REPRESSOR OF SILENCING 1 에 의한 앱시스산 유도성 유전자 *NICOTINAMIDASE 3* 의 발현 조절

FEBRUARY, 2017

JOO YOUNG LIM

MAJOR IN HORTICULTURAL SCIENCE
AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

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UNDER THE DIRECTION OF DR. JIN HOE HUH
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
SEOUL NATIONAL UNIVERSITY

BY
JOO YOUNG LIM

MAJOR IN HORTICULTURAL SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE
THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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BY THE COMMITTEE MEMBERS

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ABSTRACT

Nicotinamide adenine dinucleotide (NAD^+) is a key electron-carrier during redox reactions in all living organisms. NAD^+ also plays critical roles in ADP-ribosylation, protein deacetylation and calcium signaling. In eukaryotes, NAD^+ can be synthesized via two metabolic pathways, the *de novo* and the salvage pathways. Here I report that *NICOTINAMIDASE 3 (NIC3)* which encodes a catalytic enzyme that converts nicotinamide into nicotinic acid in the NAD^+ salvage pathway was controlled by DNA demethylase REPRESSOR OF SILENCING1 (ROS1) in response to abscisic acid (ABA). The *ros1* mutants lost ABA-inducible expression of *NIC3* due to DNA hypermethylation at the upstream region of its promoter. The *nic3* mutants exhibited ABA hypersensitivity for seed germination and root elongation, and in addition, ectopic expression of *NIC3* rescued ABA hypersensitive phenotypes of the *ros1* mutant. This results suggest that *NIC3*

expression via ROS1-mediated DNA demethylation is crucial for ABA responses. I further investigated whether NIC3 conserves similar function of yeast homolog Pyrazinamidase/nicotinamidase 1 (Pnc1). Pnc1 has been known to promote rDNA silencing by increasing the activity of Silent information regulator 2 (Sir2), a NAD^+-dependent histone deacetylase, associated with lifespan extension. Consistent with the amino acid alignment data that NIC3 contains catalytic residues responsible for Pnc1 activity, heterologous expression of *NIC3* in yeast increased rDNA stability but failed to extend lifespan. Therefore, NIC3 is presumably a partially functional homolog of yeast Pnc1 and may have evolved to be involved in ABA responses with its expression regulated by active DNA demethylation in plants.

Key Words: Nicotinamide adenine dinucleotide, Nicotinamidase, Abscisic acid, DNA demethylation, REPRESSOR OF SILENCING 1, *Arabidopsis thaliana*, *Saccharomyces cerevisae*

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LIST OF ABBREVIATIONS

NAD^+	Nicotinamide adenine dinucleotide
PARP	Poly(ADP-ribose) polymerase
NMNAT	Nicotinate/nicotinamide mononucleotide adenyltransferase
Pnc1	Pyrazinamidase/nicotinamidase 1
Sir2	Silent information regulator 2
NIC1, 2, 3	NICOTINAMIDASE 1, 2, 3
ABA	Abscisic acid
ROS1	REPRESSOR OF SILENCING 1
DME	DEMETER
DML2, 3	DEMETER-LIKE 2, 3
EPF2	EPIDERMAL PATTERNING FACTOR 2
RdDM	RNA-directed DNA methylation
wt	Wild-type
NADP	Nicotinamide adenine dinucleotide phosphate
ATP	Adenosine triphosphate
NAMPT	Nicotinamide phosphoribosyltransferase
SIRT1	Sirtuin 1
MEP	2-C-methyl-d-erythritol-4-phosphate

NCED	9-cis-epoxycarotenoid dioxygenase
P450	Cytochrome P450 monooxygenase
PA	Phaseic acid
ABA-GE	ABA glucosylester
START	StAR-related lipid-transfer
PYR1	PYRABACTIN RESISTANCE 1
PYL	PYR1-like protein
RCAR	Regulatory components of the ABA receptor
PP2C	Type 2C protein phosphatase
ABI1, 2, 5	ABA insensitive 1, 2, 5
HAB	Hypersensitive to ABA
SnRK2	Sucrose non-fermenting related kinase 2
bZIP	basic leucine zipper
ABRE	ABA-responsive element
AREB	ABRE-binding protein
ABF	ABRE-binding transcription factor
LEA	Late embryo genesis abundant
SLAC1	SLOW ANION CHANNEL-ASSOCIATED 1
KAT1	POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA 1

HMT	Histone methyltransferase
HAT	Histone acetyltransferase
HDA	Histone deacetylase
ATX1	ARABIDOPSIS TRITHORAX-LIKE PROTEIN 1
RD29A, B	RESPONSIVE TO DESICCATION 29A, B
5mC	5-methylcytosine
TET	Ten-eleven translocation
BER	Base excision repair
HhH	helix-hairpin-helix
GPD	Glycine/proline-rich loop with a conserved aspartic acid
MEA	MEDEA
FWA	FLOWERING WAGENINGEN
FIS2	FERTILIZATION INDEPENDENT SEED2
MBD7	METHYL-CpG-BINDING DOMAIN PROTEIN 7
IDM	INCREASED DNA METHYLATION
MS	Murashige and Skoog
CaMV	Cauliflower mosaic virus
RT-PCR	Reverse transcription PCR
PGK	Phosphoglycerate kinase

ADH	Alcohol dehydrogenase
SC	Synthetic complete
qRT-PCR	Quantitative RT-PCR
DAS	Day-after sowing
BS-seq	Bisulfite sequencing
TE	Transposable element
FC	Fold change
FDR	False discovery rate
DMR	Differentially methylated region
A. thaliana	*Arabidopsis thaliana*
O. sativa	*Oryza sativa*
C. elegans	*Caenorhabditis elegans*
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
Msn2, 4	Multicopy suppressor of SNF1 mutation 2, 4

INTRODUCTION

Energy and metabolic homeostasis is achieved in all living organisms under normal condition. On exposure to stresses, the energy balance between energy production and consumption is impaired (De Block and Van Lijsebettens, 2011). Due to their sessile nature, plants are constantly exposed to a variety of harsh environmental conditions such as an excess or a deficit of light, water, salt and temperature. As a result, plants are confronted by energy deprivation and growth arrest, and, eventually, plants will die if the energy level decreases below a threshold (Baena-Gonzalez and Sheen, 2008).

Photosynthesis, a process by which solar energy is converted into chemical energy, is followed by cellular respiration crucial for energy homeostasis in that it releases energy stored in fixed carbon for cellular use. Nicotinamide adenine dinucleotide (NAD^+) acts as a common electron-carrying cofactor during diverse redox reactions including cellular respiration. NAD^+ also plays vital roles in ADP-ribosylation, protein deacetylation and calcium signaling (Guse, 2015; Imai et al., 2000; Janke et al., 2015; Schreiber et al., 2006). Eukaryotes possess two metabolic pathways of NAD^+ biosynthesis, the *de novo* and salvage pathways, in order to maintain the cellular NAD^+ levels to avoid cell death (Hashida et al., 2009; Janke et al., 2015). In the *de novo* pathway, either tryptophan or aspartate serves as a precursor. Alternatively, nicotinamide, a degradation product from NAD^+ via sirtuin-mediated deacetylation or ADP-ribosylation, is converted back to NAD^+ in a series of reactions in the energy-efficient salvage pathway (Hunt et al., 2004; Janke et al., 2015; Katoh et al., 2006). In *Arabidopsis*, many enzymes involved in the NAD^+

salvage pathway such as Poly(ADP-ribose) polymerase (PARP) and Nicotinate/nicotinamide mononucleotide adenyltransferase (NMNAT) have been reported to play important roles in stress responses (De Block et al., 2005; Hashida et al., 2010; Vanderauwera et al., 2007).

Nicotinamidase is an enzyme that catalyzes the deamination of nicotinamide to generate nicotinic acid and ammonia in the NAD^+ salvage pathway. Pyrazinamidase/nicotinamidase 1 (Pnc1), identified as a nicotinamidase in yeast, has drawn attention to its function as an activator of Silent information regulator 2 (Sir2), a NAD^+-dependent histone deacetylase (sirtuin) critical for transcriptional silencing at the mating-type loci, telomeres and rDNA locus associated with aging (Blander and Guarente, 2004). Under calorie restriction condition which promotes respiration, an increase in the NAD^+/NADH ratio activates Sir2, resulting in lifespan extension via repressing rDNA recombination and reducing levels of toxic extrachromosomal rDNA circles (Lin et al., 2002; Lin et al., 2004; Sinclair and Guarente, 1997). Remarkably, the expression of Pnc1 is upregulated by stimuli that extends lifespan such as calorie restriction, heat stress, and salt stress, and therefore, the decreased levels of nicotinamide which is a noncompetitive inhibitor of Sir2 lead to increased lifespan (Anderson et al., 2003; Gallo et al., 2004; Sauve et al., 2005). These reports suggest that Pnc1-mediated Sir2 function links metabolite levels to transcriptional regulation through histone deacetylation in response to nutritional and environmental changes.

Nicotinamidase activity is conserved in bacteria, yeast, plants and invertebrates, and may play critical roles in modulating sirtuin functions (Balan et al., 2008; Ghislain et al., 2002; Janke et al; 2015; Vrablik et al., 2009). In *Arabidopsis*,

three *NICOTINAMIDASE (NIC)* genes *NIC1, NIC2* and *NIC3* are designated as homologs of yeast *PNC1* (Hunt et al., 2004). All three nicotinamidases have the catalytic activity of converting nicotinamide into nicotinic acid *in vitro* (Hunt et al., 2007; Wang and Pichersky, 2007). Moreover, *nic1* and *nic2* mutants exhibited hypersensitivity to salt and abscisic acid (ABA), a key phytohormone mediating transcriptional regulation in response to osmotic stresses involving drought and salinity (Hunt et al., 2007; Wang and Pichersky, 2007). These results suggest a biological function of nicotinamidases in ABA-mediated osmotic stress responses. Additionally, in rice (*Oryza sativa*), a recent study has reported that the increased concentration of nicotinamide which is the substrate of OsNIC inhibits the expression of *OsSRT*s, homologs of *SIR2*-like genes, leading to leaf senescence (Wu et al., 2016). To identify the link among environmental changes, metabolite levels and nicotinamidase-mediated stress responses through epigenetic process, further studies are required.

DNA methylation is a stable but reversible epigenetic mark essential for diverse biological processes such as gene imprinting, transposon silencing and X chromosome inactivation. DNA methylation primarily occurs in the CG context in mammals, whereas DNA methylation in plants takes place at cytosine residue in all sequence contexts: symmetric CG and CHG (where H indicates A, C or T) and asymmetric CHH (Law and Jacobson, 2010). In *Arabidopsis*, DNA methylation can be actively removed by the plant-specific DNA glycosylases/lyases including REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2) and DML3 (Law and Jacobson, 2010; Penterman et al., 2007). In contrast to DME which is expressed in the central cell and responsible for seed development

(Gehring, 2013; Huh et al., 2008), ROS1, DML2, and DML3 are expressed in the vegetative tissues with their biological functions poorly understood. ROS1 is required for inhibiting transcriptional silencing of transgenes and endogenous genes, and together with DML2 and DML3, prevents excessive DNA methylation at genomic regions (Gong et al., 2002; Lister et al., 2008; Penterman et al., 2007; Qian et al., 2012; Stroud et al., 2013). Recent studies have reported that ROS1 regulates the expression of *EPIDERMAL PATTERNING FACTOR 2* (*EPF2*) in stomatal development and stress-responsive genes required for *Fusarium oxysporum* resistance by opposing RNA-directed DNA methylation (RdDM) at transposable elements in their promoter regions(Le et al., 2014; Yamamuro et al., 2014). However, the molecular mechanism by which DME/ROS1 DNA glycosylases are recruited to specific target loci remains unclear (Lang et al., 2015).

Here I report that *ros1* mutants exhibited ABA hypersensitivity in seed germination and root elongation, suggesting that some ABA-responsive genes were dysregulated associated with DNA hypermethylation. Most notable among them is *NIC3*. In *ros1* mutants, an ABA-inducible expression of *NIC3* was lost due to excessive DNA methylation of transposable elements near the promoter region compared to wild-type (*wt*). The *nic3* mutants displayed hypersensitivity to ABA, and moreover, ectopic expression of *NIC3* restored normal seed germination and root growth rates in *ros1* mutants. These findings suggest that ROS1-mediated *NIC3* expression plays vital roles in ABA response. Among three nicotinamidase genes in *Arabidopsis*, the function of NIC3 has not been elucidated yet. In accordance with the fact that nicotinamidase activity is conserved in many eukaryotes, database searches revealed that catalytic residues responsible for yeast Pnc1 activity were

conserved in bacteria, worm, drosophila and plants including *Arabidopsis*. I further examined whether NIC3 conserves similar functions as Pnc1. Heterologous expression of *NIC3* increased rDNA stability in wt yeast cells, but could not extend the lifespan in wt and *pnc1Δ* yeast cells. Taken these together, NIC3 is presumed to have the partially conserved function of Pnc1 and might have evolved to play crucial roles in ABA responses with its expression regulated by ROS1 in *Arabidopsis*.

LITERATURE REVIEWS

1. Nicotinamidase

Nicotinamide adenine dinucleotide (NAD^+) and its derivative nicotinamide adenine dinucleotide phosphate (NADP) are cofactors which carry electrons in diverse metabolic redox reactions of all living organisms. NAD^+ is also involved in calcium signaling, ADP-ribosylation and protein deacetylation (Guse 2015; Imai et al., 2000; Janke et al., 2015; Schreiber et al., 2006). In order to maintain the cellular NAD^+ levels for these reactions, there are two pathways of NAD^+ biosynthesis, the *de novo* and salvage pathways. In the de novo pathway, NAD^+ is synthesized from either tryptophan or aspartate by using five adenosine triphosphates (ATPs). On the other hand, in the salvage pathway, nicotinamide which is broken down from NAD^+ via ADP-ribosylation or sirtuin-based deacetylation is recycled back to NAD^+ followed by a series of reactions (Hashida et al., 2009; Hunt et al., 2004; Janke et al., 2015; Katoh et al., 2006) (Figure 1).

Nicotinamidase is an enzyme that converts nicotinamide into nicotinic acid in the NAD^+ salvage pathway. In yeast, Pyrazinamidase/nicotinamidase 1 (Pnc1) which shows 30 percent of amino acid sequence identity with *Escherichia coli* PncA was identified as the nicotinamidase (Ghislain et al., 2002). Pnc1 has attracted attention because its product nicotinamide serves as a noncompetitive inhibitor of Silent information regulator 2 (Sir2), a NAD^+-dependent histone deacetylase (sirtuin) essential for chromatin silencing at the mating-type loci, telomeres and rDNA locus associated with longevity (Blander and Guarente, 2004). Calorie restriction is known to extend lifespan through the activation of Sir2 by promoting respiration which

increases the NAD^+/NADH ratio. The enhanced Sir2 activity inhibits rDNA recombination and thereby prevents the accumulation of toxic extrachromosomal rDNA circles (Lin et al., 2002; Lin et al., 2004; Sinclair and Guarente, 1997). In addition, Sir2 activity is regulated by the levels of nicotinamide, an inhibitor of Sir2, as well as the NAD^+/NADH ratio. Diverse stimuli such as calorie restriction, heat stress and high osmolarity are known to upregulate the level of Pnc1 (Anderson et al., 2003). The deletion of *PNC1* fails to extend lifespan due to the elevated levels of nicotinamide, whereas overexpression of *PNC1* leads to lifespan extension through Sir2-mediated rDNA silencing (Anderson et al., 2003; Gallo et al., 2004; Sauve et al., 2005). These reports have suggested that Pnc1-mediated Sir2 function links metabolite levels, in response to nutritional and environmental changes, to transcriptional regulation through histone deacetylation.

Nicotinamidase whose activity is conserved in bacteria, yeast, plants and invertebrates may play pivotal roles in modulating sirtuin functions (Balan et al., 2008; Ghislain et al., 2002; Janke et al., 2015; Vrablik et al., 2009). In mammals, instead of nicotinamidase generating nicotinic acid, nicotinamide mononucleotide by nicotinamide phosphoribosyltransferase (NAMPT) converts nicotinamide into NAD^+ via the salvage pathway (Revollo et al., 2004; Schweiger et al., 2001). However, nicotinamide levels are also crucial for mammalin sirtuin functions as in yeast. Nicotinamide negatively regulates NAD^+-dependent deacetylation of p53 tumor suppressor protein induced by the Sir2 homologue SIRT1, which results in promoted apoptosis during DNA damage and oxidative stress responses (Bitterman et al., 2002; Luo et al., 2001). Furthermore, the turnover of nicotinamide by Nampt extends the lifespan of human vascular smooth muscle cells with enhanced resistance

to oxidative stress via the optimization of Sirtuin 1 (SIRT1)-mediated p53 degradation (van der Veer et al., 2007). In addition to SIRT1, nicotinamide inhibits the activity of poly(ADP-ribose) polymerase (PARP) which catalyzes the cleavage of NAD^+ into nicotinamide and ADP-ribose and then polymerizes ADP-ribose on nuclear-acceptor proteins during DNA repair and chromatin decondensation associated with stress-inducible genes (Virag et al., 2002).

Even though the NAD^+ salvage pathway of yeast and mammals diverges at nicotinamide, plants recycle NAD^+ in the same manner as in yeast. *Arabidopsis* genome contains three *NICOTINAMIDASE (NIC)* genes *NIC1*, *NIC2* and *NIC3* which are homologs of yeast *PNC1* (Hunt et al., 2004). Both NIC1 and NIC2 have the catalytic activity of the deamination of nicotinamide to produce nicotinic acid *in vitro*, and both *nic1* and *nic2* mutants exhibited hypersensitive phenotypes to ABA and salt (Hunt et al., 2007; Wang and Pichersky, 2007). These studies suggest that nicotinamidases are important for ABA-mediated osmotic stress responses in plants. Many other enzymes in the NAD^+ salvage pathway are also known to be involved in stress responses. A decrease in activity of PARP catalyzing ADP-ribose polymerization in conjunction with the cleavage of NAD^+ into nicotinamide and ADP-ribose maintains energy homeostasis by a reduction in NAD^+ consumption, which leads to enhanced stress tolerance through the alteration of stress signal transduction including upregulated expression of ABA-responsive genes (De Block et al., 2005; Vanderauwera, 2007). Nicotinate/nicotinamide mononucleotide adenyltransferase (NMNAT) is also responsible for drought stress tolerance via ABA-mediated stomatal movements (Hashida et al., 2010). Additionally, *nic2* mutant seeds showed delayed germination with impaired germination potential and

reduced poly(ADP-ribosylation) levels resulting from decreased levels of nicotinamide, an inhibitor of PARP (Hunt et al., 2007). Remarkably, in rice (*Oryza sativa*), the increased level of nicotinamide, the substrate of nicotinamidase (OsNIC), inhibits expression of *SIR2*-like genes (Os*SRT*s), then leading to leaf senescence (Wu et al., 2016). To elucidate the link between NIC and cellular processes including stress responses in plants, further research is required.

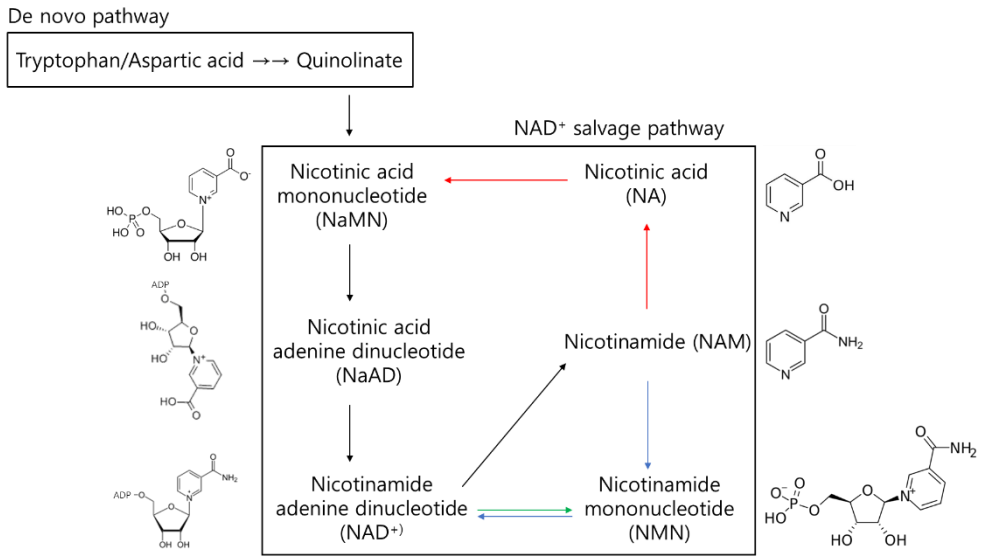

Figure 1. The NAD$^+$ biosynthetic pathways of eukaryotes.

Black arrows indicate salvage reaction reported from yeast, plants and mammals. Red and Blue arrows indicate salvage reaction reported from yeast and plants and from plants and mammals, respectively. A green arrow indicates salvage reaction reported only from plants (Hashida et al., 2009; Janke et al., 2015).

2. Abscisic acid-mediated stress responses

Abscisic acid (ABA) is a key phytohormone crucial for growth and development including seed maturation, seed dormancy, stomatal aperture and environmental stress responses and tolerance. On exposure to osmotic stresses such as drought and high salinity, ABA is accumulated in plant cells resulting from the activation of ABA synthesis and the inhibition of both ABA catabolism and formation of inactive ABA conjugates (Finkelstein, 2013; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002).

ABA biosynthesis begins with the 2-C-methyl-d-erythritol-4-phosphate (MEP) pathway in plastids, followed by the production of C_{40} carotenoids (Hirai et al., 2000). The *cis*-isomers of violaxanthin and neoxanthin are cleaved by *9-cis*-epoxycarotenoid dioxygenase (NCED) to release the C_{15} product xanthoxin, which in turn is converted to ABA via the intermediate abscisic aldehyde. The rate-limiting step of ABA biosynthesis is regarded as the cleavage step catalyzed by NCED, the expression of which is tightly regulated in response to osmotic stresses (Nambara and Marion-Poll, 2005; Qin and Zeevaart, 1999). In *Arabidopsis*, *NCED3* expression is considerably induced by dehydration, and overexpression of *NCED3* increases both ABA levels and drought tolerance (Iuchi et al., 2001; Thompson et al., 2000).

In addition to ABA biosynthesis, ABA catabolism is important for regulating ABA levels. There are two types of ABA catabolism, hydroxylation and conjugation in *Arabidopsis* (Finkelstein, 2013). Among several ABA hydroxylation pathways, the predominant catabolic pathway is the hydroxylation of ABA at C-8' position by cytochrome P450 monooxygenase, leading to the formation of 8'-OH-ABA which is then isomerized to phaseic acid (PA) (Nambara and Marion-Poll,

2005). *Arabidopsis CYP707A* which encodes an ABA 8'-hydroxylase is upregulated, and PA levels are increased upon subsequent rehydration after dehydration (Kushiro et al., 2004). Furthermore, conjugation of ABA or its metabolites with glucose, commonly existing as ABA glucosylester (ABA-GE), can inactivate ABA. In *Arabidopsis,* the activity of ß-d-glucosidase which releases ABA from ABA-GE is rapidly increased by dehydration-induced polymerization (Lee et al., 2006).

To effectively regulate ABA-mediated stress responses, it is necessary to perceive ABA and amplify the signal through signal transduction pathways. The core ABA signaling pathway was established by the identification of soluble ABA receptors of StAR-related lipid-transfer (START) protein family such as PYRABACTIN RESISTANCE 1 (PYR1), PYR1-like proteins (PYLs) and regulatory components of the ABA receptors (RCARs) (Nakashima and Yamaguchi-Shinozaki, 2013; Umezawa et al., 2010). ABA receptors negatively regulate the activity of type 2C protein phosphatases (PP2Cs) such as ABA insensitive 1 (ABI1), ABI2 and hypersensitive to ABA (HAB1) (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). In the absence of ABA, the PP2Cs can inactivate several kinases of sucrose non-fermenting related kinase 2 (SnRK2) family by direct dephosphorylation (Umezawa et al., 2009; Vlad et al., 2009). In response to environmental stresses, ABA accumulates in plant cells and binds to PYR/PYL/RCARs, thereby promoting the interaction of PYR/PYL/RCARs and PP2C. In turn, the dephosphorylation activity of PP2Cs is inhibited, leading to the activation of SnRK2s and subsequent phosphorylation of downstream factors (Nakashima and Yamaguchi-Shinozaki, 2013; Umezawa et al., 2010).

The downstream targets of SnRK2s include transcription factors and ion

channels required for ABA-responsive gene expression and rapid stomatal movements, respectively. The best-characterized transcription factors conferring ABA inducibility are basic leucine zipper (bZIP) family transcription factors such as ABI5 and ABA-responsive elements (ABRE)-binding proteins (AREBs)/ ABRE-binding transcription factors (ABFs) (Cutler et al., 2010; Nakashima and Yamaguchi-Shinozaki, 2013). The *areb1 areb3 abf3* triple mutants exhibit increased resistance to ABA with reduced drought tolerance and misregulation of stress-responsive genes including the late embryo genesis abundant (LEA) class genes (Yoshida et al., 2010). In addition, membrane proteins including ion channels such as the slow anion channel SLAC1 and the inward-rectifying potassium channel KAT1 have essential roles in stomatal closure induced by osmotic stress. SLAC1 and KAT1 are activated or repressed respectively via SnRK2-mediated phosphorylation to promote stomatal closure (Geiger et al., 2009; Sato et al., 2009). Not only genetic processes but also epigenetic mechanisms mediated by epigenetic modifiers involving ATP-dependent chromatin remodelers, histone methyltransferases (HMTs) and histone acetyltransferases (HATs)/deacetylases (HDAs) play an important role for ABA signaling and ABA-dependent gene expression (Finkelstein, 2013; Fujita et al., 2011; Kim et al., 2015). The SWI3 subunit and BRAHMA ATPase of the SWI/SNF chromatin remodeling complex can directly interact with HAB1 and ABI5/ABI3, respectively, which might regulate ABA-responsive gene expression (Saez et al., 2008; Han et al., 2012). Furthermore, under drought stress, *NCED3*, encoding a key enzyme of ABA synthesis, is activated with increased H3K4me3 marks by the *Arabidopsis* histone methyltransferase trithorax-like 1 (ATX1), the loss of which results in the reduced expression of ABA-

inducible genes such as *RESPONSIVE TO DESICCATION 29A (RD29A)* and *RD29B* (Ding et al., 2011). Moreover, overexpression of *AtHD2C* induced ABA insensitivity and enhanced salinity and drought tolerance, and the *hda6* and *hda19* mutants showed ABA hypersensitivity in germination and growth, with a decrease in ABA-responsive gene expressions (Sridha and Wu, 2006; Chen and Wu, 2010). These reports suggest that epigenetic modifications can contribute to dynamic regulation of ABA signaling and responses.

3. DNA demethylation in plants

DNA methylation, an epigenetic mark which indicates the conversion of cytosine to 5-methylcytosine (5mC), plays essential roles in diverse biological processes such as gene imprinting, transposon silencing and X-chromosome inactivation. In mammals, DNA methylation primarily takes place in the symmetric CG context. In contrast, DNA methylation in plants occurs at cytosine bases in all sequence contexts: symmetric CG and CHG (where H indicates A, C or T) and assymetric CHH (Law and Jacobsen, 2010).

DNA demethylation is a process by which DNA methylation marks are removed. DNA demethylation which is involved in diverse cellular processes of plants and animals can take place either by passively or actively. Passive DNA demethylation occurs when maintenance methyltransferase is inhibited or absent during DNA replication, whereas active DNA demethylation is the enzymatic mechanism independent of DNA replication (Wu and Zhang, 2010). In mammals, instead of direct removal of 5mC, 5mC is converted into an intermediate base via Ten-eleven translocation (TET) family-mediated hydroxylation/oxidation or AID/APOBEC family-mediated deamination, followed by the base excision repair (BER) pathway (Kohli and Zhang, 2013; Law and Jacobsen, 2010; Zhang and Zhu, 2012). In plants, however, active demethylation begins with the direct excision of 5mC by the plant-specific DNA glycosylase activity, resulting in unmethylated cytosine through the BER pathway (Zhu, 2009).

Four DNA glycosylase family members are found in *Arabidopsis* - REPRESSOR OF SILENCING 1 (ROS1), DEMETER (DME), DEMETER-LIKE 2 (DML2) and DML3 (Law and Jacobson, 2010; Penterman et al., 2007). The DNA

glycosylases, referred to as DNA demethylases, are bifunctional DNA glycosylases/lyases that break the *N*-glycosidic bond and cleave the sugar-phosphate backbone resulting in β- and δ-elimination products (Gehring et al., 2006; Mok et al., 2010). The gap must be processed to generate 3'-OH and then presumably filled via the BER pathway (Lee et al., 2014; Zhu, 2009). The ROS1/DME DNA glycosylases possess the glycosylase domain which consists of a helix-hairpin-helix (HhH) motif, a glycine/proline-rich loop with a conserved aspartic acid (GPD) and adjacent four cysteine residues that may hold a [4Fe-4S] cluster (Mok et al., 2010). Furthermore, they contain two additional conserved domains of unknown function, which is distinct from other DNA glycosylases.

DME is preferentially expressed in the central cell of the female gametophyte and plays pivotal roles in establishing imprinted gene expression (Choi et al., 2002; Gehring, 2013). DME was known to activate maternal allele-specific expression of *MEDEA* (*MEA*), *FLOWERING WAGENINGEN* (*FWA*) and *FERTILIZATION INDEPENDENT SEED2* (*FIS2*) by erasing DNA methylation in the central cell and endosperm, leading to seed development (Gehring, 2013; Huh et al., 2008; Law and Jacobsen, 2010). In contrast to *DME*, *ROS1*, *DML2* and *DML3* are broadly expressed in vegetative tissues. ROS1, along with DML2 and DML3, is essential for preventing the spreading of DNA methylation at genomic regions (Lister et al., 2008; Penterman et al., 2007; Stroud et al., 2013; Qian et al., 2012). Especially, ROS1 plays significant roles in the inhibition of transcriptional silencing of transgenes and endogenous genes (Gong et al., 2002; Zhu et al., 2007). Although the biological function of ROS1 remains unclear, recent studies suggest that ROS1-mediated active demethylation controls the expression of *EPIDERMAL*

PATTERNING FACTOR 2 (*EPF2*) for stomatal development and *Fusarium oxysporum*-responsive genes by opposing RNA-directed DNA methylation (RdDM) action at transposable elements (Yamamuro et al., 2014; Le et al., 2014). These studies also indicate that ROS1 regulates the expression of these specific genes targeted by RdDM. The current model hypothesizes that METHYL-CpG-BINDING DOMAIN PROTEIN 7 (MBD7) and INCREASED DNA METHYLATION (IDM) proteins prevent DNA methylation spread by facilitating ROS1-mediated DNA methylation. However, the molecular mechanism of how DME/ROS1 DNA glycosylases regulate the expression of specific target genes remains to be elucidated (Lang et al., 2015).

MATERIALS AND METHODS

Plant materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used as a wt line in this study. The *ros1-3* seeds were kindly provided by Prof. Robert Fischer of UC Davis (Penterman et al., 2007). The homozygous T-DNA insertion lines including *ros1-4* (SALK_045303), *nic3-1* (SALK_034040) and *nic3-2* (SALK_107343) were obtained from *Arabidopsis* Biological Resource Center (ABRC). Seeds were sterilized and stratified at 4 °C for 3 days in the dark. They were sown on Murashige and Skoog (MS) medium, then grown in a growth chamber at 22 °C under 16 h of fluorescent light at 30 ± 10 μmol m^{-2} s^{-1}.

To generate the transgenic line overexpressing *NIC3*, the *NIC3* coding sequence was PCR-amplified from the *Arabidopsis* cDNA with primers DG2597 and DG2599 and then cloned into the *Kpn* I and *Xba* I sites of the pBI101 vector under the control of cauliflower mosaic virus (CaMV) 35S promoter. The empty pBI101 vector and the verified construct were respectively transformed into the *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method. Transgenic *Arabidopsis* plants in the *ros1-4* background were generated by *Agrobacterium*-mediated floral dip method. T1 candidate seeds were screened on MS agar plates containing kanamycin as selection antibiotics and then examined for the vector sequence by genotyping with primers DG1204 and DG2404 and for *NIC3* expression levels by reverse transcription PCR (RT-PCR) with primers DG1948 and DG1949. Verified T2 lines were used for further analysis.

Yeast strains and media

Yeast strains used in this study are listed in Table 2. Strains for analyzing replicative lifespan and rDNA silencing were obtained from the EUROSCARF collection and kindly from Prof. Won-Ki Huh of Seoul National University, respectively. For the heterologous expression of *AtNIC3* in *Saccharomyces cerevisiae*, the coding sequence of *NIC3* was PCR-amplified from the *Arabidopsis* cDNA with primers DG2698 and DG2699 and then cloned into the *Eco* RI and *Xho* I sites of the pYES5 vector and the p413ADH vector, respectively. These vectors contain the *URA3* or the *HIS3* marker gene and the constitutive phosphoglycerate kinase (*PGK*) promoter or alcohol dehydrogenase (*ADH*) promoter, respectively. The pYES5 vector and the NIC3-pYES5 construct were transformed into wt (BY4742), *pnc1*Δ (Y13738) and *sir2*Δ (Y14401) strains for replicative lifespan analysis, and the p413ADH vector and the NIC3- p413ADH construct were transformed into wt (DMY2798 and DMY2804) and *sir2*Δ (HY1746 and HY1748) strains for rDNA silencing assay by lithium acetate method. Yeast cells were grown at 30 °C on rich medium (YPD; 1% yeast extract, 2% peptone, 2% glucose), calorie restriction medium containing 0.5% glucose and selective synthetic complete (SC) medium lacking appropriate amino acids.

Screening of ABA sensitivity

ABA (A1049, Sigma-Aldrich) treatment during seed germination and root elongation assays was performed. For seed germination assay, seeds were sterilized and stratified for 7 days were sown on MS medium with increasing concentrations of ABA (0 to 1.2 μM). Germination frequencies were evaluated every 4 days. For

root elongation assay, 4-day-old seedlings growing in normal MS medium were transferred to MS medium supplemented with ABA (0 to 20 μM) and were allowed to grow vertically for 14 days.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Plant and yeast samples for RNA extraction were prepared with three biological replicates. Total *Arabidopsis* RNA was isolated from 17-day-old seedlings with or without 20 μM ABA treatment using TRIzol® Reagent (Life Technologies) according to the manufacturer's protocol. For each biolocial replicate, three plants were pooled to obtain a single sample for RNA purification. Total RNA from yeast cells were isolated with the RNeasy MiniKit (QIAGEN).

The cDNA synthesis was conducted using the QuantiTech Reverse Transcription kit (QIAGEN). Quantitative RT-PCR was performed using the Roter-Gene Q (QIAGEN) with SYBR green Q-master mix (Genet Bio). *Arabidopsis UBIQUITIN10* or yeast *ACTIN1* was used as a quantitative control for the calculation of relative transcript level of target gene. Primer sets used for qRT-PCR were listed in Table 1.

Assessment of Pnc1 homologs from public databases and sequence analysis

Protein sequences of Pnc1 homologs identified from bacteria, yeast, plants and invertebrates were obtained from public databases involving EcoGene 3.0 (http://www.ecogene.org/), Saccharomyces Genome Database (SGD; http://www.yeastgenome.org/), Phytozome v11.0 (https://phytozome.jgi.doe.gov/), FlyBase (http://flybase.org/) and WormBase (http://www.wormbase.org/) (Table 1).

Amino acid alignment of Pnc1 homologs was performed using ClustalX v2.1 (Larkin et al., 2007) and GeneDoc v2.7.00 (Nicholas et al., 1997).

Replicative lifespan analysis

A single colony from each strain was picked from the selection medium and patched onto the calorie restriction medium (0.5 % glucose, 1 % yeast extract, 2 % peptone). Cells were picked and arrayed in vertical lines, then incubated at 30 °C for 3 h. After the isolation of newly budded (virgin) for lifespan analysis, twenty virgin cells were incubated at 30 °C for 2 hrs to divide at least once. The daughter cells were separated from the mother cells by gently tapping a fiber-optic glass needle on the plate, then counted and discarded using a Zeiss Tetrad Microscope. This procedure was continued until all the mother cells cease cell division.

rDNA silencing assay

Yeast cells were grown in SC media lacking histidine (SC-His) to an OD_{600} of 1.0 at 30 °C. The 10-fold serial dilutions of cell suspensions were spotted on SC-His and SC media lacking histidine and uracil (SC-His-Ura) to measure the silencing of the *mURA3* reporter gene at the rDNA region. Plates were incubated at 30 °C for 2 days.

Table 1. List of primers

Name	Sequence (5'→3')	Purpose
DG810	CGATGGATCTGGAAAGGTTC	qRT-PCR
DG811	AGCTCCACAGGTTGCGTTAG	qRT-PCR
DG853	CCAGTTAAGGACAGAACACCG	Genotyping
DG854	TCGTCTTTCGATCAAATCCAC	Genotyping
DG964	TGGAAGGGATCCGTCGTGGATTCT	Genotyping
DG965	CCCGCGACTCTTGATTGTTTCAGCAACTT	Genotyping
DG1204	GATTTCACGGGTTGGGGTTTCT	RT-PCR
DG1948	CAAGTAACCGGACCAGACG	qRT-PCR
DG1949	GGAGGCGGGTTTTGTTAAA	qRT-PCR
DG2124	CAGCATGACTTAGATGCTTTTAGC	Genotyping
DG2125	TACTGGGAACGGAAACGTATG	Genotyping
DG2404	CCCACTATCCTTCGCAAGAC	RT-PCR
DG2597	AATTGGTACCATGGCTTCCTCATCAACG	Cloning
DG2599	AATTTCTAGATTAGTTACCGAGTAGACTTC	Cloning
DG2698	AATTGAATTCATGGCTTCCTCATCAACG	Cloning
DG2699	AATTCTCGAGTTAGTTACCGAGTAGACTTC	Cloning
DG2966	TGACTGACTACTTGATGAAG	qRT-PCR
DG2967	TGCATTTCTTGTTCGAAGTC	qRT-PCR

Table 2. List of yeast strains

Strain	Genotype
BY4742	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0*
JY001	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* [pYES5]
JY002	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* [pYES5 *NIC3*]
Y13738	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 pnc1Δ*::*kanMX4*
JY003	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 pnc1Δ*::*kanMX4* [pYES5]
JY004	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 pnc1Δ*::*kanMX4* [pYES5 *NIC3*]
Y14401	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sir2Δ*::*kanMX4*
JY005	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sir2Δ*::*kanMX4* [pYES5]
JY006	*MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 sir2Δ*::*kanMX4* [pYES5 *NIC3*]
DMY2798	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3*
JY101	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3* [p413ADH]
JY102	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3* [p413ADH *NIC3*]
HY0245	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3 sir2Δ*::*TRP1*
JY107	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3 sir2Δ*::*TRP1* [p413ADH]
JY108	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 leu2*::*mURA3 sir2Δ*::*TRP1* [p413ADH *NIC3*]
DMY2804	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3*
JY201	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3* [p413ADH]
JY202	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3* [p413ADH *NIC3*]
HY0291	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3 sir2Δ*::*TRP1*
JY207	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3 sir2Δ*::*TRP1* [p413ADH]
JY208	*MAT***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11 can1-100 RDN1-NTS1*::*mURA3 sir2Δ*::*TRP1* [p413ADH *NIC3*]

Table 3. List of Pnc1 homologs used in amino acid alignments

Protein ID	Length (aa)	Organism Name
AT2G22570.1	244	*Arabidopsis thaliana*
AT5G23220.1	198	*Arabidopsis thaliana*
AT5G23230.1	198	*Arabidopsis thaliana*
Y38C1AA.3a	335	*Caenorhabditis elegans*
Y57G11C.47	316	*Caenorhabditis elegans*
FBpp0083200	357	*Drosophila melanogaster*
P21369	213	*Escherichia coli*
LOC_Os04g44420.1	158	*Oryza sativa*
YGL037C	216	*Saccharomyces cerevisiae*

RESULTS

The *ros1* mutants exhibited hypersensitivity to ABA

ABA is a major plant hormone critical for growth and development such as seed dormancy, stomatal aperture and environmental stress responses. Under osmotic stress conditions, ABA accumulates in plant cells and binds to receptors, resulting in the amplification of the signal through signal transduction pathway, and thereby regulating the transcription of ABA-responsive genes (Cutler et al., 2010; Nakashima and Yamaguchi-Shinozaki, 2013).

Recent studies reported that epigenetic mechanisms including DNA methylation, histone modification and chromatin remodeling as well as genetic processes contribute to ABA signaling and ABA-dependent gene expression in osmotic stress responses (Finkelestein, 2013; Fujita et al., 2011; Kim et al., 2015; Reinders et al., 2009). To investigate the effect of DNA methylation on transcriptional regulation of ABA-responsive genes, genetic screens were performed on *Arabidopsis* mutants defective in DNA methylation components for ABA sensitivity. Two *ros1* T-DNA insertion lines *ros1-3* and *ros1-4* were found to be hypersensitive to ABA during seed germination and root elongation (Figure 2A-E). Under mock conditions, *ros1* mutants displayed similar seed germination rates to the wt. These observations suggest ROS1 may play a key role in ABA responses.

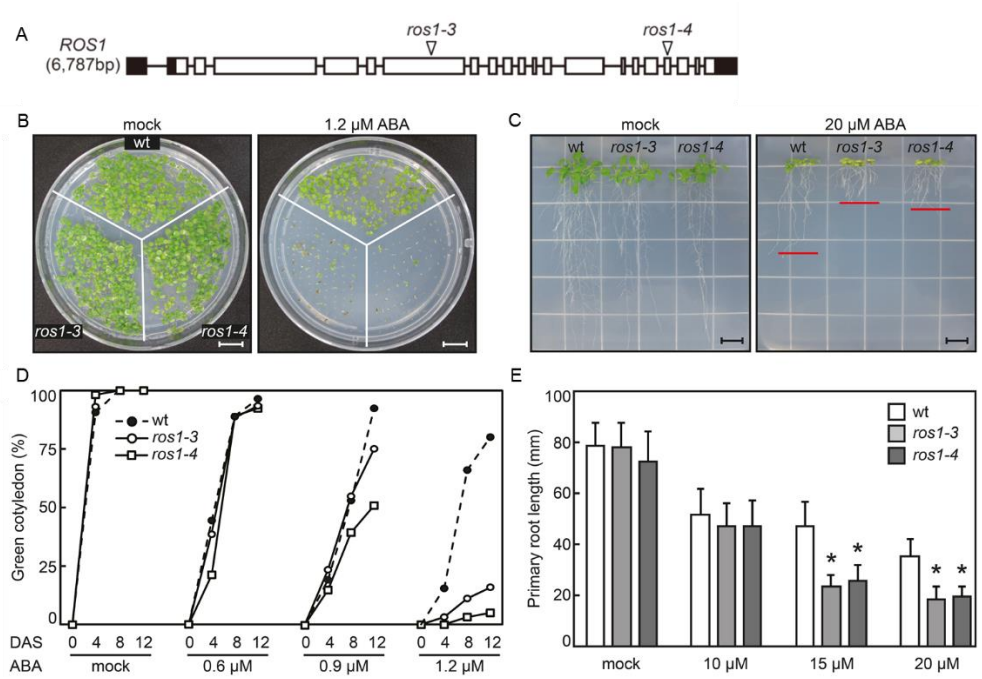

Figure 2. ABA hypersensitivity of *ros1* mutants

(A) Schematic diagram of the *ROS1* gene structure. The relative locations of T-DNA insertions (*ros1-3* and *ros1-4*) are shown. Lines indicate introns while closed and open boxes indicate untranslated regions and coding sequences, respectively. **(B)** Seed germination at 12 day-after sowing (DAS) on MS media under mock or ABA condition. **(C)** Germination frequency evaluated every 4 days. Every point refers to the percentage of green cotyledons that emerged from the total sown seeds (n>80). **(D)** Root elongation at 19 DAS on MS media under mock or ABA condition. **(E)** Primary root length at 19 DAS under variable ABA conditions. Bars refer to means ± SD (n=4). Asterisks indicate significant difference from wt in the same condition (two-tailed t test with Bonferroni-Holm correction; *P<0.05) (Courtesy of Dr. June-Sik Kim).

Scale bar = 10 mm

NIC3 was identified as a candidate gene for ABA hypersensitivity in *ros1* mutants

ROS1 is responsible for repressing transcriptional silencing of some endogenous genes including *RD29A* and *EPF2* (Gong et al., 2002; Le et al., 2004; Yammamuro et al., 2014). Based on the putative function of ROS1, it was assumed that some genes important for ABA responses might be dysregulated in *ros1* mutants due to DNA hypermethylation. To test the hypothesis, transcriptome and methylome analyses were performed. The RNA-seq data revealed that a total of 30 genes were commonly downregulated in *ros1-3* and *ros1-4* mutants compared to the wt upon ABA treatment, and 11 out of 30 genes were ABA-inducible (Figure 3A). To determine whether the expression changes of these genes were associated with DNA methylation changes, whole genome bisulfite sequencing (BS-seq) was performed. According to the methylome data, 18.7 % of all *Arabidopsis* coding genes (5,692 of 31,109; TAIR10) were proximal to hypermethylated regions in *ros1* mutants compared to the wt. However, 66.7 % of commonly downregulated genes were proximal to hypermethylated regions in *ros1* mutants (Figure 3B). These results suggest that the expressions of some ABA-responsive genes are epigenetically regulated via ROS1-mediated DNA demethylation.

Among downregulated genes in *ros1* mutants, the most notable is *NICOTINAMIDASE 3* (*NIC3*) which encodes a catalytic enzyme that converts nicotinamide into nicotinic acid in the NAD^+ salvage pathway. *Arabidopsis* genome contains three nicotinamidases, *NIC1*, *NIC2*, and *NIC3*. The *nic1* and *nic2* mutants were reported to exhibit hypersensitivity to ABA, suggesting that nicotinamidases are crucial for ABA responses (Wang and Pichersky, 2007; Hunt et al., 2007).

According to the RNA-seq data, in the absence of ABA, there was no significant difference of *NIC3* expression levels between wt and *ros1* mutants. Under ABA conditions, however, *NIC3* expression was dramatically decreased relative to the wt (Figure 4A). It was then investigated whether ABA-inducible expression of *NIC3* was lost in *ros1* mutants due to DNA hypermethylation. The whole genome BS-seq data indicated that the upstream region of *NIC3* promoter contains four transposable elements (TEs), two of which were hypermethylated in *ros1* mutants compared to the wt (Figure 4B). The local BS-seq data revealed that CG methylation was significantly increased in both TEs (AT5TE28305 and AT5TE28295), and CHH methylation (where H is A, C or T) was also increased in the AT5TE28305 (Figure 4C). These results strongly suggest that DNA hypermethylation of TEs at the upstream of *NIC3* promoter leads to the loss of ABA-inducible expression of *NIC3* in *ros1* mutants, which might be responsible for ABA hypersensitive phenotype of *ros1* mutants.

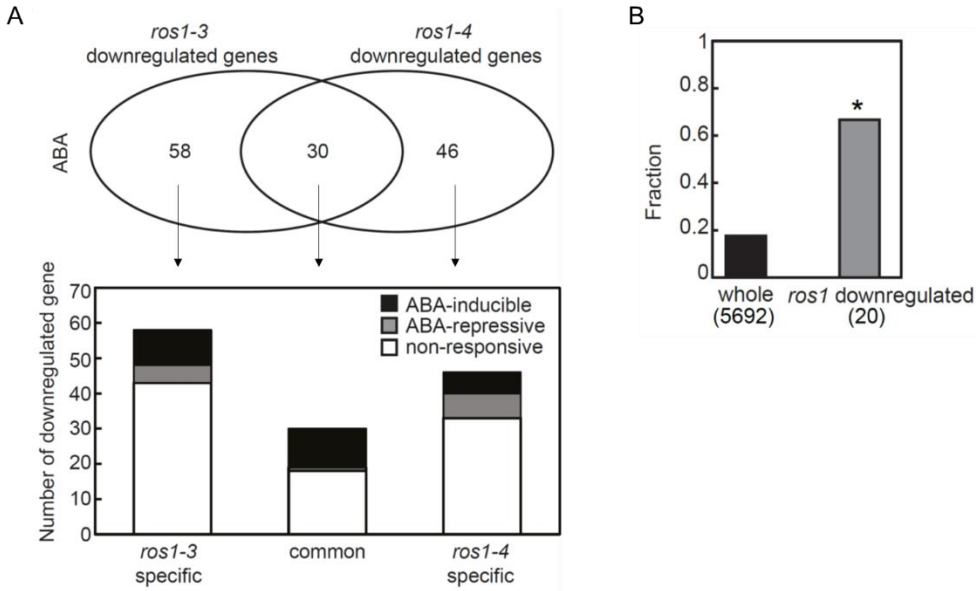

Figure 3. Transcriptome changes associated with DNA methylation changes in *ros1* mutants upon ABA treatment

(A) Downregulated genes in two *ros1* mutant lines (|Fold change (FC)|≥2; False Discovery Rate (FDR)<0.05) and their ABA responsiveness. **(B)** Fractions of the *ros1* downregulated genes proximal (≤2kb) to *ros1* hypermethylated regions. The whole indicates the fraction of all the Arabidopsis genes positioned proximal to *ros1* hypermethylated regions. The asterisk indicates significant difference from the whole (two-tailed Fisher's exact test with Bonferroni-Holm correction; *P<0.05) (Courtesy of Dr. June-Sik Kim).

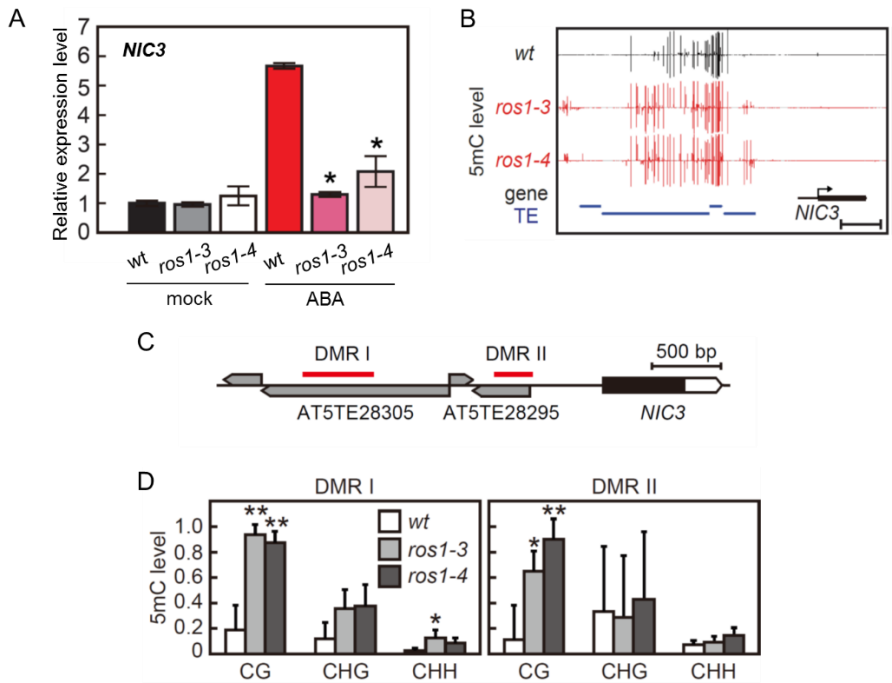

Figure 4. Suppression of ABA-inducible *NIC3* expression by DNA hypermethylation at the upstream *NIC3* promoter in *ros1* mutants

(A) The log2-scaled expressional fold-changes (Log_2FC) of *NIC3* evaluated by qRT-PCR. Asterisks indicate significant differences from the wt (two-tailed t test with Bonferroni-Holm correction; *P<0.05) **(B)** Genome browser view of the DNA methylation (5mC) levels of the upstream region of the *NIC3* promoter evaluated via BS-seq analysis. The left side of each panel is the 5'-upstream region of the allocated locus. Positive and negative bars indicate 5mC levels of single cytosine on the Watson and Crick strands, respectively. **(C)** Schematic diagram of the *NIC3* locus. Relative locations of the promoter TEs (gray boxes) and Differentially Methylated Regions (DMRs) (red bars) are shown. **(D)** 5mC levels at DMR I and DMR II (two-tailed t test with Bonferroni-Holm correction; *P<0.05, **P<0.01) (Courtesy of Dr. June-Sik Kim).

Scale bars= 500 bp

Dysregulation of *NIC3* expression conferred ABA hypersensitivity

To determine whether *NIC3* expression is critical for ABA responses, ABA responses of two *nic3* T-DNA insertion lines *nic3-1* and *nic3-2* were examined for seed germination and root growth (Figure 5A). While weaker than *ros1* mutants, *nic3* mutants was hypersensitive to ABA for seed germination compared to the wt (Figure 5B). Moreover, in the root elongation assay, *nic3* mutants exhibited similar inhibition of root growth compared to *ros1* mutants upon ABA treatment (Figure 5C). Thus, *nic3* mutants showed ABA hypersensitive phenotypes with respect to seed germination and root growth.

It was also tested whether *NIC3* overexpression could restore the ABA hypersensitive phenotype of *ros1* mutant. The *ros1-4* mutant line overexpressing *NIC3* (*NIC3*ox) under the control of CaMV 35S promoter was generated along with the wt and *ros1-4* mutant lines carrying the empty vector. Upon the treatment with 0.6 μM and 0.9 μM ABA, the *NIC3*ox line restored normal seed germination rates relative to the wt (Figure 6A, B). In response to 1.2 μM ABA, the *NIC3*ox line exhibited decreased germination compared to the wt but its germination rates were significantly increased compared to the *ros1* mutant (Figure 6A, B). The root elongation assay also demonstrated that ectopic expression of *NIC3* rescued normal root growth (Figure 7A, B). The qRT-PCR data supported these results in that the expression level of *NIC3* in the *NIC3*ox line was significantly higher than the *ros1* mutant but less than wt (Figure 8). Taken these together, ectopic expression of *NIC3* restored the ABA hypersensitive phenotypes of the *ros1* mutant for seed germination and root growth, suggesting that misregulation of *NIC3* expression is responsible for ABA hypersensitivity in *ros1* mutants.

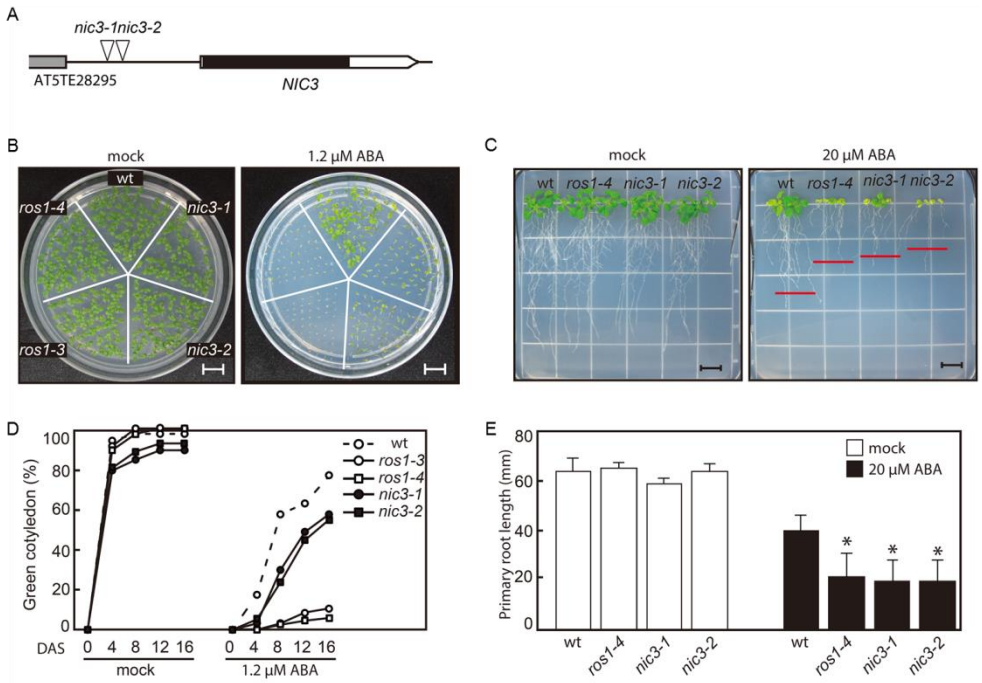

Figure 5. ABA hypersensitivity of *nic3* mutants

(A) Schematic diagram of the *NIC3* locus. The relative locations of T-DNA insertions (*nic3-1* and *nic3-2*) and the promoter TE (gray box) are shown. Lines indicate intergenic regions while closed and open boxes indicate untranslated regions and coding sequences, respectively. **(B)** Seed germination at 12 DAS on MS media under mock or ABA condition. **(C)** Germination frequency measured every 4 days. Every point refers to the percentage of green cotyledons that emerged from the total sown seeds (n>70). **(D)** Root elongation at 19 DAS on MS media under mock or ABA condition. **(E)** Primary root length at 19 DAS under various ABA concentrations. Bars indicate means ± SD (n=4). Asterisks indicate significant difference from wt in the same condition (two-tailed t test with Bonferroni-Holm correction; *P<0.05) (Courtesy of Dr. June-Sik Kim).

Scale bars = 10 mm

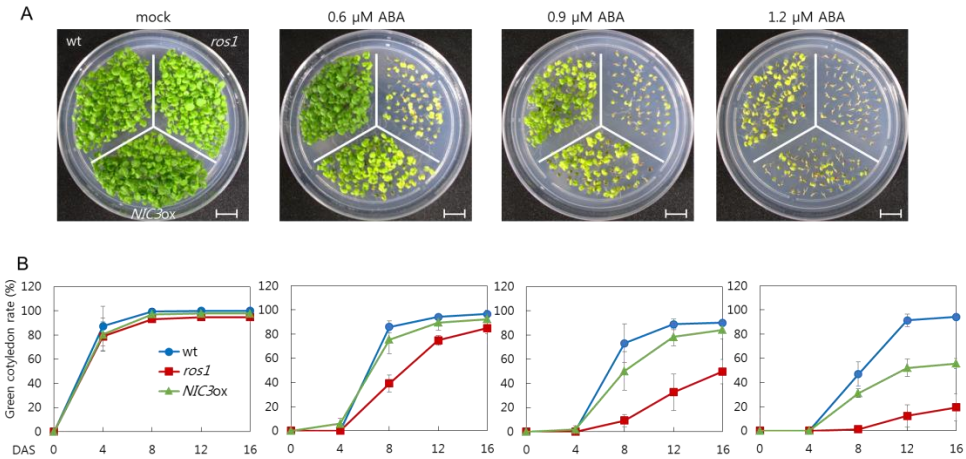

Figure 6. Restored seed germination by ectopic expression of *NIC3* in the *ros1* mutant

(A) Seed germination at 16 DAS in both the wt and the *ros1-4* mutant carrying the empty vector and the *NIC3*ox line (*ros1-4+35S*:*NIC3*) under various ABA concentrations. **(B)** Germination frequency measured every 4 days. Every point refers to the percentage of green cotyledons that emerged from the total sown seeds (n>50).

Scale bars= 10 mm

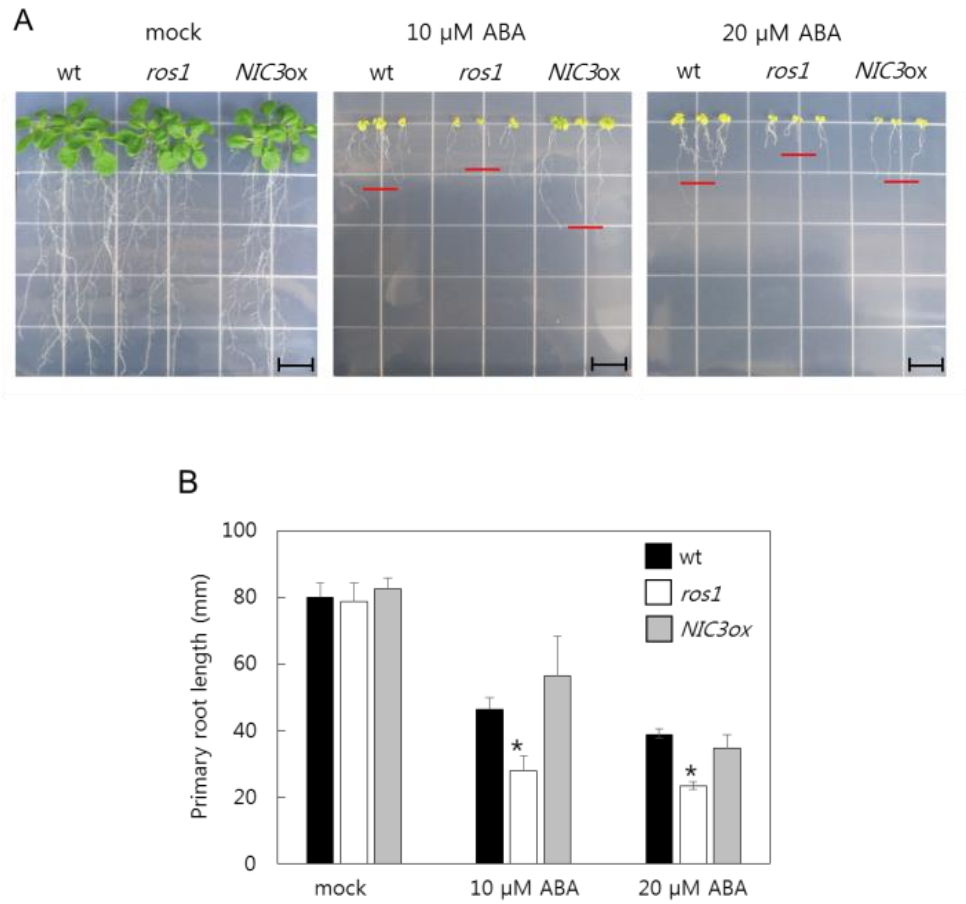

Figure 7. Restored root growth by ectopic expression of *NIC3* in the *ros1* mutant

(A) Root elongation at 18 DAS on MS media under various ABA concentrations. **(B)** Primary root length at 18 DAS. Bars refer to means ± SD (n=4). Asterisks indicate significant difference from wt in the same condition (two-tailed t test with Bonferroni-Holm correction; *P<0.05).

Scale bars= 10 mm.

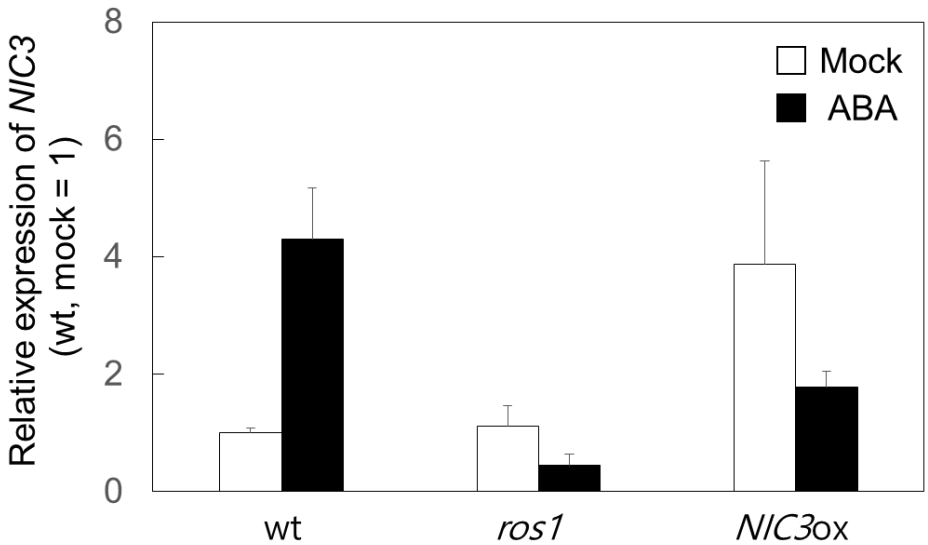

Figure 8. Increased expression of *NIC3* in the *NIC3*ox line

Relative expression levels of *NIC3* in the wt, the *ros1-4* mutant and the *NIC3*ox line.

Bars refer to means ± SD (n=3).

Catalytic residues of yeast Pnc1 are conserved in *Arabidopsis* NIC3

Bacteria, yeast, plants and invertebrates have nicotinamidases which convert nicotinamide into nicotinic acid in the NAD^+ salvage pathway (Balan et al., 2008; Ghislain et al., 2002; Vrablik et al., 2009; Wang and Pichersky, 2007). To determine whether amino acid residues important for catalysis are conserved among these species, amino acid sequences were aligned for Pnc1 homologs. A total of eight Pnc1 homologs were obtained from *A. thaliana*, *O. sativa*, *C. elegans*, *D. melanogaster* and *E. coli* (Table 3). C167, K122, and D8 were reported as the catalytic triad residues of yeast Pnc1, and especially, the cysteine residue was proposed to be functionally important, as its thiol group attacks the carbonyl carbon of nicotinamide (Hu et al., 2007). In accordance with the previous report that nicotinamidase activity is conserved across various species, both cysteine and aspartic acid residues are conserved in all Pnc1 homologs, and the lysine residue is also highly conserved, suggesting that Pnc1 function might be evolutionarily conserved (Figure 9). Notably, *Arabidopsis* NIC3 contains all three catalytic residues, suggesting that NIC3 might be a functional homolog of yeast Pnc1.

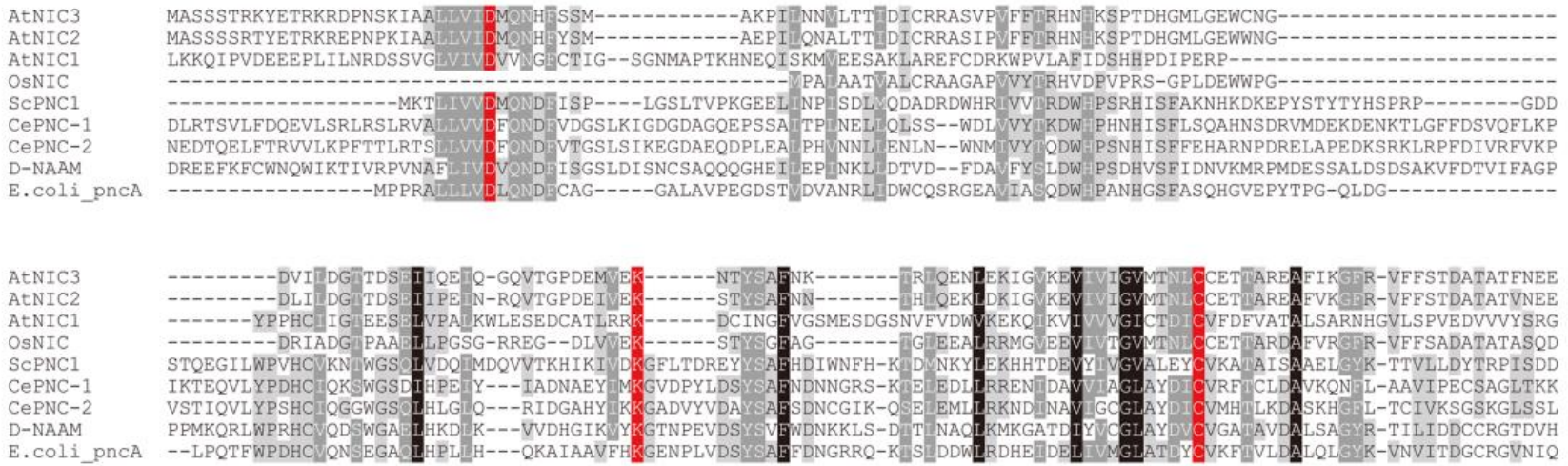

Figure 9. Amino acid sequence alignments of yeast Pnc1 homologs

NIC3 sequence was compared with homologs in *A. thaliana*, *O. sativa*, *S. cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *E. coli*. Catalytic residues are colored in red. Alignments were performed using ClustalX v2.1 and GeneDox.

Heterologous expression of *NIC3* increased rDNA silencing but failed to extend lifespan in yeast

Sir2 is well known for silencing the rDNA loci associated with lifespan (Sinclair and Guarente, 1997; Lin et al., 2000). Pnc1 increases the activity of Sir2 by lowering the level of nicotinamide, which leads to an increase in rDNA stability and lifespan in response to calorie restriction (Anderson et al., 2003; Gallo et al., 2004). To determine whether NIC3 has a conserved function of Pnc1, the rDNA silencing assay and the replicative lifespan analysis were performed by heterologous expression of *NIC3* in yeast. In the rDNA silencing assay, consistent with the previous report, transcriptional silencing at the rDNA locus was decreased in *sir2Δ* cells compared to wt cells (Figures 10A, B). As expected, constitutive expression of *NIC3* increased rDNA silencing in wt cells but showed no difference in *sir2Δ* cells, suggesting that *NIC3* overexpression upregulates the rDNA silencing activity of Sir2 (Figures 10A, B).

It was next tested whether overexpression of *NIC3* could increase the lifespan by upregulating Sir2 activity. Consistent with the previous reports, *pnc1Δ* and *sir2Δ* cells expressing the empty vector exhibited a shorter lifespan compared to the wt under calorie restriction condition (Figure 11A). In contrast to the above rDNA silencing assay, *NIC3* overexpression could not increase but rather decrease the lifespan in wt and *pnc1Δ* cells, suggesting that NIC3 failed to serve the similar function of Pnc1 for lifespan extension (Figure 11B). Hence, these results suggest that NIC3 has a conserved function of Pnc1 for rDNA silencing, but contributed little to lifespan extension.

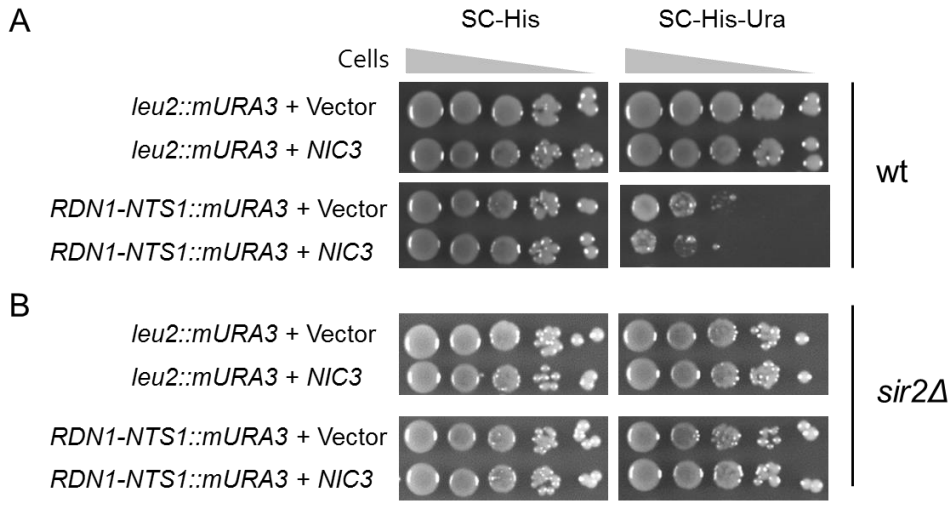

Figure 10. Increased rDNA silencing by heterologous expression of *NIC3* in yeast

The *mURA3* reporter gene was inserted at the NTS1 region of the rDNA locus or outside the rDNA array (*leu2*::*mURA3*). Silencing at the rDNA region was determined by cell growth on SC medium without histidine and uracil. Cell growth on SC medium without histidine was used as a control. The spotting assay was performed with cells containing an empty vector or expressing *NIC3* on the p413ADH vector (A) and *sir2Δ* cells containing an empty vector or expressing *NIC3* on the p413ADH vector (B).

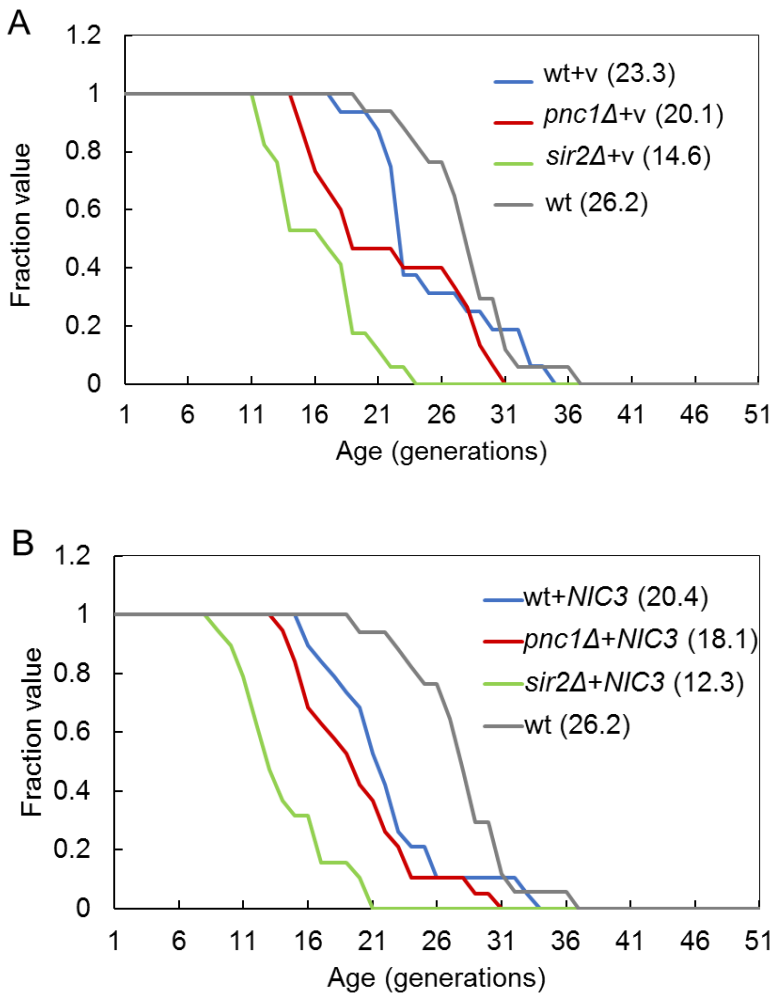

Figure 11. Decreased replicative lifespan by heterologous expression of *NIC3* in yeast

For calorie restriction, cells were grown on YPD containing 0.5 % glucose. The replicative lifespan assay was performed with wt, *pnc1Δ* and *sir2Δ* cells containing an empty vector (A) and wt, *pnc1Δ* and *sir2Δ* cells expressing *NIC3* on the pYES5 vector (B). Average lifespans are shown in parentheses.

Discussion

DNA methylation generally represses the expression of proximal genes associated with transposable elements (Cokus et al., 2008; Diez et al., 2014; Lippman et al., 2004). ROS1, a member of the DNA glycosylase family in *Arabidopsis*, has been reported to prevent DNA hypermethylation at genomic regions and repress transcriptional silencing of transgenes and endogenous genes (Gong et al., 2002; Le et al., 2014; Lister et al., 2008; Penterman et al., 2007; Yamamuro et al., 2014). This study demonstrated that ROS1-initiated DNA demethylation at transposable elements in upstream *NIC3* promoter is crucial for *NIC3* expression in response to ABA, suggesting the role of NAD^+ biosynthesis and dynamic nicotinamide level changes in ABA responses (Figure 4, 6, and 7). Based on the functional importance of Pnc1-mediated Sir2 function in yeast, it was next examined whether NIC3 is a functional homolog of Pnc1. Heterologous expression of *NIC3* in yeast increased rDNA silencing but failed to rescue the shorter lifespan of *pnc1Δ* cells. (Figure 9 and 10). Therefore, these findings suggest that NIC3 might serve a partially conserved function of Pnc1 and have evolved to function divergently in ABA-mediated osmotic stress responses with its expression controlled by ROS1 in *Arabidopsis*.

While *DME* was known to be expressed in the central cell for seed development in *Arabidopsis*, *ROS1* is expressed in all vegetative tissues with biological functions remained elusive (Choi et al., 2002; Law and Jacobsen, 2010). The *ros1* mutants did not exhibit obvious developmental abnormalities, but ROS1 was known to regulate the ABA- and stress-responsive *RD29A::LUCERIFERASE*

(*RD29A::LUC*) transgene and the endogenous *RD29A* gene (Gong et al., 2002; Penterman, 2007). This study revealed that *ros1* mutants were hypersensitive to ABA for seed germination and root elongation, suggesting that ROS1 is required for pruning DNA methylation patterns in response to ABA or osmotic stresses (Figure 2). Although the mechanism by which ROS1 responds to environmental changes such as ABA or osmotic stresses remains to be determined, ROS1 seems to link environmental inputs with gene expression changes. Therefore, in response to ABA, ROS1 activates the expression of *NIC3* by erasing DNA methylation marks, implying stress adaptations in plants.

While genome-wide DNA demethylation is well established in mammals, it has been reported that DNA demethylases are targeted to specific loci in plants (Zhu, 2009). Recent studies have reported that ROS1 regulates the expression of *EPF2* for stomatal development and stress-responsive genes for *Fusarium oxysporum* resistance associated with transposable elements in their promoters by counteracting the function of RdDM (Le et al., 2014; Yamamuro et al., 2014). In accordance with these reports, this study revealed that ROS1 regulates the expression of an ABA-inducible gene *NIC3* by preventing DNA hypermethylation at transposable elements in the upstream region of its promoter for ABA responses (Figures 4 and 8). Further study is required to understand how ROS1 is targeted to the *NIC3* promoter regions or determine whether ROS1 directly or indirectly regulates the expression of *NIC3*.

Nicotinamidase is a catalytic enzyme that converts nicotinamide into nicotinic acid in the NAD^+ salvage pathway. Many enzymes in the NAD^+ salvage pathway such as PARP and NMNAT are known to be involved in stress responses

(De Block et al., 2005; Hashida et al., 2010; Vanderauwera et al., 2007). Consistent with previous reports, it was found that *nic3* mutants displayed hypersensitive phenotypes to ABA, and *NIC3* overexpression in the *ros1* mutant background rescued ABA hypersensitivity for seed germination and root elongation (Figure 5-7). These results suggest that dynamic levels of nicotinamide and NAD^+ might be associated with ABA responses. Further investigation on dynamic levels of metabolites in the NAD^+ salvage pathway in ABA responses will be required.

NIC3 was identified as a homolog of yeast nicotinamidase *PNC1* (Hunt et al., 2004). Pnc1 is considered an activator of a NAD^+-dependent histone deacetylase Sir2 by decreasing the level of nicotinamide (Anderson et al., 2003; Gallo et al., 2004). While *NIC3* expression is regulated by ROS1-initiated DNA demethylation in response to ABA, the stress-responsive transcription factors, Multicopy suppressor of SNF1 mutation 2 (Msn2) and Msn4, are known to promote the expression of *PNC1* by hyperosmotic shocks (Ghislain et al., 2002; Medvedik et al., 2007). Accordingly, unlike yeast which does not possess the DNA methylation system, plants are able to regulate *NIC3* expression via ROS1-intiated DNA demethylation in a more flexible manner. Furthermore, it was investigated whether NIC3 conserves similar functions as Pnc1. In this study, heterologous expression of *NIC3* increased rDNA silencing in wt yeast cells, but it could not extend lifespan in wt and *pnc1Δ* cells (Figure 10 and 11). One possibility is that constitutive *NIC3* expression has a negative impact on the NAD^+ salvage pathway by accumulation or depletion of NAD^+ or other metabolites, resulting in toxicity to yeast cells. Alternatively, heterologous expression of *NIC3* unexpectedly affects another mechanism contributing to longevity. To determine whether NIC3 is a functional

homolog of Pnc1, further experiments are required to investigate whether NIC3 regulates the activity of histone deacetylase Sir2 or other NAD^+-dependent enzymes. However, given the finding that NIC3 has a conserved function of increasing rDNA silencing but contributed little to lifespan extension, NIC3 might be a partially functional homolog of yeast Pnc1.

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초 록

모든 생명체에서 니코틴아마이드 아데닌 다이뉴클레오타이드(NAD^+)는 다양한 산화환원 반응에 관여하는 주요 전자전달자이다. NAD^+는 ADP 라이보실화, 단백질 탈아세틸화, 칼슘 신호전달 등에서 중요한 역할을 한다고도 알려져 있다. 진핵생물에서 NAD^+는 신규 생합성(*de novo*) 경로와 재이용(salvage) 경로를 거쳐 합성될 수 있다. 모델식물인 애기장대에서 NICOTINAMIDASE 3는 NAD^+ 재이용 경로에서 니코틴아미드를 니코틴산으로 전환하는 효소이다. 본 연구는 *NIC3* 유전자의 발현이 앱시스산에 반응하여 DNA 탈메틸 효소인 ROS1에 의해 조절된다는 사실을 밝혔다. 야생형에서는 앱시스산에 의해 *NIC3* 유전자의 발현이 유도되지만, ROS1의 돌연변이체에서는 *NIC3* 유전자의 프로모터 부분에 메틸화 수준이 증가하여 앱시스산에 의해 *NIC3* 유전자의 발현이 증가하지 않았다. 또한 앱시스산 처리 시 ROS1의 돌연변이체와 유사하게 NIC3의 돌연변이체는 종자 발아와 뿌리 신장이 억제되었고, ROS1의 돌연변이체에 *NIC3* 유전자를 과발현시켰을 때 정상적인 종자 발아와 뿌리 신장을 보이며 표현형이 회복되는 것을 확인하였다. 따라서 ROS1이 매개하는 DNA 탈메틸화에 의한 *NIC3* 발현이 앱시스산 반응에서 필수적임을 알 수 있다. 그러나 *NIC3* 유전자의 기능 연구는 보고된 바가 없어 NIC3가 모델생물인 효모에서의 상동 단백질인 Pnc1과 유사한 기능을 수행하는지 확인하였다. 효모에서

애기장대 *NIC3*의 발현을 유도했을 때 rDNA 안정성이 증가하였으나 수명은 감소하는 현상을 보임으로써 NIC3가 Pnc1과 유사하지만 다소 상이한 기능을 수행할 가능성을 시사하였다. 그러므로 *NIC3*는 효모 유전자 *PNC1*의 기능을 어느 정도 보존하고 있으며 동시에 식물에서 DNA 탈메틸화에 의해 발현이 조절되어 앱시스산 반응에 관여하도록 진화해온 것으로 추정된다.

주요어: 니코틴아마이드 아데닌 다이뉴클레오타이드, Nicotinamidase, 앱시스산, DNA 탈메틸화, REPRESSOR OF SILENCING 1, 애기장대, 효모

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