이학박사 학위논문

Molecular genetic and physiological studies on the role of reactive oxygen species under abiotic stress conditions in *Arabidopsis*

환경 스트레스 하에서 활성 산소의 역할에 대한 분자 유전학적 및 생리학적 연구

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화학부

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ABSTRACT

When plants are exposed to environmental stresses, such as drought and heat conditions, reactive oxygen species (ROS) are produced. ROS are also generated in plant cells mainly as byproducts of aerobic energy metabolism. Therefore, plants have developed diverse ROS-detoxifying machinery to cope with ROS accumulation. Under prolonged-stress conditions, ROS will exceed the capacity of scavenging machinery, causing oxidative damage to cellular components, including DNA, proteins, and lipids. However, it is largely unknown how ROS metabolism is linked with stress responses.

Accumulating evidence indicates that salicylic acid (SA), in addition to its role in mediating disease resistance responses, also plays an important role in developmental processes and abiotic stress responses, such as heat and high salinity. Here, I report that physiological concentrations of SA promote germination under high salinity by reducing reactive oxygen species (ROS) in *Arabidopsis*. Germination of the SA-deficient *sid2* mutant seeds was hypersensitive to high salinity. The inhibitory effect of high salinity was significantly reduced in the presence of physiological concentrations (<50 μM) of SA. Under high salinity, the endogenous contents of H_2O_2 were elevated in the wild-type and *sid2* seeds but reduced to the original levels after treatments with 1 μM SA. Together, my

observations indicate that although SA is not required for germination under normal growth conditions, it reduces ROS to promote germination under high salinity.

Responses to adverse environmental conditions are regulated by gene regulatory networks, in which transcription factors play a central role in plants. In this study, I show that a NAC transcription factor, NTL4/ANAC053, is required for ROS generation under drought stress in *Arabidopsis*. *NTL4* gene is induced by abscisic acid (ABA), drought, and heat stress. NTL4 protein has transcriptional activity and binds directly to the promoters of genes encoding ROS biosynthetic enzymes under drought stress conditions, leading to the ROS production. Elevated ROS levels induce leaf senescence. Leaf senescence was accelerated in the 35S:*4ΔC* transgenic plants overexpressing an bioactive form of NTL4 under drought conditions. The transgenic plants were highly sensitive to drought stress, and ROS accumulated in the transgenic leaves. In contrast, ROS levels were reduced in the *NTL4*-deficient *ntl4* knockout mutants that exhibited delayed leaf senescence and enhanced drought resistance. These observations indicate that NTL4 regulates leaf senescence under drought stress by modulating ROS generation.

ROS also cause plant growth retardation under heat stress by inducing oxidative damage to various cellular constituents. However, it remains unknown

how ROS metabolism and signaling is linked with thermotolerance response. Here, I demonstrate that the RNA-binding protein FCA, a key component of the flowering genetic pathways in *Arabidopsis*, is also required for the acquisition of thermotolerance. Whereas the transgenic plants overexpressing the *FCA* gene, 35S:*FCA*, were resistant to heat stress, the *fca-9* mutant lacking functional FCA was hypersensitive to heat stress. The ROS level was reduced in the 35S:*FCA* transgenic plants but elevated in the *fca-9* mutant under heat stress. I found that the levels of antioxidants, such as ascorbate and anthocyanins, were significantly elevated in the 35S:*FCA* transgenic plants but reduced in the *fca-9* mutant. These observations indicate that FCA contributes to the acquisition of thermotolerance by reducing ROS under heat stress.

key words : Reactive oxygen species (ROS), abiotic stresses, *Arabidopsis*, phytohormones, *NTL4*, *FCA*

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ABBREVIATIONS

ABA	Abscisic acid
ABI5	ABA-INSENSITIVE 5
BiFC	Bimolecular fluorescence complementation
CaMV	Cauliflower mosaic virus
cDNA	Complementary DNA
ChIP	Chromatin immunoprecipitation
DAB	3,3'-diaminobenzidine
GO	Gene ontology
GUS	β-glucuronidase
HR	Hypersensitive response
LD	Long day
MS	Murashige-Skoog
MTF	Membrane-bound transcription factor
NAC	NAM, ATAF1/2, CUC2
NTL	NTM1-Like
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PER1	1-cys peroxiredoxin

RBOH	RESPIRATORY BURST OXIDASE HOMOLOG
ROS	Reactive oxygen species
RT	Reverse transcription
SA	Salicylic acid
SID2	SA-INDUCTION DEFICIENT 2
TBARS	Thiobarbituric acid reactive substances
TM	Transmembrane
TTC	2,3,5-triphenyl tetrazolium chloride

INTRODUCTION

1. Abiotic stress responses in plants

All through a life cycle, sessile plants have been under various stress conditions including biotic and abiotic stresses. Diverse plant developmental processes such as germination, root elongation, senescence and flowering time are affected by environmental changes, particularly cold, heat, drought and high salinity (Buchanan-Wollaston et al., 2005; Jiang et al., 2009; Park et al., 2009; Chung et al., 2010). Previous studies show that plant adaptation to environmental stresses is modulated by complex gene regulatory networks, in which growth hormones play crucial roles through extensive signaling crosstalks (Seo et al., 2009; Divi et al., 2010). However, it is largely unknown the molecular mechanisms underlying plant growth and development controls of environmental stress responses.

Abscisic acid (ABA) is a crucial plant hormone that mediates plant responses to abiotic stresses, including drought, cold, heat, and high salinity. ABA induces cellular and physiological acclimation responses to readjust developmental processes, such as remodeling of root growth, leaf senescence, and abscission, under abiotic stress conditions (Sharp and LeNoble, 2002; Yang et al., 2003). Recently, numerous genes and signaling molecules that mediate ABA responses have been identified through molecular genetic and physiological studies (Cutler et

al., 2010; Hauser et al., 2011). Of particular interest are reactive oxygen species (ROS). Accumulating molecular genetic, cell biological, and physiological evidence supports the role of ROS in ABA signaling networks (Pei et al., 2000; Jiang and Zhang, 2002).

Recent studies indicate that SA also regulates diverse aspects of plant responses to abiotic stresses through extensive signaling crosstalks with other growth hormones (Achard et al., 2006; Horvath et al.,2007; Spoel and Dong, 2008; Tuteja and Sopory, 2008; Vlot et al., 2009; Wolters & Jürgens, 2009). It has been shown that SA plays a role in plant adaptive responses to osmotic stress (Singh and Usha, 2003), chilling and drought (Senaratna et al., 2000), high temperatures (Clarke et al., 2004), and to high salinity (Khodary, 2004).

High salinity causes plant growth retardation, such as inhibition of seed germination (Borsani et al., 2001). It has been reported that SA is involved in seed germination under high salt conditions in maize (Guan & Scandalios, 1995), *Arabidopsis* (Nishimura et al., 2005), and in barley (Xie et al., 2007). A germination study using transgenic *Arabidopsis* plants that overexpress the *NahG* gene encoding a salicylate hydroxylase has shown that lack of SA promotes germination under high salinity and osmotic stress (Borsani et al., 2001), suggesting the inhibitory role of SA. In contrast, it has been observed that germination of the SA-deficient plants is more severely delayed compared to that

of wild-type seeds in the presence of high salt and that the delayed germination is recovered by exogenous application of SA (Rajjou et al., 2006). Recently, it has been found that seed germination of the *sid2* mutant is more sensitive to salt stress (Alonso-Ramírez et al., 2009), supporting a promotive role of SA.

When plants are exposed to drought conditions, ABA signals trigger ROS accumulation, and leaf senescence is induced (Bhattacharjee, 2005). Drought-induced leaf senescence contributes to the maintenance of water balance in whole plant body and the remobilization of nutrients from senescing leaves to youngest leaves or sink organs (Munné-Bosch and Alegre, 2004). Accordingly, suppression of ROS production markedly delays leaf senescence and enhances drought resistance (Rivero *et al.*, 2007). However, it is currently unclear how ROS metabolism is linked with ABA signaling in inducing leaf senescence.

Among abiotic stresses, heat stress leads to the disruption of cellular homeostasis and growth retardation in plants, which cause severe crop loss in agriculture (Wang et al., 2003). Therefore, plants are forced to spend valuable resources to adjust their growth and development and prevent heat stress-induced cellular and metabolic damage, a process referred as heat acclimation (Larkindale and Huang, 2005). The heat acclimation and adaptive response to heat stress is regulated through complex signaling networks that include heat-shock proteins (HSPs), heat stress transcription factors (HSFs), stress hormones, and signaling

molecules, such as Ca^{2+} and reactive oxygen species (ROS) (Larkindale et al., 2005; Volkov et al., 2006). However, it is largely unknown how plants sense the changes in ambient temperature and cope with the cellular and physiological damage caused by heat stress.

2. Regulation of ROS metabolism under stress conditions

ROS are toxic chemicals that cause oxidative damage to DNA, proteins, and membrane lipids. However, they also function as essential signaling molecules in mediating stress adaptation responses to biotic and abiotic stresses (Apel and Hirt, 2004; Mittler et al., 2011). ROS are produced primarily as byproducts of normal metabolic processes, such as respiration and photosynthesis, in chloroplasts, mitochondria, and peroxisomes (Apel and Hirt, 2004). They are also generated in apoplasts by the activity of NADPH oxidases under stress conditions (Mittler et al., 2004). A group of NADPH oxidases and respiratory burst oxidase homologs (Rbohs) has been identified in *Arabidopsis* (Sagi and Fluhr, 2006). The roles of *Atrboh* genes have been demonstrated in the ABA-mediated stomatal closing, defense responses, and plant developmental processes, such as root hair growth and seed germination (Torres *et al.*, 2002; Kwak *et al.*, 2003). In this work, I demonstrated that ROS production by a subset of Atrboh enzymes directly links osmotic stress response with leaf senescence. Under drought conditions, the NTL4

transcription factor activates the *Atrboh* genes, resulting in ROS accumulation that triggers leaf senescence. I propose that NTL4 regulation of ROS production underlies the leaf senescing process occurring under drought conditions.

ABA is closely coupled with ROS metabolism in plants. It affects the expression of genes that constitute ROS regulatory networks, such as *CAT1* (*catalase 1*) and *GR1* (*glutathione reductase 1*) in maize (Zhang et al., 2006) and *APX1* (*ascorbate peroxidise 1*) and *CAT1* in *Arabidopsis* (Davletova et al., 2005; Xing et al., 2008). In addition, molecular genetic studies on the *Atrboh* genes have shown that *AtrbohD* and *AtrbohF* genes are required for hypersensitive response (HR) and ABA regulation of stress responses (Torres et al., 2002; Kwak et al., 2003). ROS are also involved in ABA regulation of stomatal aperture (Pei et al., 2000), supporting the linkage between ABA and ROS signals.

When plants are exposed to biotic and abiotic stresses, ROS levels are elevated not in whole plant body but in local plant parts (Torres et al., 2005; Duan et al., 2010). A well-known example of local ROS accumulation is provided by the HR occurring during plant disease response. Recent physiological insights and experimental evidence also support the notion that localized ROS accumulation in specific tissues or cells underlies plant adaptation responses to drought stress. It has been reported that ROS accumulate and programmed cell death is induced in the root tip area under drought conditions (Duan et al., 2010). It has also been shown

that drought-induced senescence in the affected leaves improves the chance of plant survival under drought conditions by preventing the spread of the damages to the rest of plant body through remobilization of nutrients and prevention of water loss (Munné-Bosch and Alegre, 2004), similar to what observed during the HR in infected plants (Alvarez et al., 1998; Apel and Hirt, 2004).

Oxidative damages caused by increasing ROS levels under high salt conditions have been studied extensively in plants. Although ROS is generally perceived as toxic molecules, it has been shown that ROS also acts as signaling molecules when present at low concentrations (Mittler et al., 2004). Previous studies have demonstrated that SA is closely related with ROS under stress conditions. A representative example is hypersensitive response (Durrant and Dong, 2004). When plants are infected by pathogens, SA stimulates ROS biosynthesis to induce cell death on the infected region. However, the functional relationship between SA and ROS is poorly understood in plant responses to abiotic stresses. In *Arabidopsis*, it has been suggested that SA is linked with ROS-mediated damages under abiotic stress conditions. It has been shown that SA increases ROS-mediated oxidative damage and induce H_2O_2 production (Harfouche et al., 2008). However, SA induces resistance to diverse abiotic stresses, suggesting that SA reduces ROS-mediated oxidative damages (Singh and Usha, 2003; Vlot et al., 2009).

Under heat stress, the intracellular levels of ROS, such as superoxide,

hydrogen peroxide, and hydroxyl radical, are significantly elevated in plants (Møller et al., 2007; Zhou et al., 2012). An oxidative burst occurs shortly after exposure to high temperatures, obviously owing to the NADPH oxidase activity (Miller et al., 2009). It has been recently found that pretreatments with H_2O_2 leads to the induction of thermotolerance, and NADPH oxidase-defective mutants, *Arabidopsis respiratory burst oxidase homolog B* (*atrbohB*) and *atrbohD*, are susceptible to heat stress (Larkindale and Huang, 2004; Larkindale et al., 2005).

Plants have developed an array of ROS scavenging/detoxifying enzymes and various antioxidants to deal with the accumulated ROS under stressful conditions. Superoxide dismutase (SOD) converts superoxide into hydrogen peroxide. Ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) decompose hydrogen peroxide to water (Mittler, 2002). Ascorbate and glutathione (GSH) act as antioxidants. *Arabidopsis* mutants that are defective in antioxidant production, such as *ascorbate peroxidase 1* (*apx1*), *apx2*, *vitamin C defective 1* (*vtc1*), and *vtc2*, exhibit symptoms of increased oxidative damage when exposed to abiotic stresses (Larkindale et al., 2005; Suzuki et al., 2013). On the other hand, peroxiredoxins, which are thiol-based peroxidases, enhance plant tolerance against oxidative and heat stresses (Kim et al., 2010). In this work, I demonstrate that FCA, plant-specific RNA-binding protein, regulates thermotolerance by modulating antioxidant activity under heat stress conditions.

The contents of antioxidants, such as ascorbate and anthocyanins, were reduced in *fca* mutants but elevated in *FCA*-overexpressing plants. These observations indicate that FCA plays a role in the stimulation of thermotolerance by enhancing the antioxidant activity under heat stress conditions, providing a novel role of FCA in plant stress response.

MATERIALS AND METHODS

1. Plant materials and growth conditions

All *Arabidopsis thaliana* lines used were in Columbia (Col-0) background. Plants were grown in a controlled culture room at 23°C with relative humidity of 55% under long day conditions (16-h light and 8-h dark) with white light illumination (120 μmol photons/s/m^2) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). The *sid2* mutant and the *NahG* transgenic plant have been described (Gaffney et al., 1993; Wildermuth et al., 2001). The 35S:*NTL4* and 35S:*4ΔC* transgenic plants have been described (Kim et al., 2010). Two T-DNA insertional *NTL4*-deficient mutants, *ntl4-1* (SALK-009578C) and *ntl4-2* (SALK-007900), were isolated from a mutant pool deposited in the ABRC at Ohio State University. The *fca-9*, *fca-11*, and *abi5-3* mutants and 35S:*FCA* transgenic plants have been described previously (Bäurle et al., 2007; Piskurewicz et al., 2008; Jung et al., 2012).

2. Seed germination assays

Routinely, *Arabidopsis* seeds air-dried for 2 weeks were used for germination assays. Seeds were imbibed on 1/2 X Murashige & Skoog (MS)-agar plates (hereafter referred to as MS-agar plates) at 4°C for 3 days in complete darkness and

allowed to germinate at 22°C under LDs. Emergence of visible radicles was used as a morphological marker for germination. Forty-fifty seeds were counted for each measurement and averaged.

To examine the effects of NaCl, KCl, and LiCl on germination, MS-agar plates supplemented with 100-200 mM NaCl, 150 mM KCl, or with 65 mM LiCl were used for cold imbibition and germination. To examine the effects of osmotic stress on germination, MS-agar plates supplemented with 300 mM mannitol or 300 mM sorbitol were used.

To determine how SA affects seed germination under high salinity, MS-agar plates supplemented with 150 mM NaCl and 0-5 mM SA were used for cold imbibition and germination. Catechol was used at a final concentration of 10 μM. Ascorbic acid was included in the germination assays at a final concentration of 1 mM.

3. Transcript level analysis

Transcript levels were examined by qRT-PCR. Total RNA samples were extracted from appropriate plant materials using the RNeasy plant total RNA isolation kit (Qiagen, Valencia, CA). Prior to qRT-PCR assays, total RNA samples were pretreated with an RNase-free DNaseI to remove any contaminating genomic DNA. The first-strand cDNA was synthesized from 1 to 2 μg of total RNA in a 20 μl

reaction volume using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). One μl of the reaction mixture was used for each qRT-PCR run.

qRT-PCR was performed in 96-well blocks with an Applied Biosystems 7500 Real-Time PCR System (Foster City, CA) using the SYBR Green I master mix in a volume of 20 μl. The PCR primers were designed using the Primer Express software installed in the system and listed in Table 1. The two-step thermal cycling profile and processing of qRT-PCR data were carried out as described previously (Seo et al., 2009).

4. Histochemical assays

The GUS-coding sequence was fused in-frame to the 3' end of the *NTL4* gene with its own promoter consisting of approximately 1 kb upstream of the transcription start site, and the fusion construct was transformed into Col-0 plants. For histochemical detection of GUS activities, 10-day-old transgenic plants grown on MS-agar plates were fixed in 90% acetone for 20 min on ice and washed twice with rinsing solution [50 mM sodium phosphate, pH 7.2, 0.5 mM $K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$]. The plant materials were subsequently incubated at 37^{o}C for 20-24 h in fresh rinsing solution containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Duchefa, Harlem, The Netherlands). They were then dehydrated through a series of ethanol dilutions, ranging from 15 to 80%, mounted on slide glasses, and visualized using a DIMIS-

M digital camera (JMTECH, Seoul, Korea).

5. Determination of ROS levels

Superoxide radical was detected by NBT staining, and hydrogen peroxide was detected by DAB staining, as described previously (Ramel et al., 2009) but with a few modifications. Plant samples were incubated incubated in the NBT staining solution (0.5 mg/ml) for 8 h or in the DAB staining solution (0.5 mg/ml) for 24 h at room temperature in darkness. The plant samples were destained by incubating the samples in 95% ethanol at 70^{o}C for 15 min. Quantitation of the staining density was performed by the LabWorks software (UVP, Upland, CA).

Endogenous contents of hydrogen peroxide were measured as described previously (Lee et al., 2010). The Amplex Red hydrogen peroxide assay kit according to the procedure provided by the manufacturer (Molecular Probes, Eugene, OR) were used for assays. Briefly, 100 mg of leaf samples was ground in liquid nitrogen, and 200 μl of 20 mM sodium phosphate buffer (pH 6.5) was added and thoroughly mixed. The mixture was centrifuged at 9500 X g for 10 min at 4^{o}C, and the supernatant was used for subsequent assays. The Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with H_2O_2 in a 1:1 stoichiometry, producing a red-fluorescent compound, resorufin. Since resorufin has absorption and fluorescence emission maxima at approximately 571 nm and 585 nm and its

extinction coefficient is high (54,000/cm/M), the assays can be performed through fluorometry and spectrophotometry. Fluorescence measurements were carried out using the Cary Eclipse fluorescence spectrophotometer (Varian Associates, Palo Alto, CA).

Thiobarbituric acid reactive substances (TBARS) assay was performed using the OXI-TEK TBARS assay kit (ZeptoMetrix, Buffalo, NY). Approximately 50 mg of plant materials were ground in liquid nitrogen and then resuspended in 500 μl of 1X phosphate buffered saline (PBS) buffer, pH 7.4 (27 mM potassium chloride, 137 mM sodium chloride, and 1.76 mM potassium phosphate). The crude solution of 100 μl was mixed with 2.5 ml of the reaction buffer containing 0.5% (w/v) thiobarbituric acid and 20% (v/v) trichloroacetic acid in 1X PBS buffer, pH 7.4. The mixture was boiled for 1 h and then centrifuged for 30 min at 4000 X g. The supernatant was used for measuring absorbance at 532 nm. The TBARS contents were calculated by comparing the absorbance to the standard curve generated with 12.5-100 μM malondialdehyde standards.

6. Detection of cell death

Dead cells in rosette leaves were visualized by trypan blue staining, as described previously (Koch and Slusarenko, 1990). Whole leaf mounts were stained with lactophenol-trypan blue (10 ml of lactic acid, 10 ml of glycerol, 10 g of phenol, 10

mg of trypan blue, dissolved in 10 ml of deionized water). The leaf samples were boiled for 1 min and left overnight in the staining solution at room temperature. The leaves were distained by incubating in the destaining solution (1:2, lactophenol:ethanol) for 30 min at room temperature.

For electrolyte leakage assays, one-week-old plants grown on MS-agar plates were used. The aerial parts of 5 seedlings were floated on deionized water for 12 h in complete darkness before measuring the sample conductivity using the Orion 5-star conductivity meter (Thermo, Beverly, MA). Then, plant samples were boiled in the same solution for 5 min, and total conductivity of the solution was measured. Electrolyte leakage is represented by the relative conductivity that is calculated by dividing sample conductivity by total conductivity.

The root vitality was examined as described previously (Zhou et al., 2012). Ten-day-old heat-treated plants were incubated in 0.6% (w/v) triphenyltetrazolium chloride solution for 20 h at 23^{o}C. The plant materials were washed with deionized water three times, and 1-cm tips of the primary roots of 20 plants were cut off and homogenized in 95% (v/v) ethanol. The homogenate was incubated in water bath for 10 min at 80^{o}C to extract formazan and centrifuged at 4000 X g for 5 min, and the supernatant was used to measure the absorbance at 530 nm.

7. Measurements of antioxidant contents

Ascorbate contents were measured as described previously (Gillespie et al., 2007). Heat-treated whole plants were ground in liquid nitrogen before adding 1 ml of 6% (w/v) trichloroacetic acid (TCA) per 40 mg of plant leaves. The crude extract was centrifuged at 4^{o}C for 10 min at 16000 X g. The supernatant was taken and used for the measurements of ascorbate contents. The reaction mixture contained the 200 μl of the supernatant, 100 μl of 75 mM phosphate buffer, 500 μl of 10% (w/v) TCA, 400 μl of 43% (v/v) H_3PO_4, 400 μl of 4% (w/v) α-α'-bipyridyl, and 200 μl of 3% (w/v) $FeCl_3$. Prior to adding to the reaction mixture, the supernatant was mixed with 100 μl of 10 mM dithiothreitol and incubated for 10 min, and 100 μl of 0.5% (w/v) *N*-ethylmaleimide was added to the incubation mixture for the measurement of total ascorbates. The absorbance was measured at 525 nm after incubation for 1 h. The ascorbate contents were calculated by comparing the absorbance to the standard curve generated with 0.15-10 mM ascorbate standards.

For the measurements of glutathione contents, heat-treated whole plants were ground in liquid nitrogen before adding 1 ml of 6% (w/v) TCA per 40 mg whole plants. The crude extract was centrifuged at 4^{o}C for 10 min at 16000 X g. The supernatants were subject to the measurements, as described previously (Queval et al., 2007). The reaction mixture contained 200 μl of the supernatant, 700 μl of 0.3 mM NADPH, 100 μl of 6 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), and 10 μl of glutathione reductase enzyme stock in 125 mM phosphate

buffer (50 units/ml). For the measurements of glutathione disulfide (GSSG) contents, the supernatant was mixed with 20 μl of 2-vinylpyridine and incubated at 25^{o}C for 60 min, prior to mixing with the reaction mixture. The absorbance at 412 nm was monitored at appropriate time intervals until it exceeds 2.0. The glutathione contents were calculated by comparing the absorbance to the standard curve generated with 6.25-50 μM glutathione standards.

Anthocyanin contents were measured as described previously (Solfanelli et al., 2006). Heat-treated whole plants were ground in liquid nitrogen. Anthocyanins were extracted by incubation of the ground plants at 4^{o}C for 16 h in 1 ml of methanol containing 1% (v/v) HCl per 40 mg of plant materials. The mixture was centrifuged at 4^{o}C for 10 min at 16000 X g. The absorbance of the supernatant was measured at 530 nm and 657 nm using a diode array spectrophotometer (WPA Biowave, Cambridge, UK). The anthocyanin contents were calculated by solving the equation $A_{530} - 0.25 \times A_{657}$.

8. Measurements of chlorophyll contents

Measurements of chlorophyll contents were carried out as described previously (Oh et al., 1997). Chlorophylls were extracted with N,N-dimethylformamide, and the extracted solution was incubated at 4^{o}C for 2 h in complete darkness. Chlorophyll contents were assayed by measuring absorbance at 652nm, 665nm,

and 750nm using a diode array spectrophotometer (WPA Biowave, Cambridge, UK).

9. Protein-protein interaction assays

The BD Matchmaker system (Clontech, Mountain View, CA) was used for the yeast-two hybrid assays. The pGADT7 vector was used for GAL4 AD (activation domain), and the pGBKT7 vector was used for GAL4 BD (DNA binding domain). The yeast strain AH109 (Leu-, Trp-, Ade-, His-), which has chromosomally integrated reporter genes *lacZ* and *HIS* under the control of the GAL1 promoter, was used for transformation. Yeast transformants were cultured in liquid medium without Leu and Trp for 10 h, and the culture was dotted on the agar-medium without Leu, Trp, His and Ade after diluted to an OD_{600} of 0.1.

The nYFP-FCA and ABI5-cYFP vectors were used for bimolecular fluorescence complementation (BiFC) assays. The vectors cotransfected into *Arabidopsis* mesophyll protoplasts by the polyethylene glycol (PEG)-calcium transfection method (Yoo et al., 2007). The transfected protoplasts were incubated at 23^{o}C for 15 h. The subcellular localization of FCA-ABI5 protein complexes were monitored using differential interference contrast microscopy and fluorescence microscopy. Reconstitution of YFP fluorescence was observed using a Zeiss LSM510 confocal microscope (Carl Zeiss, Yena, Germany) with the

following YFP filter set up: excitation 515 nm, 458/514 dichroic, and emission 560- to 615-nm band-pass filter.

10. Protoplast transfection assays

For transcriptional activation activity assays, a series of reporter and effector vectors was constructed. In the reporter vector, 4 copies of the GAL4 upstream activation sequence (UAS) were fused to the *β-glucuronidase* (*GUS*) gene. The *ABI5* or *NTL4ΔC* gene sequences were fused to the GAL4 DNA-binding domain-coding sequence driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter in the effector vector. The *FCA* gene was subcloned into the expression vector harboring the CaMV 35S promoter. The reporter, effector, and expression vectors were cotransfected into *Arabidopsis* mesophyll protoplasts by the PEG-calcium transfection method (Yoo et al., 2007). The CaMV 35S promoter-luciferase construct was also cotransfected as an internal control. GUS activity was measured by the fluorometric method as described previously (Lee et al., 2012), and luciferase assay was performed using the Luciferase Assay System kit (Promega, Madison, WI).

For the transient expression assays, the promoter sequence regions harboring the ABRE elements of *EM6* and *PER1* genes were subcloned into the reporter vector. The *ABI5* or *FCA* gene sequence was subcloned into the effector

vector. The reporter and effector vectors were cotransfected into *Arabidopsis* mesophyll protoplasts. The luciferase expression construct was included as an internal control in the transfection. GUS and luciferase activity assays were performed after incubation for 16 h as described above.

11. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out as described previously (Lee et al., 2012) using one-week-old plants grown on MS-agar plates. The whole plants were vacuum-infiltrated with 1% (v/v) formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin preparations were sonicated into 0.4- to 0.7-kb fragments. An anti-MYC antibody (Millipore, Billerica, MA) was added to the chromatin solutions, which were precleared with salmon sperm DNA/ Protein A agarose beads (Roche, Indianapolis, IN). The precipitates were eluted from the beads. Cross-links were reversed, and residual proteins were removed by incubation with proteinase K. DNA was recovered using the QIA quick spin column (Qiagen, Valencia, CA). Quantitative PCR was used to determine the amounts of genomic DNA enriched in the chromatin preparations. The primers used are listed in Table 1. An *eIF4A* DNA fragment was used as an internal control for normalizing the amounts of the chromatin preparations used.

12. Western blot analysis

A MYC-coding sequence was fused in-frame to the 5' end of the *NTL4* gene, and the fusion construct was transformed into Col-0 plants. The 35S:*MYC-NTL4* transgenic plants grown for 2 weeks on MS-agar plates were used to examine the effects of drought and ABA on NTL4 processing. Protein extraction and immunological assays were performed as described previously (Kim et al., 2006). Briefly, harvested plant materials were ground in liquid nitrogen, and total cellular extracts were suspended in SDS-PAGE sample loading buffer. The protein samples were then analyzed on 10% SDS-PAGE gels and blotted onto Hybond-P^+ membranes (Amersham-Pharmacia, Amersham, UK). The blots were hybridized with an anti-MYC antibody (Santa Cruz Biotech, Santa Cruz, CA).

Primers	Sequences	Usage
eIF4A-F	5′-TGACCACACAGTCTCTGCAA	qRT-PCR
eIF4A-R	5′-ACCAGGGAGACTTGTTGGAC	〃
FCA-F	5′-GCTCTTGTCGCAGCAAACTC	〃
FCA-R	5′-GATCCAGCCCACTGTTGTTTAC	〃
PER1-F	5′-CGTGCCCTTCATATTGTTGG	〃
PER1-R	5′-GACGCCATCAACAACGAGTC	〃
EM6-F	5′-AGCCGAGGAGGGCAAACTCG	〃
EM6-R	5′-GGTCCTGAATTTGGATTCGT	〃
RD29B-F	5′-AGGGGAAAGGACATGGTGAG	〃
RD29B-R	5′-TACCACCGAGCCAAGAAGTG	〃
RAB18-F	5′-GTTGCCAGGTCATCATGATC	〃
RAB18-R	5′-CACCGGGAAGCTTTTCCTTG	〃
NIA1-F	5′-ACCACCAGGAGAAACCGAAC	〃
NIA1-R	5′-GAAAGACTCGTCCCAGGCTC	〃
GolS4-F	5′-CACGTGGCCAGAAGATATGG	〃
GolS4-R	5′-CCAAAGCATAGCCAAAACCA	〃
eIF4a-F	5′-TGACCACACAGTCTCTGCAA	〃
eIF4a-R	5′-ACCAGGGAGACTTGTTGGAC	〃
NTL4-F	5′-AAGGAATTGGATGTGGGGTC	〃
NTL4-R	5′-GAAACGCCTCACCAATCAAA	〃
AtrbohA-F	5′-AGGGGTCGTTTGACTGGTTC	〃
AtrbohA-R	5′-CTCGTAAACGCTGGTGCAGT	〃
AtrbohC-F	5′-CGTGGACCTCACGGTAGATG	〃
AtrbohC-R	5′-GACGGTTTCGTTTCAGCAAA	〃
AtrbohE-F	5′-CACGAGAGGAAATAGCTGCG	〃
AtrbohE-R	5′-AATCGTCTCGGGCGAGTAAT	〃
AtrbohC-A-F	5′-TACCCAAATCGTCCTCATGC	ChIP
AtrbohC-A-R	5′-TTTTGGCGTAAATAAAATTAGTAAA	〃
AtrbohC-B-F	5′-TGGACAAACATAAGAATTACTATAACA	〃
AtrbohC-B-R	5′-GCTGTGAATCTTTAGCAGCGA	〃
AtrbohC-C-F	5′-CCAAGTCCGACCACGTCTCT	〃
AtrbohC-C-R	5′-GGAGAATGAGAAAATGGATTCG	〃
AtrbohE-D-F	5′-TCTTCTTCTTCTTAGCCAGCTCAT	〃
AtrbohE-D-R	5′-GGAGGAGGAAGAGAGTGGGA	〃
AtrbohE-E-F	5′-TTCTGTCATCTAAGTATCTACGTGGC	〃
AtrbohE-E-R	5′-TGAATATGCAAAGGCAAGGG	〃
AtrbohE-F-F	5′-AGAAAACAACTGTGAAAACCACG	〃
AtrbohE-F-R	5′-CTTCTCAACCGATTGATACAAAAA	〃

Table 1. Primers used in qRT-PCR and ChIP assays.

F, forward primer; R, reverse primer.

CHAPTER 1

Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in *Arabidopsis*

RESULTS

SA affects seed germination in a dosage-dependent manner

I set up an extensive set of germination assays using different *Arabidopsis* genotypes to obtain as to how SA affects seed germination. I first examined the effects of different concentrations of SA (0-5 mM). Whereas SA of 0-10 μM had no discernible effects, higher concentrations of SA inhibited germination in a dosage-dependent manner (Fig. 1).

I employed two SA-deficient *Arabidopsis* plants that are frequently used in the SA-related assays; the *sid2* mutant and the *NahG* transgenic plant. The *SID2* (*SA-induction deficient*) gene encodes an isochorishmate synthase, and the *sid2* mutant is defective in SA biosynthesis (Nawrath & Métraux, 1999). The *NahG* transgenic plant overexpresses the *NahG* gene encoding a salicylate hydroxylase that catabolizes SA to catechol (Gaffney et al., 1993). The overall plant growth and development of the SA-deficient plants were indistinguishable from those of wild-type plants (Col-0) (Fig. 2). Their germination phenotypes were also similar to that of wild-type plants when germinated on MS-agar plates (Fig. 3), indicating that SA is not essential for germination under normal growth conditions.

Notably, the SA-deficient plants exhibited distinct germination phenotypes when SA was included in the media. Seeds of the wild-type and SA-

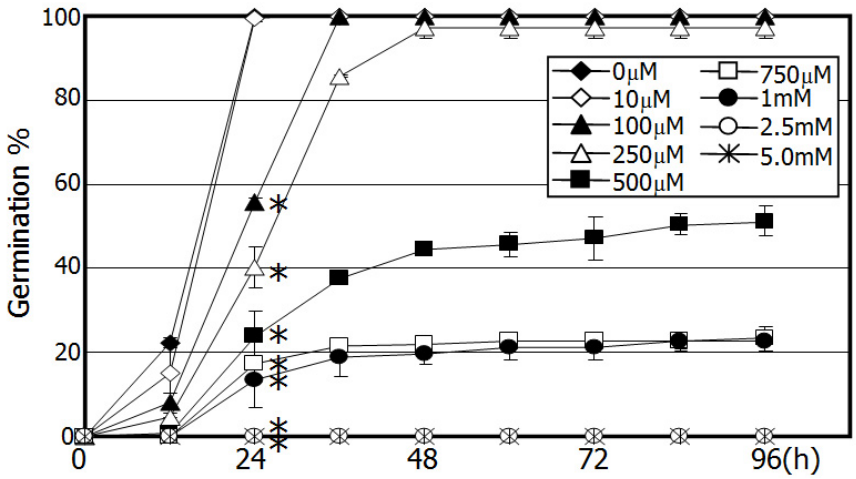

Figure 1. SA delays germination in a dosage-dependent manner.

For germination assays, seeds air-dried for 2 weeks were imbibed at 4°C on MS-agar plates for 3 days in complete darkness and allowed to germinate at 22°C under long days. Appearance of visible radicles was used as a morphological marker for germination. Three measurements, each consisting of 40-50 seeds, were averaged and statistically treated using a student's *t*-test. Bars indicate standard error of the mean. h, hours after cold-imbibition. To examine the effects of SA on germination, the seeds of the Col-0 plants were cold-imbibed and germinated on MS-agar plates supplemented with various concentrations of SA. Germination was significantly delayed at 24 h after cold-imbibition in a dosage-dependent manner in the presence of 100 μM-5 mM SA (*P<0.01).

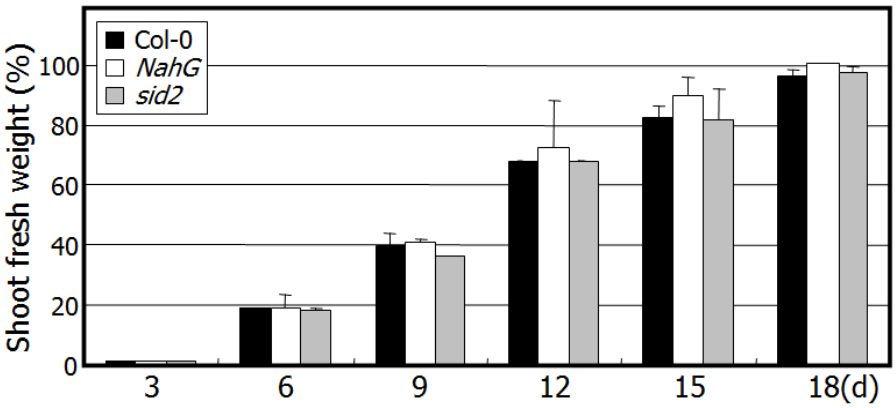

Figure 2. Shoot growth of the Col-0, *NahG*, and *sid2* plants.

Fresh weights of 30 shoots grown on MS-agar plates under normal growth conditions for up to 18 days after cold-imbibition were measured and averaged.

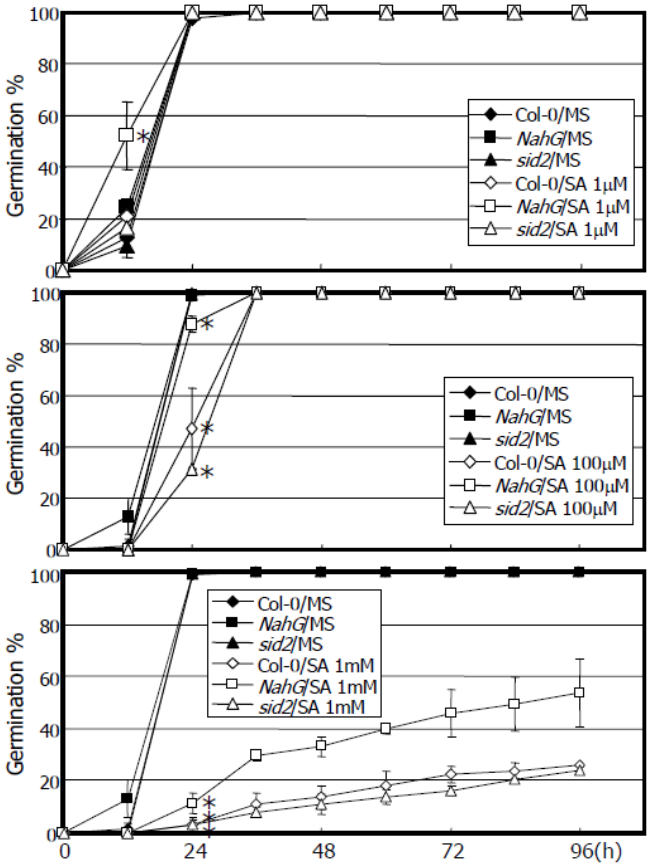

Figure 3. Effects of SA on germination of the Col-0, *NahG*, and *sid2* seeds. Seeds were cold-imbibed and allowed to germinate in the presence of 1 μM (upper panel), 100 μM (middle panel), or 1 mM of SA (lower panel). In the presence of 1 μM SA, germination percentage of the *NahG* seeds was significantly higher than that without SA at 12 h after cold-imbibition (*P<0.01). Meanwhile, in the presence of 100 μM or 1 mM SA, germination percentages of the Col-0, *NahG*, and *sid2* seeds were significantly higher than those without SA at 24 h after cold-imbibition (*P<0.01).

deficient plants germinated at a similar rate on MS-agar plates supplemented with 1 μM SA. When higher than 100 μM SA was added to the media, germination of wild-type seeds was delayed (Fig. 3). Germination of the *sid2* seeds was also delayed to a degree similar to that of wild-type seeds, showing that high concentrations of SA have a negative effect on germination. Meanwhile, germination of the *NahG* seeds was less sensitive to SA. Since both the *sid2* and *NahG* plants have defects in SA accumulation, the differential germination response of the *NahG* seeds to SA would be attributable to a SA degradation product, such as catechol.

SA reduces the inhibitory effects of high salinity on germination

SA affects seed germination under high salinity and osmotic stress (Borsani et al., 2001; Rajjou et al., 2006; Alonso-Ramírez et al., 2009). I therefore decided to examine the effects of SA on germination under high salinity.

Germination of wild-type seeds was significantly delayed by NaCl, with more severe inhibition at higher concentrations (Fig. 4A), as previously reported (Saleki et al., 1993). I examined the effects of SA on germination in the presence of 150 mM NaCl. The results showed that germination was further inhibited when SA of higher than 100 μM was added in the media (Fig. 4B). In contrast, lower concentrations of SA (1-10 μM) compromised the inhibitory effects of high salinity

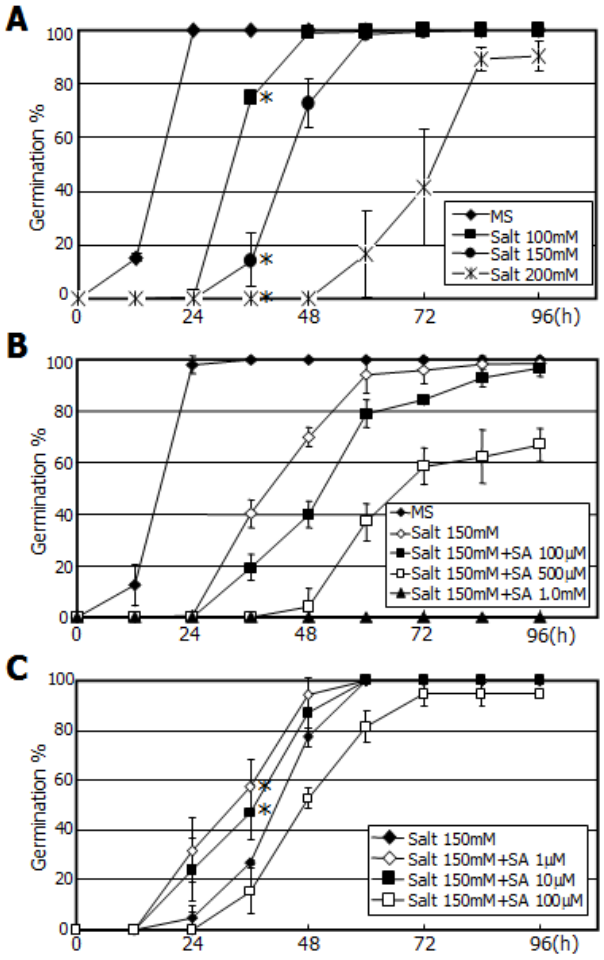

Figure 4. SA promotes germination under high salinity.

Germination assays were carried out as described in Figure 1. Statistical significance of the measurements was determined using a student's *t*-test. Bars indicate standard error of the mean. h, hours after cold-imbibition.

(A) Effects of NaCl on germination of the Col-0 seeds. Seeds were cold-imbibed and germinated on MS-agar plates supplemented with various concentrations of NaCl. All the concentrations of NaCl examined significantly delayed germination

at 36h after cold-imbibition (*P<0.01).

(B) Effects of high concentrations of SA on germination of the Col-0 seeds under high salinity. Seeds were cold-imbibed and germinated on MS-agar plates supplemented with 150 mM NaCl (calculated water potential ψ_w of -0.83 MPa; Verlues et al., 2006) and various concentrations (100 μM-1 mM) of SA.

(C) Effects of low concentrations of SA on germination of the Col-0 seeds under high salinity. SA at the concentration of 1 μM or 10 μM significantly promoted germination (*P<0.01).

(Fig. 4C). These observations indicate that lower concentrations of SA, which are close to the estimated physiological concentrations of SA in *Arabidopsis* plants (Preston et al., 2009), eliminate the inhibitory effects of high salinity on germination.

Germination of the *sid2* seeds is hypersensitive to high salinity

Our data indicate that SA plays a promotive role in germination under high salinity. However, this observation is in contrast to a previous germination study using the *NahG* seeds (Borsani et al., 2001). One possible reason for the discrepancy would be that the effects of SA on germination vary depending on *Arabidopsis* genotypes examined.

To examine the hypothesis, I carried out germination assays using seeds of the *sid2* mutant and the *NahG* plant in the presence of 150 mM NaCl and varying concentrations of SA. Whereas germination of the *NahG* seeds was delayed by high salinity to a similar degree as that observed with wild-type seeds, that of the *sid2* seeds was more significantly delayed (Fig. 5), showing that germination of the *sid2* seeds is hypersensitive to high salinity. When 500 μM SA was included in the assays, germination of the wild-type and *sid2* seeds was further delayed (Fig. 6A). In contrast, germination of the *NahG* seeds was uninfluenced by SA, which is certainly due to SA degradation occurring in the *NahG* plant.

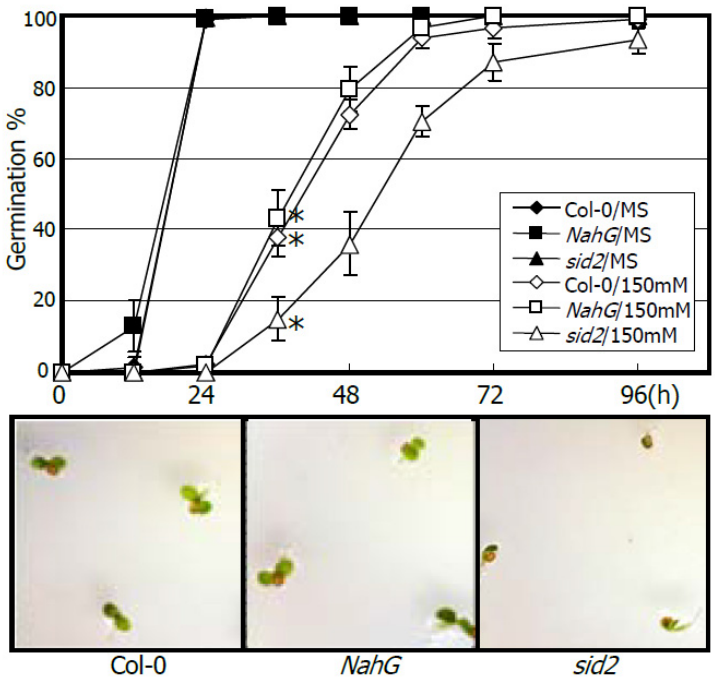

Figure 5. Germination of the *sid2* seeds is hypersensitive to high salinity. Germination assays were carried out as described in Figure 1. Statistical significance of the measurements was determined using a student's *t*-test. Bars indicate standard error of the mean. h, hours after cold-imbibition. Seeds were cold-imbibed and germinated on MS-agar plates supplemented with 150 mM NaCl. Germination was significantly delayed in the presence of NaCl (*P<0.01). The bottom panel shows very young seedlings right after germination.

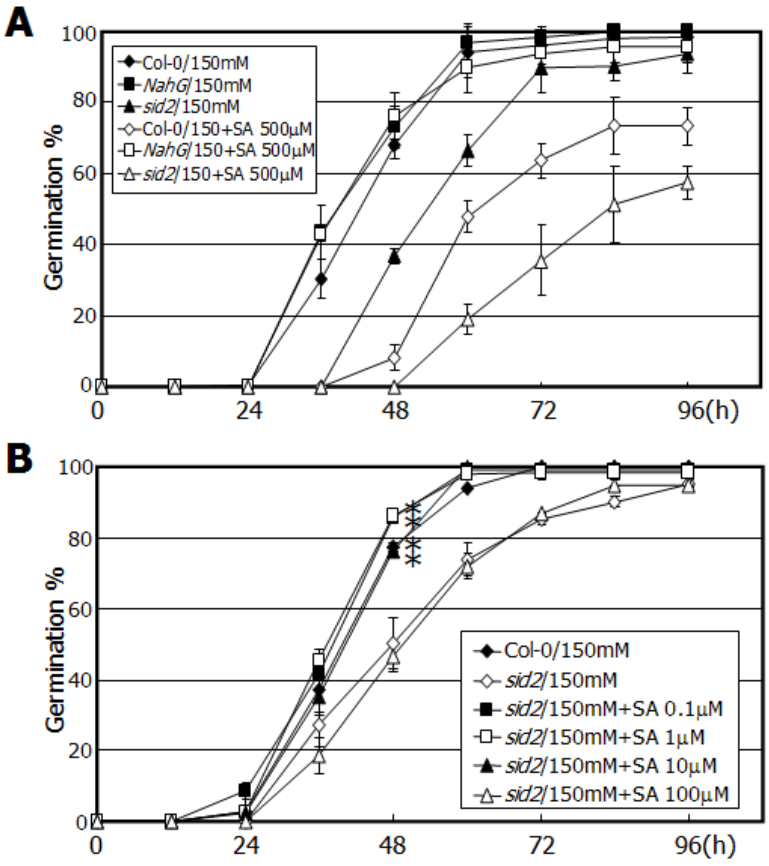

Figure 6. Low concentration of SA promotes seed germination under high salinity.

(A) Effects of 500 μM SA on germination of the Col-0, *sid2*, and *NahG* seeds under high salinity.

(B) Effects of low concentrations of SA on germination of the *sid2* seeds under high salinity Germination was significantly promoted by 10 μM or lower concentrations of SA (*P<0.01).

It is estimated that physiological concentrations of SA are far below 50 μM in *Arabidopsis* plants (Preston et al., 2009). I therefore examined the effects of lower concentrations of SA on the germination of the *sid2* seeds under high salinity. Interestingly, lower concentrations of SA, such as 0.1 μM, 1 μM, and 10 μM, promoted the germination of the *sid2* seeds (Fig. 6B). These observations indicate that SA, at its concentrations close to those in physiological conditions, promotes germination under high salinity.

SA is related with osmotic stress imposed by high salinity on germination

It has been reported that both high salinity and osmotic stress confer an inhibitory effect on seed germination (Zhu, 2000). I also observed that high salinity delays germination. However, the inhibitory effect is reduced by low concentrations of SA.

To gain insight into how SA compromises the inhibitory effects of high salinity, I examined the biochemical nature of salt effects on germination. Germination of wild-type seeds and of the *sid2* and *NahG* seeds was delayed by 150 mM KCl (calculated water potential ψ_w of -0.83 Mpa; Verslues et al., 2006) to a similar degree as with 150 mM NaCl (Fig. 7A). Since high concentrations (>65 mM) of Li^+ are toxic to plant growth, the effects of LiCl were examined at 65 mM (calculated water potential ψ_w of -0.43 MPa). The effects of 65 mM LiCl were also similar to those of 150 mM NaCl (Fig. 7B).

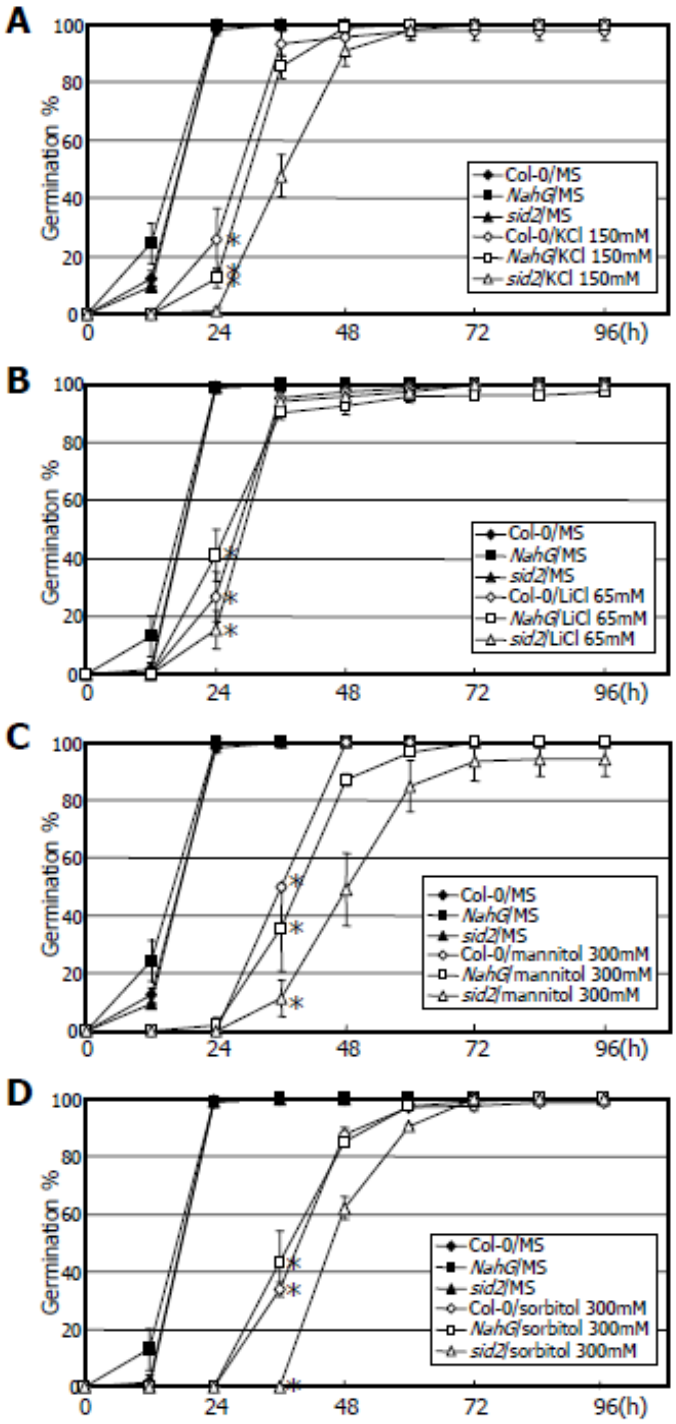

Figure 7. High salinity imposes osmotic stress on germination.

Germination assays were carried out as described in Figure 1. Statistical

significance of the measurements was determined using a student's *t*-test. Bars indicate standard error of the mean. h, hours after cold-imbibition. Water potentials were calculated as previously described (Verlues et al., 2006).

(A-D) Germination responses of the Col-0, *sid2*, and *NahG* seeds to 150 mM KCl (calculated water potential ψ_w of -0.83 MPa) (A), 65 mM LiCl (calculated water potential ψ_w of -0.43 MPa) (B), 300 mM mannitol (calculated water potential ψ_w of -0.83 MPa) (C), and 300 mM sorbitol (calculated water potential ψ_w of -0.83 MPa) (D). Germination was significantly delayed by LiCl and KCl at 24 h after cold-imbibition or by mannitol and sorbitol 36 h after cold-imbibition (*P<0.01).

I next examined the effects of 300 mM mannitol (calculated water potential ψ_w of -0.83 Mpa). Germination of wild-type seeds and of the *sid2* and *NahG* seeds was delayed in a pattern similar to that observed in the presence of 150 mM NaCl (Fig. 7C). Furthermore, similar inhibitory effects were observed when the media was supplemented with a nonionic osmolite, sorbitol (300 mM, calculated water potential ψ_w of -0.83 Mpa) (Fig. 7D). Together, these observations support that the role of SA in germination under high salinity is related with osmotic stress rather than ionic toxicity.

Catechol promotes germination under high salinity

Germination percentage of the *sid2* seeds was significantly lower than those of the *NahG* seeds under high salinity (Fig. 8A). I hypothesized that the differential effects of high salinity on germination would be caused by catechol possessing an antioxidant activity (Rice-Evans et al., 1997).

To examine the hypothesis, we analyzed the effects of varying concentrations of catechol on germination. Treatments of wild-type seeds with catechol at concentrations of 1 μM to 1 mM did not confer any visible effects on germination under normal growth conditions (Fig. 8B). We found that catechol at concentrations higher than 100 μM affects seed viability, as evidenced by dark-brown coloring and lack of germination. I therefore used 10 μM catechol in the

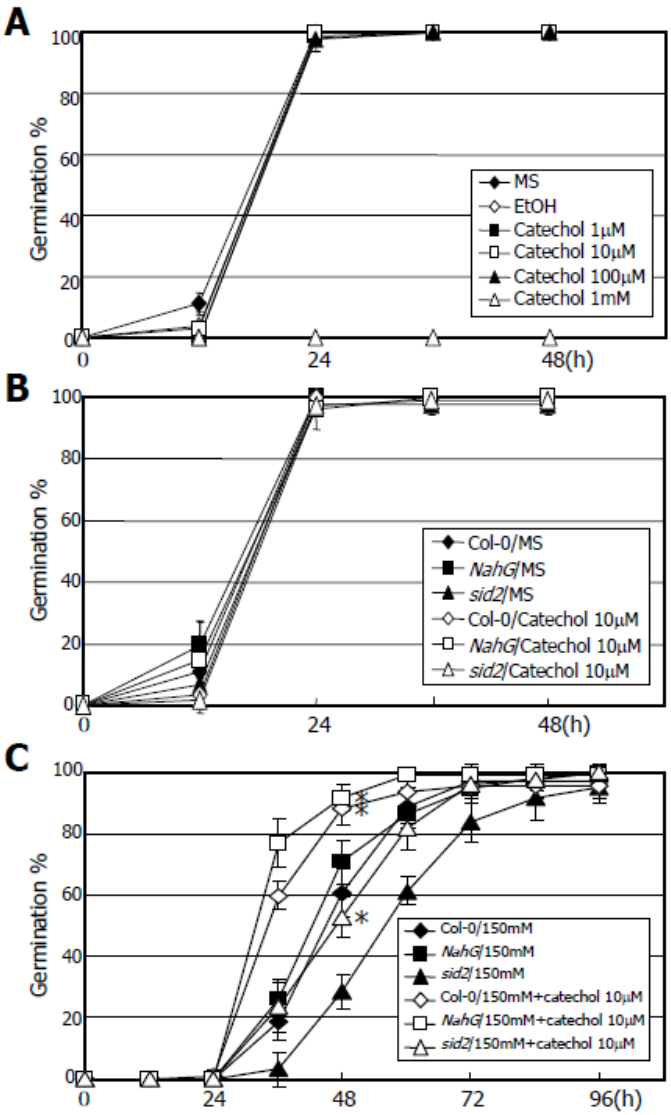

Figure 8. Catechol promotes germination under high salinity.

Germination assays were carried out as described in Figure 1. Statistical significance of the measurements was determined using a student's *t*-test. Bars indicate standard error of the mean. h, hours after cold-imbibition.

(A) Germination of the Col-0 seeds in the presence of various concentrations of catechol.

(B) Germination of the Col-0, *sid2*, and *NahG* seeds in the presence of 10 μM catechol.

(C) Germination of the Col-0, *sid2*, and *NahG* seeds in the presence of 150 mM NaCl with or without 10 μM catechol. Germination of the Col-0, *sid2*, and *NahG* seeds was significantly promoted by 10 μM catechol at 48 h after cold-imbibition under high salinity (*P<0.01).

subsequent assays.

I examined the effects of 10 μM catechol on the germination of the *sid2* and *NahG* seeds. Again, there were no apparent effects of catechol on the germination of the SA-deficient seeds (Fig. 8C). Seedlings grown on MS-agar plates containing 10 μM catechol for upto 2 weeks were also morphologically identical to wild-type seedlings, indicating that catechol does not affect seedling growth as well as germination under normal growth conditions.

Interestingly, 10 μM catechol promoted the germination of both wild-type and SA-deficient seeds under high salinity (Fig. 8C). The germination percentage of the *sid2* seeds on MS-agar plates containing 150 mM NaCl and 10 μM catechol was close to those of the wild-type and *NahG* seeds on MS-agar plates supplemented with 150 mM NaCl alone. It was therefore evident that the germination response of the *NahG* seeds to high salinity is caused catechol.

Catechol acts as an antioxidant

The promotive effects of catechol on seed germination under high salinity suggested that it might act as an antioxidant that reduces the endogenous level of ROS produced under high salinity (Lekse et al., 2001).

I assayed the germination rates of wild-type and SA-deficient seeds in the presence of a representative ROS, hydrogen peroxide (H_2O_2). Germination of wild-

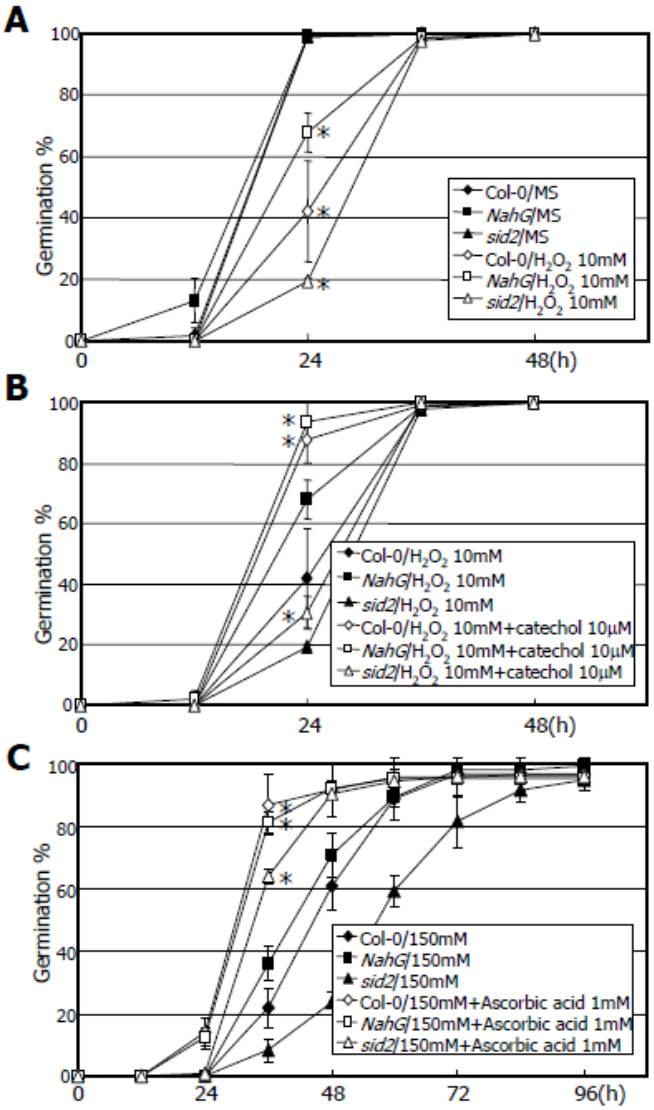

Figure 9. **Catechol acts as an antioxidant.**

Germination assays were carried out as described in Figure 1. Statistical significance of the measurements was determined using a student's *t*-test. Bars indicate standard error of the mean. h, hours after cold-imbibition.

(A-C) Germination of the Col-0, *sid2*, and *NahG* seeds in the presence of 10 mM

hydrogen peroxide (A), 10 mM hydrogen peroxide with or without 10 μM catechol (B), and 150 mM NaCl with or without 1 mM ascorbic acid (C). Germination of the Col-0, *sid2*, and *NahG* seeds was significantly delayed by 10 mM hydrogen peroxide but promoted by 10 μM catechol at 24 h after cold-imbibition (**P*<0.01). One mM ascorbic acid also significantly promoted germination under high salinity (**P*<0.01).

type seeds was delayed in proportion to the concentrations of H_2O_2 (data not shown). I therefore used 10 mM H_2O_2 in the germination assays. I found that whereas germination of the *NahG* seeds was affected to a lesser degree by H_2O_2 compared to its effect on the germination of wild-type seeds, germination of the *sid2* seeds was more severely affected by H_2O_2 (Fig. 9A), suggesting that catechol acts as an antioxidant. It was also envisioned that SA promotes seed germination by reducing the endogenous level of ROS under high salinity.

I therefore included catechol into the germination assays. Germination of the wild-type and *NahG* seeds was significantly promoted by 10 μM catechol in the presence of 10 mM H_2O_2 (Fig. 9B), supporting the role of catechol as an antioxidant. Germination of the *sid2* seeds was also promoted by catechol but to a lesser degree, suggesting that SA plays a role in H_2O_2 metabolism. I subsequently examined the effects of ascorbic acid, a well-known, naturally occurring antioxidant, on germination under high salinity. Germination of the wild-type and *NahG* seeds was markedly promoted by 1 mM ascorbic acid (Fig. 9C). These observations demonstrate that catechol acts as an antioxidant by reducing H_2O_2 levels in germination under high salinity.

SA modulates H_2O_2 levels in germination under high salinity

Germination of the *sid2* seeds is hypersensitive to H_2O_2 (Fig. 9A). The inhibitory

effects of H_2O_2 are reduced by antioxidants, particularly ascorbic acid (Fig. 9C). In addition, germination of the *sid2* seeds was promoted by catechol under high salinity. It was therefore hypothesized that the endogenous H_2O_2 level would be higher in the *sid2* seeds than in wild-type seeds.

I measured the endogenous contents of H_2O_2 in the germinating seeds. As expected, the endogenous levels of H_2O_2 were gradually elevated in the germinating seeds of both the wild-type and *sid2* plants but with overall higher levels in the *sid2* seeds during the time course of upto 48 hours after cold-imbibition (Fig. 10A). When the seeds were germinated on MS-agar plates containing 150 mM NaCl, the levels of H_2O_2 were further elevated by approximately 50% in both the wild-type and *sid2* seeds throughout the time course (Fig. 10B). When wild-type seeds were germinated on MS-agar plates supplemented additionally with 1 μM SA, the levels of H_2O_2 were reduced to the levels comparable to those measured in the germinating seeds on MS-agar plates (Fig. 10C). These observations strongly support that SA plays a role in reducing the H_2O_2 levels under high salinity.

It was unexpected that the endogenous level of H_2O_2 was higher in the germinating *NahG* seeds than in the germinating wild-type and *sid2* seeds on MS-agar plates (Fig. 10A) but was reduced in the presence of 150 mM NaCl unlike the elevations in the wild-type and *sid2* seeds (Fig. 10B). In addition, the level was

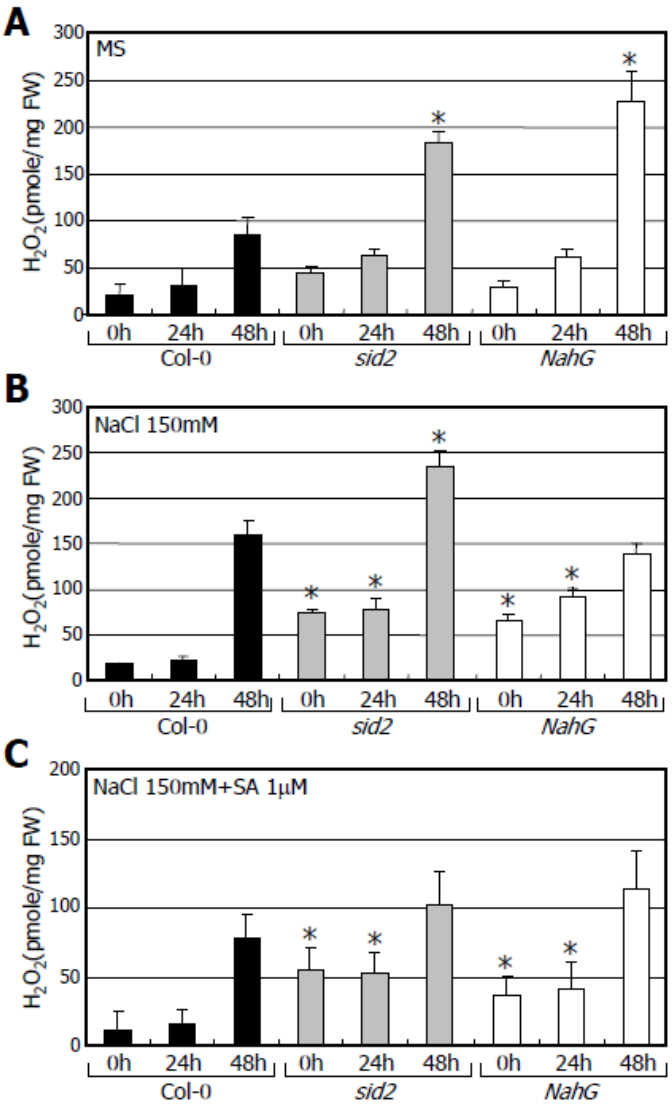

Figure 10. Endogenous contents of hydrogen peroxide are reduced by SA. Hydrogen peroxide contents were measured as previously described (Shin & Schachtman, 2004). Approximately 100 mg of seeds was used for each assay. Three measurements were averaged and statistically treated using a student's *t*-test (**P*<0.01). Bars indicate standard error of the mean. To examine the effects of NaCl and SA on endogenous contents of hydrogen peroxide, seeds of the Col-0,

NahG, and *sid2* plants were cold-imbibed and germinated on MS-agar plates supplemented with 150 mM NaCl and 1 μM SA. h, hours after cold-imbibition. (A-C) Endogenous contents of hydrogen peroxide in the germinating seeds on MS-agar plates (A), MS-agar plates supplemented with 150 mM NaCl (B), and MS-agar plates supplemented with 150 mM NaCl and 1 μM SA (C).

only slightly reduced in the SA-treated *NahG* seeds under high salinity (Fig. 10C). The high level of H_2O_2 in the germinating *NahG* seeds on MS-agar plates would be due to SA deficiency. Under high salinity, the SA content is elevated (Molina et al., 2002; Xiong et al., 2002), which contributes to an increase of the endogenous content of catechol and reduces the H_2O_2 level.

I next measured the peroxidase activity, which converts H_2O_2 to water, in germinating seeds in either the presence or absence of 150 mM NaCl. It was uninfluenced by salt stress in the germinating wild-type seeds (Fig. 11), indicating that H_2O_2 metabolism is not regulated by the peroxidase activity under high salinity. The peroxidase activity was higher in the germinating SA-deficient seeds on MS-agar plates (Fig. 11A) but reduced to a level comparable to that observed in wild-type seeds on MS-agar plates containing 150 mM NaCl (Fig. 11B), possibly because of feedback regulation by the altered levels of H_2O_2 in the SA-deficient seeds. Therefore, it seems that regulation of H_2O_2 by high salinity and SA is not directly linked with the peroxidase activity.

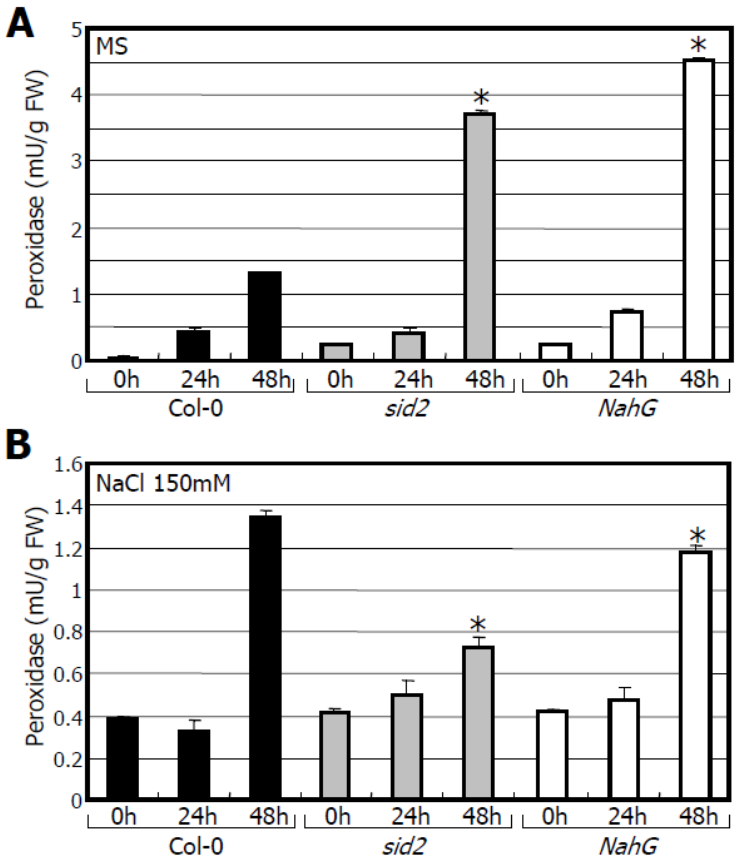

Figure 11. Peroxidase activity is elevated in the germinating seeds of the *sid2* mutant.

Peroxidase activity assays on the germinating seeds were carries out as previously described (Shin and Schachtman, 2004). Three measurements of the activities were averaged. Bars indicate standard error of the mean (*t*-test, **P*<0.01).

(A and B) Peroxidase activity in the germinating seeds of the Col-0, *NahG*, and *sid2* plants on MS-agar plates (A) and MS-agar plates supplemented with 150 mM NaCl (B).

DISCUSSION

SA in seed germination

To further define the roles of SA in seed germination, I carried out an extensive set of germination assays using two SA-deficient plants in addition to wild-type plants in the presence different combinations of SA, NaCl, catechol, ascorbic acid, and H_2O_2. We found that SA displays both the promotive and inhibitory effects on germination, depending on its concentrations applied and assay conditions.

Most previous assays on the role of SA on seed germination have been carried out using SA concentrations of higher than 100 μM (Rajjou et al., 2006; Xie et al., 2007). It is known that a physiological concentration of SA in *Arabidopsis* is lower than 50 μM (Alonso-Ramírez et al., 2009). Furthermore, high concentrations of SA impose toxic effects on plant growth and development, possibly by inducing ROS biosynthesis (Rao et al., 1997). It is therefore supposed that the effects of SA on germination should be carefully interpreted. The inhibitory effects of high concentrations of SA in the previous reports and my own observations may not be the physiological role of SA but may be caused by toxic effects.

The contrasting roles of SA on seed germination in the previous observations are also explained by the differential germination responses of *sid2* and *NahG* seeds to high salinity. The *sid2* and *NahG* plants are common in that both lack

endogenous SA. However, catechol accumulates to a high level in the *NahG* plants (Gaffney et al., 1993). I found that catechol significantly reduces the inhibitory effects of high salinity. It also promoted germination in the presence of H_2O_2. It was therefore concluded that the variable effects of high salinity on the germination of the *sid2* and *NahG* seeds is due to catechol, which acts as an antioxidant removing H_2O_2 synthesized under salt stress.

The minute differences of experimental conditions, such as methods for treatments of chemicals and how fresh seeds were used, can also cause contradictory results. In the previous experiments, the plants were submerged in liquid for the treatment of compounds or the seeds were not kept fresh and set for the same stage after harvest (Borsani et al., 2001; Rajjou et al., 2006). These differences brought unreliable results. Seed germination is very sensitive to experimental conditions. Therefore, the seed germination assay must be more carefully examined.

SA regulation of H_2O_2 levels under osmotic stress

ROS is intimately related with germination process. Several reports have shown that H_2O_2 promotes germination. In *Zinnia elegans*, seed germination is promoted by H_2O_2, which decompose germination inhibitor(s), such as ethanol-soluble compounds (Ogawa & Iwabuchi, 2001). The inhibitory effects of ABA on germination and seedling development are also overcome by H_2O_2 (Sarath et al.,

2007). My measurements of endogenous H_2O_2 contents in germinating seeds support the previous observations.

However, it is evident that ROS, such as H_2O_2, affects germination process differentially under high salinity. ROS accumulated under abiotic stress conditions cause oxidative damage during plant development, including germination. I also observed that H_2O_2 accumulates to a higher level in the germinating seeds under high salinity, where germination is profoundly delayed. The inhibitory effects of high salinity were eliminated by antioxidants, such as catechol and ascorbic acid. Notably, SA lowers the level of H_2O_2 in the germinating seeds under high salinity. Altogether, my data strongly support that SA promotes germination by reducing osmotic damage. This view is also in good agreement with the role of SA in plant resistance responses to diverse abiotic stresses by reducing the oxidative damage forced by ROS (Yang et al., 2004; Tuteja and Sopory, 2008).

It is notable that ascorbic acid displayed a promotive role in germination under high salinity. However, the effect should be interpreted with care, since ascorbic acid has more functions than antioxidant, such as possible roles in cell expansion (Schopfer, 2001) and as an essential enzyme cofactor in various cellular reactions (Linster and Clarke, 2008). In addition, it needs to be synthesized during early stages of germination as there is very little in dry seeds (Badejo et al., 2009). It is therefore possible that ascorbic acid developmentally affects germination rather

than by acting as an antioxidant under high salinity.

Considering the genetic and biochemical complexity of ROS metabolism, a plausible explanation is that multiple antioxidant metabolic enzymes as well as peroxidase activities are mediated by SA signals under high salinity. Germination assays on higher-order mutants and extensive measurements of ROS species and antioxidants would clarify the uncertainty.

CHAPTER 2

A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in *Arabidopsis*

RESULTS

NTL4 is a transcriptional activator

The NTL4 protein (At3g10500) is structurally distinct from nuclear NAC transcription factors in that it has a strong α-helical transmembrane (TM) motif in the C-terminal region (Kim et al., 2007). The 35S:*NTL4* transgenic plants overexpressing a full-size NTL4 form were phenotypically indistinguishable from Col-0 plants (Fig. 12A). In contrast, the 35S:*4ΔC* transgenic plants overexpressing a truncated NTL4 form that lacks the TM motif exhibited distinct phenotypes, such as slightly reduced growth and curled leaves with asymmetric leaf axis and serrated margin. These observations indicate that membrane release is essential for NTL4 function and the 4ΔC polypeptide is closely related with a biologically active NTL4 form.

I obtained two independent knockout mutants, *ntl4-1* (SALK-009578C) and *ntl4-2* (SALK-007900) that contain a single copy of T-DNA element in the 3rd exon of *NTL4* gene (Fig. 12B). Gene expression assays by quantitative real-time RT-PCR (qRT-PCR) confirmed no detectable expression of the *NTL4* gene in the *ntl4* mutants (Fig. 12C). They did not exhibit any visible phenotypes when grown under normal conditions.

To examine whether the NTL4 protein possess transcriptional activity, the full-size *NTL4* and truncated *4ΔC* sequences were fused in-frame to the GAL4

DNA binding domain-coding sequence, and the fusion constructs were transformed into yeast cells (Kim et al., 2006). The yeast cells expressing either the NTL4 or 4ΔC construct grew equally well on the His^- Ade^- selective media and showed elevated β-galactosidase activity (Fig. 13A).

I also employed a GAL4 transient expression assay using *Arabidopsis* protoplasts (Miura et al., 2007). The effector plasmid, the reporter plasmid containing the GUS (β-glucuronidase) reporter gene, and the plasmid containing the *Renilla* luciferase gene (Yoo et al., 2007), which was used to normalize the measurements, were cotransformed into *Arabidopsis* protoplasts (Fig. 13B, left panel). The assays revealed that transient expressions of the *NTL4* and *4ΔC* genes enhanced the GUS activity approximately 3-fold compared to vector control (Fig. 13B, right panel), indicating that the NTL4 protein is a transcriptional activator.

NTL4 gene is induced under drought and heat conditions

To obtain insights into the role played by the NTL4 transcription factor, I examined the spatial and temporal expression patterns of the *NTL4* gene. Expression levels of the *NTL4* gene was induced rapidly in older plants that exhibit symptoms of senescing process (Fig. 14A). Gene expression studies revealed that the *NTL4* gene is expressed to a relatively high level in the roots (Fig. 14B).

The phenotypes of 35S:*4ΔC* transgenic plants, such as reduced growth and

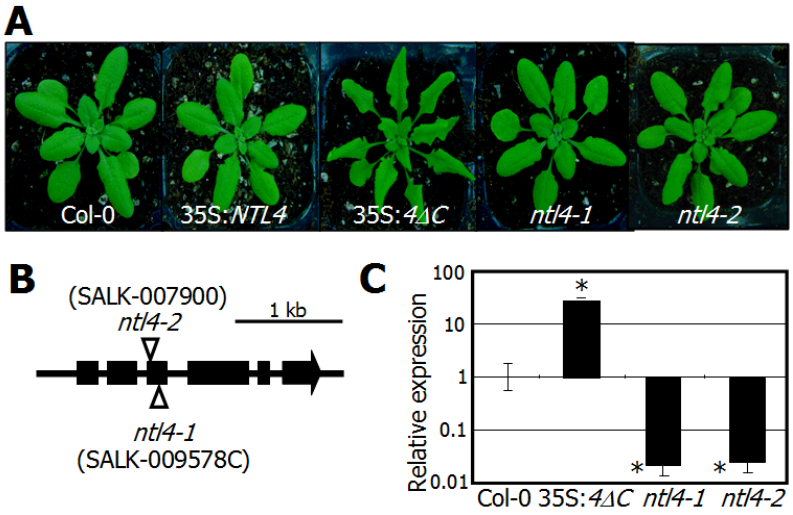

Figure 12. 35S:*NTL4ΔC* transgenic plants exhibited abnormal phenotypes under normal conditions.

(A) Phenotypes of 35S:*NTL4* and 35S:*4ΔC* transgenic plants and *ntl4* mutants. Plants were grown in soil at 23°C under long days for 4 weeks before taking photographs.

(B) Mapping of T-DNA insertion sites in *ntl4* mutants. kb, kilobase.

(C) Levels of *NTL4* gene transcripts. Transcript levels were determined by qRT-PCR. Biological triplicates were averaged and statistically treated using a student *t*-test (*P<0.01). Bars indicate standard deviation (SD).

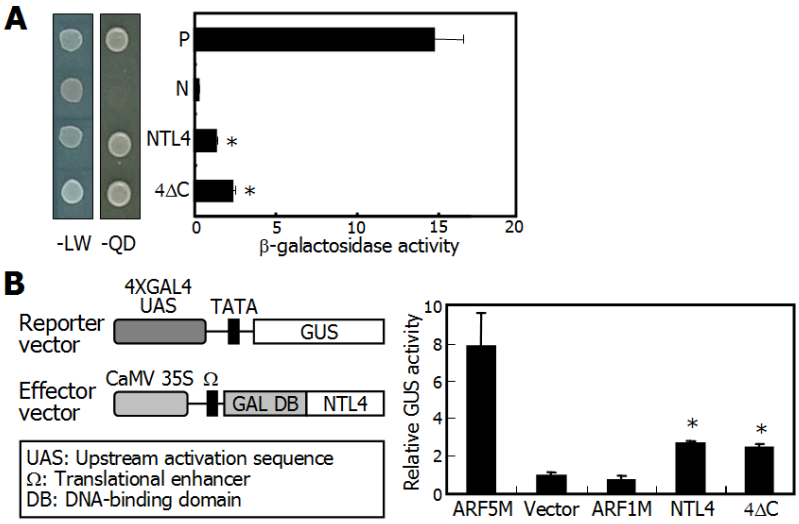

Figure 13. NTL4 is a transcriptional activator.

(A) Transcriptional regulation activity assays in yeast cells. Transcriptional regulation activities were examined by cell growth on selective media (left panel) and β-Gal activity assays (right panel). -LW indicates Leu and Trp drop-out plates. -QD indicates Leu, Trp, His, and Ade drop-out plates. P, positive control (full-size GAL4); N, negative control (DNA-binding domain alone). Four measurements were averaged and statistically treated using a student *t*-test (**P*<0.01). Bars indicate SD.

(B) Transcriptional regulation activity assays in *Arabidopsis* protoplasts. The reporter and effector vectors used were diagrammed (left panel). The GAL4 transient expression assays were carried out using *Arabidopsis* protoplasts (right panel). Vector control, the effector vector without gene inserts; ARF5M and

ARF1M, the effector vectors containing the *ARF5M* gene (activator control) and the *ARF1M* gene (repressor control), respectively (Tiwari et al., 2003). Four measurements were averaged and statistically treated using a student *t*-test (*$P<0.01$). Bars indicate SD.

altered leaf morphology, were similar to those observed in plants exposed to environmental stresses (Heil and Baldwin, 2002). I therefore examined the effects of abiotic stresses on the expression of *NTL4* gene. The *NTL4* gene was induced more than 2-fold by drought and heat treatments (Fig. 14C). I also examined whether the *NTL4* gene is influenced by growth hormones. The *NTL4* gene was induced approximately 3-fold by ABA (Fig. 14D). These observations suggest that the *NTL4* gene plays a role in drought and heat stress responses that are perhaps mediated by ABA.

To further examine the effects of drought on *NTL4* gene expression, the GUS-coding sequence was fused in-frame to *NTL4* gene promoter consisting of approximately 1-kb sequence region upstream, and the promoter-GUS fusion was transformed into Col-0 plants. GUS activity was detected mainly in the roots under normal conditions (Fig. 15). When the transgenic plants were exposed to drought stress, the GUS activity was significantly elevated in the aerial plant parts, primarily in the distal leaf area where leaf senescing process initiates under adverse growth conditions (Lim et al., 2007), suggesting that the *NTL4* gene would be related with leaf senescence under drought conditions.

Seedling growth of *ntl4-1* mutant is less sensitive to ABA

I found that *NTL4* gene is induced by ABA. I therefore examined the response of

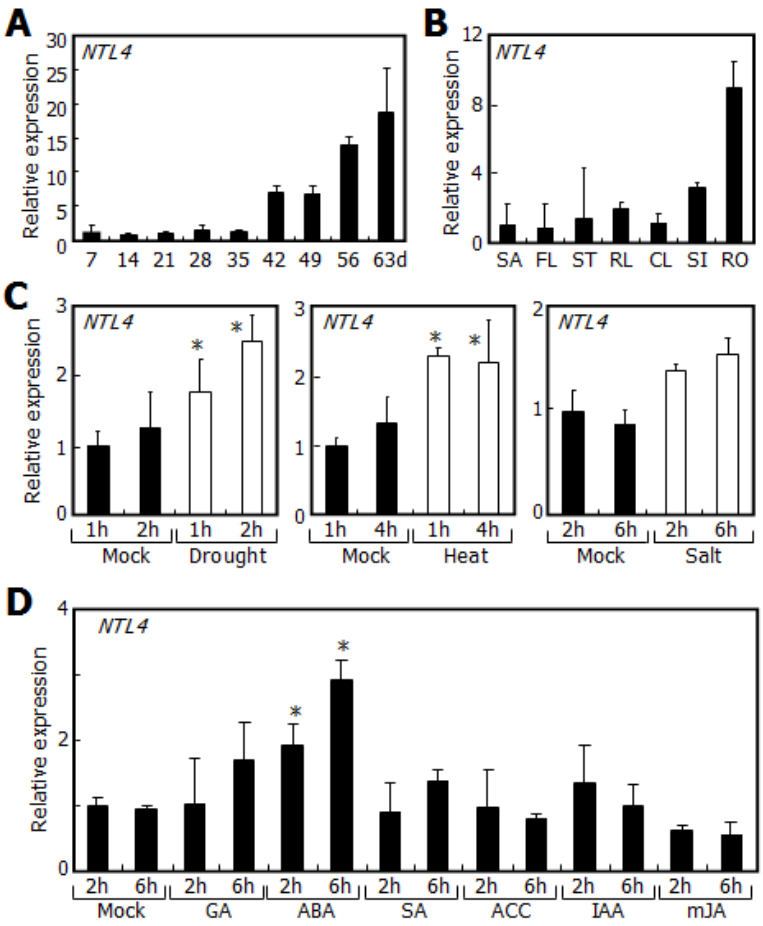

Figure 14. *NTL4* gene is induced under drought and heat conditions.

In (A)-(B), transcript levels were determined by qRT-PCR as described in Figure 12C. Bars indicate SD (*t*-test, **P*<0.01).

(A) Growth stage-dependent expression of *NTL4* gene. d, days after germination.

(B) Tissue-specific expression of *NTL4* gene. SA, shoot apical regions; FL, flowers; ST, stems; RL, rosette leaves; CL, cauline leaves; SI, siliques; RO, roots.

(C) Effects of abiotic stresses on *NTL4* gene expression. Two-week-old plants

grown on MS-agar plates were exposed to drought, heat (42^{o}C), or 200 mM NaCl for the indicated time durations. h, hour.

(D) Effects of growth hormones on *NTL4* gene expression. Two-week-old plants grown on MS-agar plates were transferred to MS liquid cultures containing different growth hormones, such as ABA (20 μM), IAA (20 μM), GA (50 μM), SA (100 μM), mJA (20 μM), and ACC (20 μM), and incubated for up to 6 h.

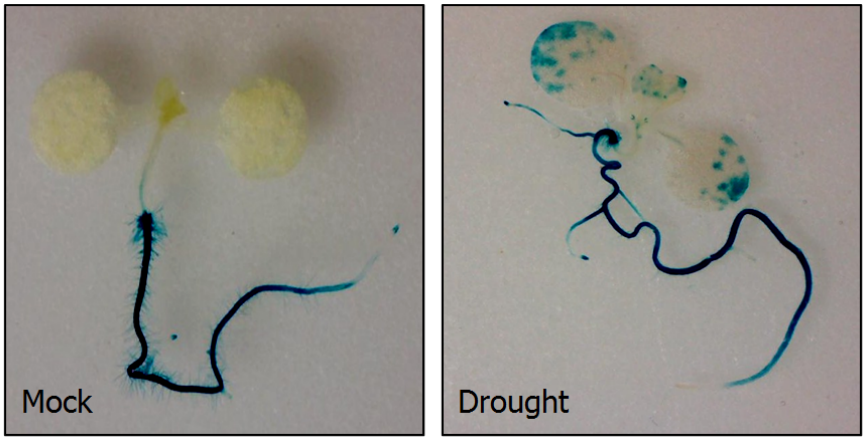

Figure 15. Effects of drought on the promoter activity of *NTL4* gene.

Ten-day-old transgenic plants expressing the p*NTL4-GUS* fusion grown on MS-agar plates were put on dry 3MM paper for 1 h and subject to GUS staining.

35S:*4ΔC* transgenic plants and *ntl4-1* mutants to ABA. Whereas the primary root growth of the 35S:*4ΔC* transgenic plants was influenced more severely by ABA, that of the *ntl4-1* mutant was less affected by ABA (Fig. 16). In the presence of 10 μM ABA, the primary root length was reduced by approximately 30% in Col-0 plants. Notably, whereas it was reduced by 60% in the transgenic plants, that of the *ntl4-1* mutant was reduced by approximately 24% under identical conditions. Lateral root growth was also influenced by ABA in a similar pattern. Whereas lateral root formation was more severely influenced by ABA in the 35S:*4ΔC* transgenic plants, it was less influenced in the *ntl4-1* mutant compared to that of Col-0 plants (Fig. 16, bottom panel). These observations support that the *NTL4* gene is involved in ABA-mediated abiotic stress responses.

ntl4 mutants exhibit enhanced drought resistance

I next carried out drought resistance assays. Plants were grown in soil for 2 weeks under normal watering conditions, and watering was halted until symptoms of wilting and necrosis are visible. The plants were then rewatered, and survived plants were counted.

Whereas the 35S:*4ΔC* transgenic plants were hypersensitive to drought, the *ntl4* mutants were relatively resistant to drought (Fig. 17), supporting the role of NTL4 in drought stress response.

I also examined the effects of drought on seedling growth. Primary root growth of the transgenic plants was significantly inhibited in the presence PEG, but the inhibitory effects of PEG were greatly reduced in the mutants (Fig. 18).

Leaf senescence is delayed in *ntl4* mutants under drought conditions

Recent increasing evidence indicates that delayed leaf senescence goes along with enhanced drought resistance (Rivero et al, 2007). I found that *NTL4* gene is rapidly induced in senescing plants. It was therefore assumed that the *NTL4* gene might be related with leaf senescence.

Leaf senescing processes of Col-0 plants, 35S:*4ΔC* transgenic plants, and *ntl4* mutants were not discernibly different when the plants were grown under normal conditions (Fig. 12A). In contrast, under drought conditions, whereas leaf senescence was accelerated in the 35S:*4ΔC* transgenic plants, it was notably delayed in the *ntl4* mutants (Fig. 19A). The *NTL4* gene was induced more than 4-fold under identical conditions (Fig. 19B). I also measured chlorophyll contents of the rosette leaves. Under drought conditions, whereas the chlorophyll contents decreased in the transgenic leaves, they were elevated slowly but steadily in the mutant leaves (Fig. 19C), indicating that the *NTL4* gene is associated with leaf senescence under drought conditions. Together, these observations indicate that the *NTL4* gene plays a role in leaf senescing process occurring under drought

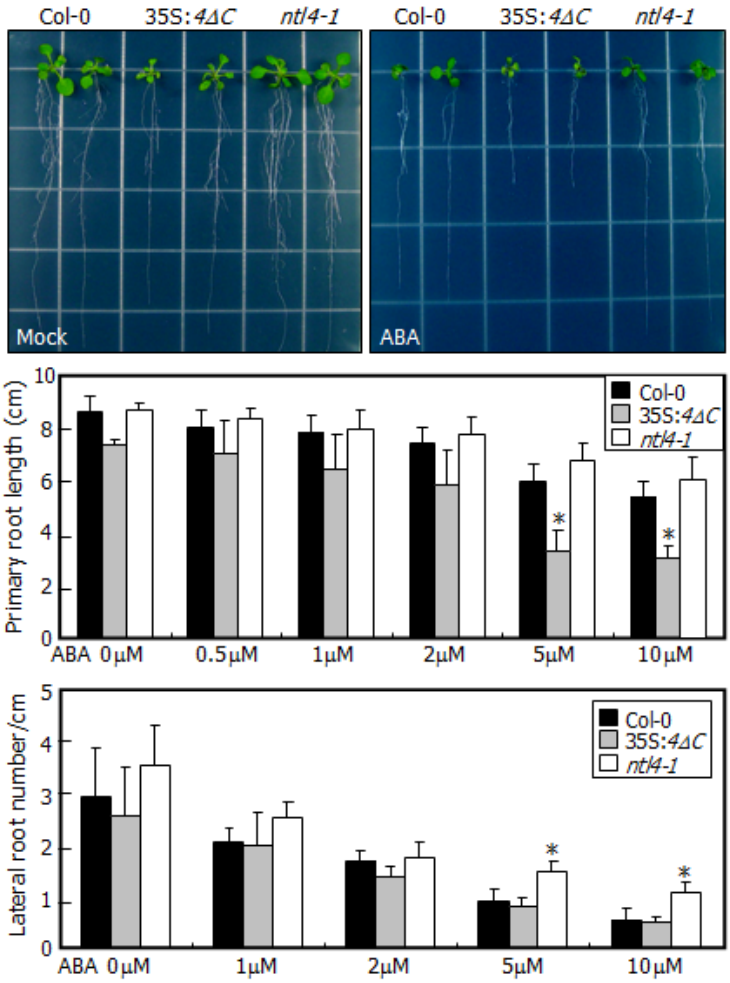

Figure 16. Effects of ABA on root growth.

Five-day-old plants grown on MS agar plates were transferred to vertical MS-agar plates containing various concentrations of ABA, and further grown for 1 week before taking photographs (upper panel, 5 μM ABA). Primary root lengths (middle panel) and lateral root numbers (lower panel) of 24 seedlings were measured and averaged for each plant genotype. Statistical significance of the measurements was determined using a student *t*-test (**P*<0.01). Bars indicate SD.

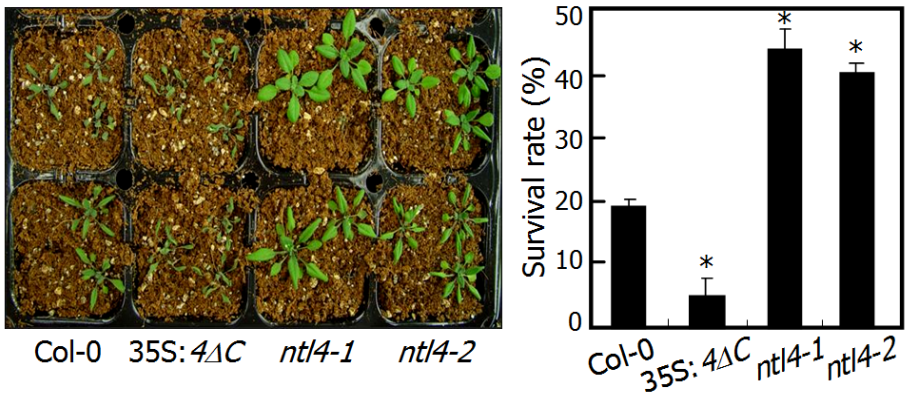

Figure 17. *NTL4* gene mediates drought stress response.

Drought resistance assays. Survival rates were calculated using 30 plants for each plant genotype and averaged. Statistical significance of the measurements was ``determined using a student *t*-test (**P*<0.01). Bars indicate SD.

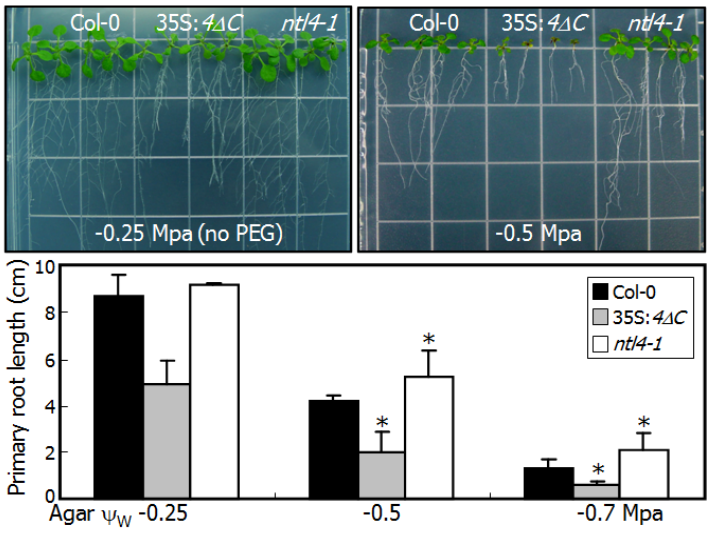

Figure 18. Seedling growth assays under osmotic stress.

Four-day-old plants grown on MS agar plates were transferred to vertical MS-agar plates having low Ψ_w that was imposed by PEG and further grown for 2 weeks before taking photograph (upper panel). Primary root lengths were measured and averaged using 24 plants for each plant genotype (lower panel). Bars indicate SD (*t*-test, **P*<0.01).

conditions.

NTL4 regulates ROS accumulation

ROS play a role in drought-induced leaf senescence (Munné-Bosch and Alegre, 2004; Bhattacharjee, 2005). My data showed that *NTL4* gene is induced in the distal leaf area under drought conditions. In addition, the 35S:*4ΔC* transgenic plants were hypersensitive to drought stress. It was therefore hypothesized that ROS might be related with NTL4 function.

I examined the levels of endogenous H_2O_2 by 3,3'-diaminobenzidine (DAB) staining (Ramel et al., 2009). It was observed that the 35S:*4ΔC* transgenic leaves were more strongly stained with DAB, but the staining density of the *ntl4-1* mutant leaves was lower than that of Col-0 leaves (Fig. 20, left panel). Under drought conditions, the densities of brown color were elevated in all plant genotypes examined. However, the relative density was higher in the 35S:*4ΔC* leaves but lower in the *ntl4-1* leaves compared to that in Col-0 leaves. I also measured the contents of H_2O_2 in the leaves. As inferred from the DAB staining assays, drought treatments induced rapid accumulation of H_2O_2 in the plants examined. However, the level was higher in the 35S:*4ΔC* leaves but detectably lower in the *ntl4-1* leaves (Fig. 20, right panel). The differential ROS levels are also consistent with the altered drought stress responses and leaf senescence in the

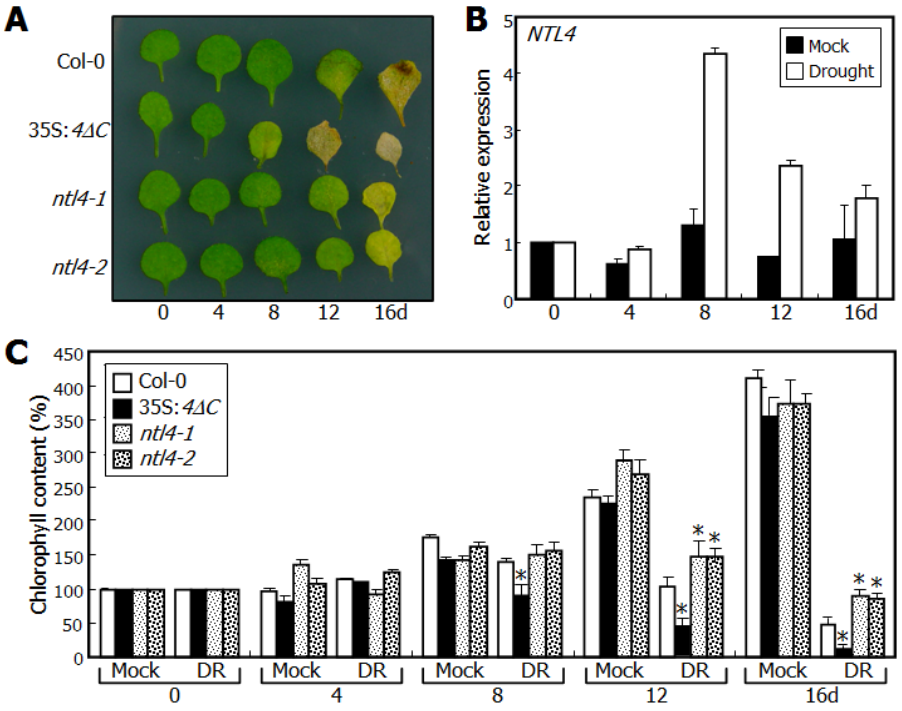

Figure 19. Leaf senescence in 35S:*4ΔC* transgenic plants and *ntl4* mutants under drought conditions.

(A) Comparison of leaf senescence. The 4th rosette leaves of plants grown in soil under drought conditions were photographed. d, days after halting watering.

(B) Kinetic expression pattern of *NTL4* gene.

(C) Measurements of chlorophyll contents. The 4th rosette leaves of 20 plants grown in soil were harvested at the indicated time points. Three measurements were averaged and statistically treated using a student *t*-test (*P<0.01). Bars indicate SD. d, days after halting watering. DR, drought.

35S:*4ΔC* transgenic plants and *ntl4* mutants.

NTL4 positively regulates *Atrboh* genes

My data showed that ROS accumulation is correlated with overexpression of an active NTL4 form. Therefore, a question was how NTL4 induces ROS accumulation. I first postulated that NTL4 promotes ROS production. ROS are produced via diverse biosynthetic pathways, among which the NADPH oxidase-mediated pathway is the most prominent one (Apel and Hirt, 2004; Sagi and Fluhr, 2006). qRT-PCR assays revealed that the transcript levels of *AtrbohA*, *AtrbohC*, and *AtrbohE* genes were higher more than 9-fold in the 35S:*4ΔC* transgenic plants than in Col-0 plants under normal conditions (Fig. 21). Under drought conditions, expression of the *Atrboh* genes was further induced at least 2-fold in both Col-0 plants and 35S:*4ΔC* transgenic plants, showing that NTL4-mediated drought stress signals upregulate a subset of the *Atrboh* genes. Consequently, the inductive effects of drought on the *Atrboh* gene expression diminished in the *ntl4-1* mutant.

NTL4 binds to *Atrboh* gene promoters

The next question was whether NTL4 regulates directly the *Atrboh* genes. Web-based sequence analysis revealed that the *Atrboh* gene promoters contain conserved sequence motifs (Fig. 22A), which are similar to the known NAC-binding

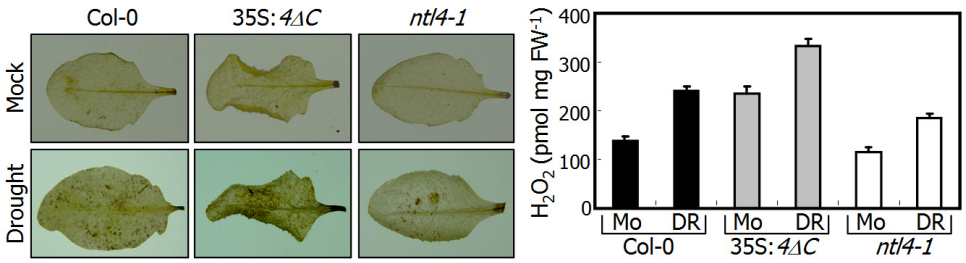

Figure 20. NTL4 promotes ROS production.

DAB staining. In the right panel, measurements of 5 representative rosette leaves were averaged for each plant genotype and statistically treated using a student *t*-test (**P*<0.01). Bars indicate SD. Mo, Mock; DR, drought.

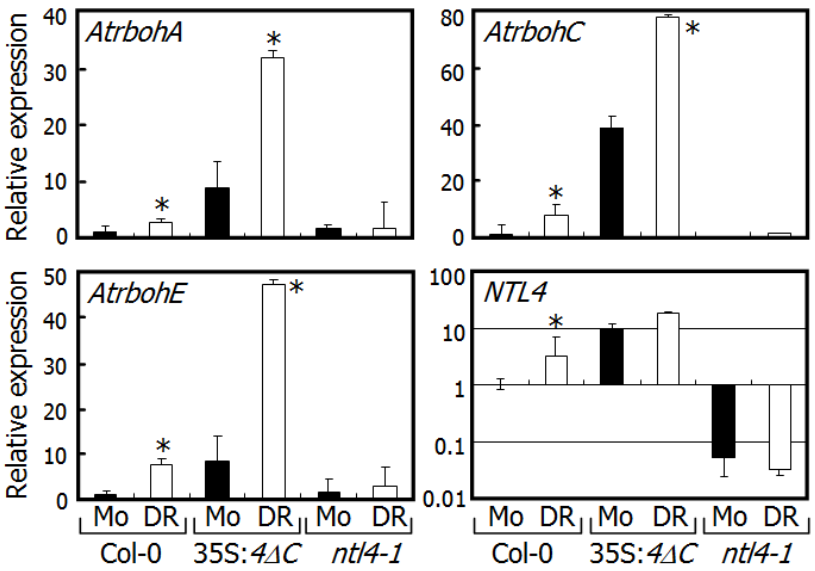

Figure 21. Expression of genes encoding ROS biosynthetic enzymes.

Bars indicate SD (*t*-test, **P*<0.01). Mo, mock; DR, drought.

sequence ([TA][TG][TAGC]CGT[GA]) (Olsen et al., 2005). The *AtrbohC* and *AtrbohE* gene promoters contained 9 and 4 putative NAC binding motifs, respectively, within the 1.5-kb sequence region from the transcriptional start site. I employed chromatin immunoprecipitation (ChIP) assays to investigate whether NTL4 binds to the sequence motifs using the 35S:*MYC-4ΔC* transgenic plants. Quantitative ChIP-PCR assays using an anti-MYC antibody revealed that the 4ΔC protein binds efficiently to the conserved sequence motifs existing in the *Atrboh* gene promoters (Fig. 22B). In addition, the 4ΔC binding to the promoter elements was further elevated after exposure to drought conditions (Fig. 22C). These observations demonstrate that the NTL4 transcription factor regulates the *Atrboh* genes by binding directly to the gene promoters *in planta*.

Cell viability is altered in 35S:*4ΔC* transgenic plants and *ntl4-1* mutant

ROS cause oxidative damage to membrane lipids, resulting in fatal leakage of cellular ions (Apel and Hirt, 2004). I therefore anticipated that NTL4-induced ROS production might influence cell viability.

Electrolyte leakage assays are commonly employed to estimate relative amounts of cell death in plant tissues (Coll et al., 2010). I analyzed the degrees of electrolyte leakage from the 35S:*4ΔC* transgenic leaves and *ntl4-1* mutant leaves. Under normal conditions, the degrees of electrolyte leakage were not significantly

different in the leaves examined (Fig. 23A). However, under drought conditions, the degrees of electrolyte leakage were higher in the 35S:*4ΔC* transgenic leaves but lower in the *ntl4-1* mutant leaves compared to those in Col-0 leaves.

I also carried out lactophenol trypan blue staining assays, which selectively visualize dead cells (Koch and Slusarenko, 1990). Upon treatments with drought, whereas the 35S:*4ΔC* transgenic leaves were densely stained as dark blue, the *ntl4-1* mutant leaves were weakly stained, relative to the staining intensity in Col-0 leaves (Fig. 23B). These observations show that cell death increases in the 35S:*4ΔC* transgenic leaves but decreases in the *ntl4-1* mutant leaves under drought conditions.

NTL4 processing is influenced by drought

I have recently reported that although the NTL4 protein having a TM motif is mainly associated with the plasma membranes, it is also detected in the nucleus (Kim et al., 2010). Therefore, a question was whether NTL4 activity is modulated at the protein processing step in addition to the gene transcriptional control under drought conditions.

I next examined whether NTL4 processing is influenced under drought conditions. First, the 35S:*MYC-NTL4* transgenic plants were treated with ABA. Overall, the NTL4 proteins were relatively unstable, and thus they were easily

degraded during the protein purification steps, as has been observed with other NTL proteins (Kim et al., 2006; Seo et al., 2008). Immunological assays revealed that the intensity of a protein band that has an estimated molecular mass close to that of the MYC-4ΔC fusion (10.5 + 46.2 kDa) was significantly elevated in ABA-treated plants (Fig. 24A). When the plants were treated with a potent proteasome MG132, both of the potentially full-size and processed NTL4 forms increased, suggesting that the ubiquitin-mediated degradation pathway is responsible for the observed rapid turnover of the NTL4 proteins.

I also examined the patterns of NTL4 processing in drought-treated plants. The intensity of the processed NTL4 form was markedly elevated (Fig. 24B), which is similar to what observed in ABA-treated plants. These observations indicate that the NTL4 activity is also regulated at the protein processing step in response to ABA-mediated drought stress signals.

Altogether, my data illustrate that ABA-mediated drought stress signals promote NTL4 activity at both the transcriptional and protein levels. The activated NTL4 transcription factor induces a subset of *Atrboh* genes, resulting in ROS accumulation. The elevated ROS production triggers leaf senescence (Fig. 24C), which is also intimately interrelated with drought resistance response. It is envisaged that when the imposed stress is expanded, the NTL4-mediated signals may cause the HR and leaf necrosis, as was observed in the 35S:*4ΔC* transgenic

plants exposed to drought stress.

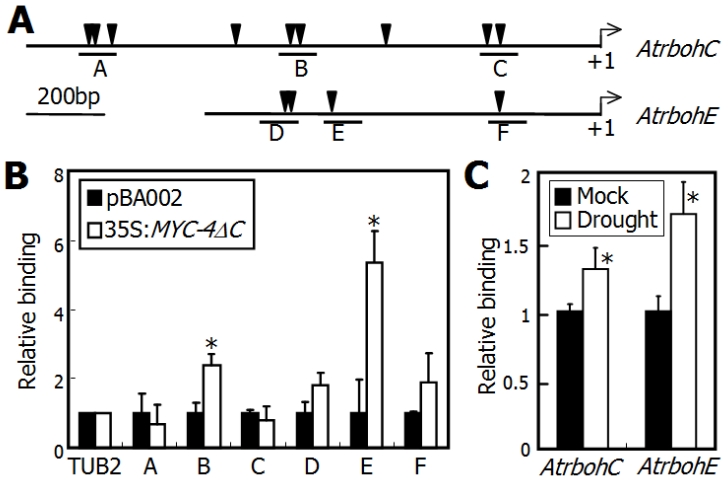

Figure 22. NTL4 binds to the promoters of *Atrboh* genes.

(A) NAC binding motifs in *AtrbohC* and *AtrbohE* gene promoters. The putative NAC binding motifs were indicated by arrowheads. The sequence regions used for ChIP assays were marked (A – F).

(B) ChIP assays. Three measurements were averaged for individual assays. Bars indicate SD (*t*-test, **P*<0.01). The values in Col-0 plants were set to 1 after normalization against *TUB2* for quantitative PCR analysis.

(C) Effects of drought on 4ΔC binding to *Atrboh* promoters. The ChIP assays were carried out as described in (B) using plants treated with drought by halting watering for 12 d and primer sets 'B' and 'E'.

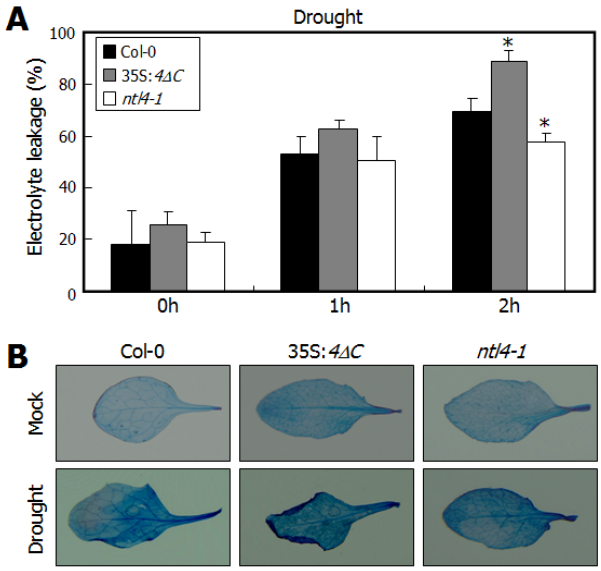

Figure 23. Cell viability in 35S:*4ΔC* and *ntl4-1* leaves under drought conditions.

(A) Electrolyte leakage assays. Three-week-old plants grown in soil were subject to drought treatments. Five measurements were averaged and statistically treated using a student *t*-test (**P*<0.01). Bars indicate SD. h, hour.

(B) Trypan blue staining. Plant materials exposed to drought, as described in (A), were used for trypan blue staining. The 6th rosette leaves were photographed.

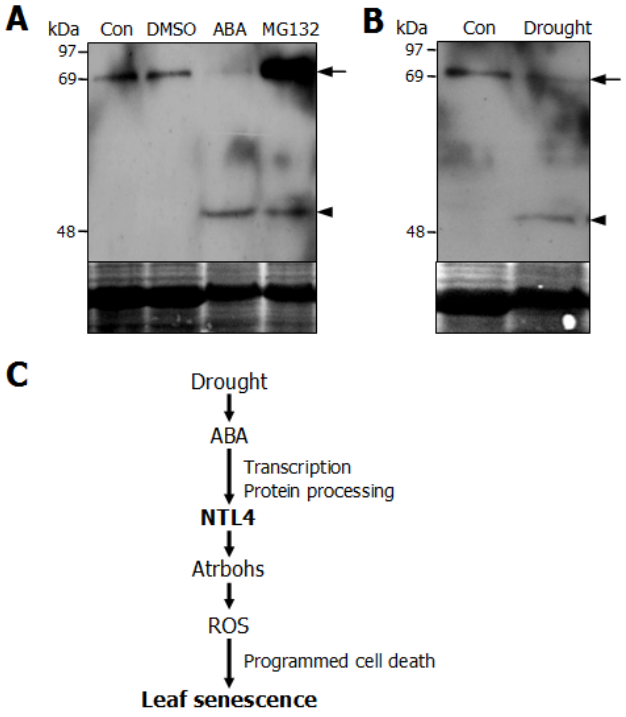

Figure 24. NTL4 mediates drought-induced leaf senescence by promoting ROS production.

(A) Effects of ABA and MG132 on NTL4 processing. The 35S:*MYC-NTL4* transgenic plants were grown for 10 d on MS-agar plates and treated for 24 h with 1% DMSO, 20 μM ABA, or 50 μM MG132. The full-size (arrow) and processed (arrowhead) NTL4 forms are indicated. Parts of Coomassie blue-stained gels are shown at the bottom as loading control. kDa, kilodalton.

(B) Effect of drought on NTL4 processing. Two-week-old plants grown in soil were subject to drought treatments by halting watering for 12 d. The full-size (arrow) and processed (arrowhead) NTL4 forms are indicated. Parts of Coomassie

blue-stained gels are shown at the bottom as loading control. kDa, kilodalton.

(C) Schematic model for NTL4 function. NTL4 promotes ROS production by binding directly to the promoters of *Atrboh* genes in response to ABA-mediated drought stress signals. The elevated ROS production triggers leaf senescence via programmed cell death.

DISCUSSION

NTL4 activation of ROS production under drought conditions

In this work, I demonstrated that NTL4 links ABA-mediated drought stress signals with ROS metabolism in inducing leaf senescence. The NTL4 transcription factor regulates the expression of *Atrboh* genes encoding ROS biosynthetic enzymes by directly binding to the gene promoters.

Biochemical and physiological roles of the ROS-generating NADPH oxidases have been extensively studied (Foreman et al., 2003; Torres et al., 2005). The roles of *Atrboh* genes have been demonstrated in the ABA-mediated stomatal closing, defense responses, and plant developmental processes, such as root hair growth and seed germination (Torres et al., 2002; Kwak et al., 2003). I found that NTL4 promotes ROS biosynthesis by inducing several *Atrboh* genes, such as *AtrbohA*, *AtrbohC*, and *AtrbohE*, and induces leaf senescence under drought conditions. My data provide a molecular basis underlying drought-induced leaf senescence.

NTL4 activity is regulated at both the gene transcriptional level and the protein processing step by ABA-mediated drought stress signals. I found that the *NTL4* gene is transcriptionally induced in the leaves by drought in an ABA-dependent manner. ABA-mediated drought stress signals also triggers the

membrane release of the NTL4 transcription factor, indicating that the NTL4 activity is modulated through controlled membrane release and thus nuclear localization, as has been shown in the cold-induced processing of the NTL6 transcription factor (Seo et al., 2010).

Although *NTL4* gene is induced in senescing leaves, leaf senescence is not discernibly affected in the 35S:*4ΔC* transgenic plants and in the *NTL4*-deficient mutants under normal growth conditions, suggesting that the *NTL4* gene does not mediate natural leaf senescence. Notably, the transgenic plants and mutant plants exhibit accelerated and delayed leaf senescence, respectively, under drought conditions. In addition, both *NTL4* gene transcription and NTL4 protein processing are induced by drought stress. Therefore, it is likely that the NTL4 transcription factor plays a role in leaf senescence specifically under drought conditions. It has been known that controlled proteolytic processing of membrane-bound transcription factors ensures rapid adaptation responses to abrupt environmental changes in plants (Seo et al., 2008). Based on the previous and my own data, I believe that induction of NTL4 protein processing, rather than *NTL4* gene induction, plays a major role in the drought-induced leaf senescence.

Physiological relevance of NTL4 function in drought-induced leaf senescence

The phenotypes of *NTL4*-deficient mutants, such as enhanced drought resistance

and delayed leaf senescence, and the reduction of ROS levels, are reliable with the correlationship between ROS accumulation and leaf senescence under drought conditions (Munné-Bosch and Alegre, 2004; Bhattacharjee, 2005; Rivero et al., 2007). Yet, the *NTL4* gene is highly expressed in senescing leaves. It is also induced mostly in the leaves under drought conditions, raising a question as to the physiological significance of NTL4 function.

I found that whereas 35S:*4ΔC* transgenic plants are susceptible to abiotic stresses, *ntl4* mutants exhibit enhanced stress resistance. In contrast, transgenic plants overexpressing other membrane-bound NAC proteins, such as NTL6, NTL8, NTL9, and NTM1, exhibit enhanced stress resistance, but gene knockout mutants exhibit stress-susceptible phenotypes (Kim et al., 2006; Kim et al., 2008; Yoon et al., 2008; Seo et al., 2010). These seemingly contrasting phenotypes are explained by the unique role of NTL4 in ROS production.

If ROS loads exceed over the capacity of metabolism and storage in plant cells, they cause cell death and necrosis in the affected area. When plants were infected with pathogens, the infected plants rapidly induce defense responses, including rapid accumulation of ROS at the site of infection (Alvarez et al., 1998; Apel and Hirt, 2004). The infected area eventually undergoes programmed cell death that blocks the spread of pathogens to other parts of plants (Alvarez et al., 1998). Recent studies also showed that remodeling of the root system through

programmed cell death contributes to drought tolerance (Duan et al., 2010).

I found that *NTL4* gene is expressed at the basal level in the aerial plant parts during the vegetative growth stages. However, it is rapidly induced in the leaves under drought conditions. The drought induction of the *NTL4* gene is more evident in the distal leaf area, where leaf senescence and cell death initiate upon exposure to drought stress (Lim et al., 2007). ROS accumulation and cell death also initiated in the distal area in senescing leaves under drought conditions. These observations strongly support that the localized induction of the *NTL4* gene and ROS accumulation in the distal leaf area is critical for NTL4 function. In this view, it is likely that the widespread cell death in whole leaf area and drought-susceptible phenotype of the 35S:*4ΔC* transgenic plants are due to ectopic expression of the *NTL4* gene by the strong 35S promoter and do not reflect the physiological role of NTL4.

Based on the previous and my own data, the most plausible explanation would be that the physiological role of the NTL4 transcription factor is to promote ROS production in the leaves, which triggers programmed cell death to induce leaf senescence under drought conditions. This response will help plants to remobilize nutrients and metabolites from the senescing leaves to the sink organs and newly formed leaves and minimize water loss from the leaves through transpiration. Under extreme drought conditions, the *NTL4* expression and ROS accumulation

would be further expanded throughout the whole plant body, leading to whole-plant necrosis, as observed in the 35S:*4ΔC* transgenic plants under drought conditions.

CHAPTER 3

The *Arabidopsis* RNA-binding protein FCA regulates thermotolerance by modulating antioxidant accumulation

RESULTS

FCA mediates thermotolerance response in an ABA-dependent manner

FCA mediates the effects of ambient temperature on the onset of flowering through the thermosensory flowering pathway in *Arabidopsis* (Blázquez et al., 2003). Recently, it has been reported that FCA regulates miR172 processing in flowering time control in response to temperature changes (Jung et al., 2012). Notably, the FCA activity is regulated by ambient temperature at both the transcriptional and protein levels, suggesting that FCA plays a role in plant responses other than flowering under fluctuating temperatures.

I first examined the effects of high temperatures on plant growth using the *FCA*-overexpressing transgenic plants (35S:*FCA*) and *FCA*-deficient mutants (*fca-11* and *fca-9*). Whereas the 35S:*FCA* transgenic plants exhibited enhanced resistance to heat treatments (45^{o}C), the *fca-11* and *fca-9* mutants displayed reduced heat resistance (Fig. 25).

Gene expression analysis under various abiotic stress conditions showed that the *FCA* gene was induced more than 3-fold by heat treatments but suppressed by approximately 60% by cold treatments (4^{o}C) (Fig. 26A). In contrast, it was not influenced to a discernible level by drought and high salt. I also examined the effects of ABA and SA on the *FCA* gene expression. The *FCA* was induced

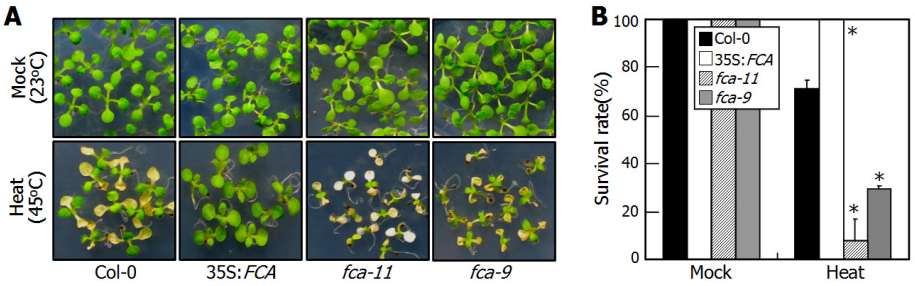

Figure 25. FCA mediates thermotolerance response.

(A and B) Measurements of survival rates. One-week-old plants grown on MS-agar plates were exposed to heat (45°C, 90 min) and allowed to recover at 23°C for 3 days (A). Survival rates were calculated using 50 plants and averaged (B). statistical significance of the measurements was determined using Student *t*-test (**P*<0.01). The bars indicate standard error of the mean.

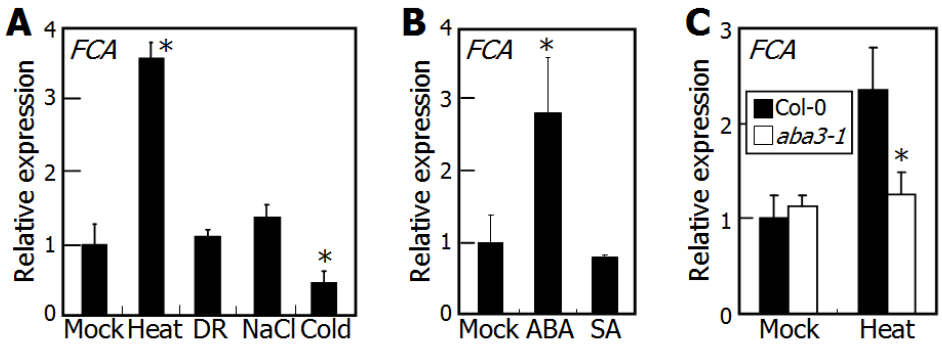

Figure 26. Effects of abiotic stresses and hormones on *FCA* expression.

In (A-C), statistical significance of the measurements was determined using Student *t*-test (**P*<0.01). The bars indicate standard error of the mean.

(A) Effects of abiotic stresses on *FCA* expression. Two-week-old plants grown on MS-agar plates were exposed to heat, drought (DR), 150 mM NaCl, or cold (4^{o}C). Transcript levels were determined by qRT-PCR. Biological triplicates were averaged.

(B) Effects of ABA on *FCA* expression. Two-week-old plants grown on MS-agar plates were transferred to MS liquid cultures containing ABA (20 μM) or SA (100 μM) and incubated for 24 h.

(C) Expression of *FCA* gene in *aba3-1* mutant under heat stress. One-week-old plants grown on MS-agar plates were exposed to heat.

approximately 3-fold by ABA but was not influenced by SA (Fig. 26B). In addition, the effects of heat on the expression of the *FCA* gene disappeared in the ABA-deficient *aba3-1* mutant (Fig. 26C), showing that the heat induction of the *FCA* gene depends on ABA.

It is known that exogenous application of ABA induces chlorophyll degradation (Nagira et al., 2006). I therefore examined the ABA responses of the *fca* mutants. Under normal growth conditions, the leaves of the *fca* mutants were green similar to the Col-0 leaves (Fig. 27, left panel). When grown in the presence of ABA, the *fca* mutant leaves were still green unlike the pale green leaves of Col-0 plants. Measurements of the chlorophyll contents revealed that the chlorophyll contents were significantly reduced in the Col-0 leaves but only slightly reduced in the *fca* mutant leaves (Fig. 27, right panel). These observations indicate that ABA sensitivity is greatly reduced in the *fca* mutants.

Cell death is accelerated in the *fca* mutants under heat stress

Heat stress disrupts the integrity of cellular membranes and leads to the inevitable leakage of inorganic and organic solutes from the cell, causing cell death (Liu et al., 2000). My data show that FCA mediates plant response to heat stress in an ABA-dependent manner.

To further examine the involvement of FCA in the thermotolerance

response, I performed the electrolyte leakage assays using the 35S:*FCA* and *fca-9* leaves after heat treatments (45^{o}C, 90 min). The degrees of electrolyte leakage were not detectably different in the Col-0, 35S:*FCA*, and *fca-9* leaves under normal conditions (Fig. 28A). However, after exposure to heat, the degrees of electrolyte leakage were significantly lower in the 35S:*FCA* leaves but markedly higher in the *fca-9* mutant leaves in comparison to those in the Col-0 leaves. I also visualized the cell death by lactophenol trypan blue staining, which selectively stains dead cells (Koch and Slusarenko, 1990). The *fca-9* leaves were stained dark blue, but the 35S:*FCA* leaves were stained light blue (Fig. 28B), indicating that cell death was reduced in the 35S:*FCA* leaves but elevated in the *fca-9* leaves compared to that in the Col-0 leaves after heat treatments.

I also performed a cell vitality assay by measuring 2,3,5-triphenyl tetrazolium chloride (TTC) reduction activity. TTC reflects the status of cellular respiration in living cells (Block and Brouwer, 2002). Under heat stress conditions, the TTC reduction activity was reduced by ~30% in the Col-0 roots (Fig. 29A). The TTC reduction activity was reduced by 54% in the *fca-9* roots but by 18% in the 35S:*FCA* roots under the identical conditions, confirming that cell death is accelerated in the *fca-9* roots but is lessened in the 35S:*FCA* roots under heat stress conditions.

ROS accumulates in the *fca* mutants under heat stress

During the hypersensitive responses caused by pathogen infection, high light, and exposure to drought and heat, ROS rapidly accumulates in the plant cells (Breusegem and Dat, 2006; Mittler et al., 2004). Under heat stress conditions, the accumulated ROS cause oxidative damage, leading to cell death (Breusegem and Dat, 2006). Considering the hypersensitivity of the *fca-9* mutants to heat and ABA, we hypothesized that ROS would accumulate to a higher level in the *fca-9* mutants under heat stress conditions.

To examine the oxidative damage caused by ROS after exposure to heat, we measured the degree of lipid peroxidation using thiobarbituric acid as reagent. Malondialdehyde (MDA) is formed from the oxidation of polyunsaturated lipids and frequently used as an indicator of the ROS-triggered oxidative damage (Zhang et al., 2009). MDA, which is one of the thiobarbituric acid reactive substances (TBARS), generates a red fluorescent derivative after condensation with thiobarbituric acids (Janero, 1990). Heat-treated plants (45^{o}C, 90 min) were allowed to recover by growing at 23^{o}C for 2 days. The contents of TBARS were lower in the 35S:*FCA* transgenic plants but higher in the *fca-9* mutant compared to those in *Col-0* plants (Fig. 29B), indicating that oxidative damage occurs to a high level, possibly because of high ROS accumulation, in the *fca-9* mutant.

I next measured the levels of endogenous H_2O_2, a representative ROS by

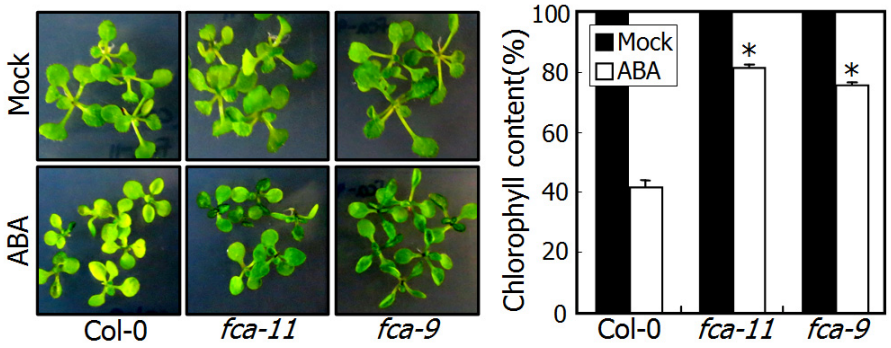

Figure 27. Measurements of chlorophyll contents after ABA treatments.

Three-day-old plants grown on MS-agar plates were transferred to MS-agar plates containing 10 μM ABA and further grown for 2 weeks (left panel), and the aerial plant parts were harvested for the measurements of chlorophyll contents (right panel). Three measurements were averaged.

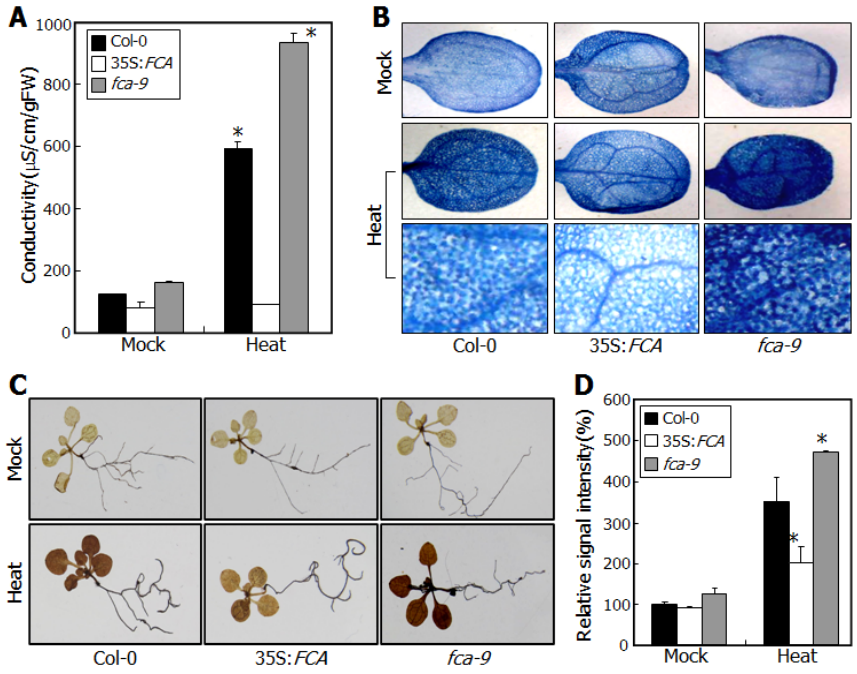

Figure 28. Cell death is accelerated in *fca* mutants under heat stress.

In (A) and (C), statistical significance of the measurements was determined using Student *t*-test (**P*<0.01). The bars indicate standard error of the mean.

(A) Electrolyte leakage assays. One-week-old plants grown on MS-agar plates were exposed to heat (45°C, 90 min). The aerial parts of plants were used for the assays. Five measurements were averaged.

(B) Trypan blue staining. The third rosette leaves of the heat-treated plants were used for trypan blue staining (B).

(C and D) DAB staining. Two-week-old, heat-treated plants were subject to DAB staining (C). Quantitation of 15 representative rosette leaves were averaged (D).

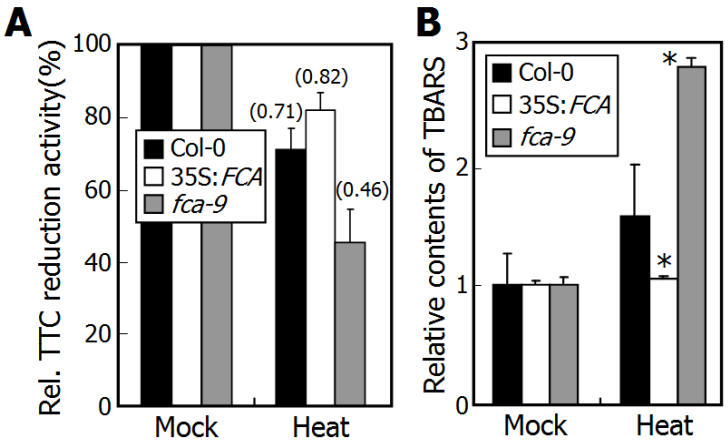

Figure 29. TTC reduction and TBARS assays.

(A) Ten-day-old plants grown on MS-agar plates were exposed to heat. The roots were used for the measurements of TTC reduction. Five measurements were averaged.

(B) TBARS assays. One-week-old plants grown on MS-agar plates were exposed to heat and allowed to recover at 23^{o}C for 2 days. Whole plants were used for the assays. Five measurements were averaged.

the 3,3'-diaminobenzidine (DAB) staining method (Torres et al., 2005). As expected, heat treatments rapidly induced H_2O_2 accumulation in Col-0 plants (Fig. 28C and 28D). However, whereas H_2O_2 accumulation was more prominent in the *fca-9* mutant, the level of H_2O_2 lower in the 35S:*FCA* plants. These observations indicate that FCA mediates ROS metabolism under heat stress conditions.

FCA modulates ROS detoxification

A question was whether FCA modulates ROS production or detoxification. To answer the question, I first examined the oxidative stress tolerance of the 35S:*FCA* transgenic plants and the *fca-9* mutant by growing the plants in the presence of paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride), a chemical that is known to induce ROS production in the plant cells. The results showed that whereas the growth of the 35S:*FCA* seedlings was less sensitive to paraquat, that of the *fca-9* seedlings was more sensitive to paraquat in comparison to the paraquat sensitivity of Col-0 plants (Fig. 30A). These observations indicate that FCA is not related with the ROS production but mediates the ROS detoxification process.

Based on the role of FCA in the ROS detoxification process, I postulated that the antioxidant activity is altered in the 35S:*FCA* transgenic plants and *fca-9* mutant. Plants produce various antioxidants, such as ascorbate and glutathione, to cope with the oxidative stress caused by ROS (Mittler et al., 2004). Measurements

of the ascorbate levels revealed that the contents of both total and reduced ascorbates were higher in the 35S:*FCA* transgenic plants but lower in the *fca-9* mutant compared to those in Col-0 plants after heat treatments (Fig. 30B). Glutathione contents were also slightly higher in the 35S:*FCA* transgenic plants under heat stress conditions. Anthocyanins are also important antioxidants in plants (Gould et al., 2002). Whereas the endogenous levels of anthocyanins were significantly higher in the 35S:*FCA* transgenic plants but relatively lower in the *fca-9* mutant under both normal and heat conditions (Fig. 30C). These observations support the notion that FCA regulates ROS accumulation by modulating the endogenous levels of various antioxidants.

FCA modulates ABI5-mediated ABA signaling under heat stress

My data showed that the *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*) gene is induced under heat stress conditions in a FCA-dependent manner (Fig. 30D). Thus, the next question was how FCA regulates the *PER1* gene induction. It has been suggested that the *PER1* gene is a putative target of the ABI5 transcription factor (Haslekås et al., 2003). It was therefore anticipated that FCA would be involved in the ABI5-mediated ABA signaling under heat stress conditions.

I first examined whether FCA interacts with ABI3 and ABI5. Yeast two-hybrid assays showed that FCA interacts with ABI5 but not with ABI3 (Fig. 31A).

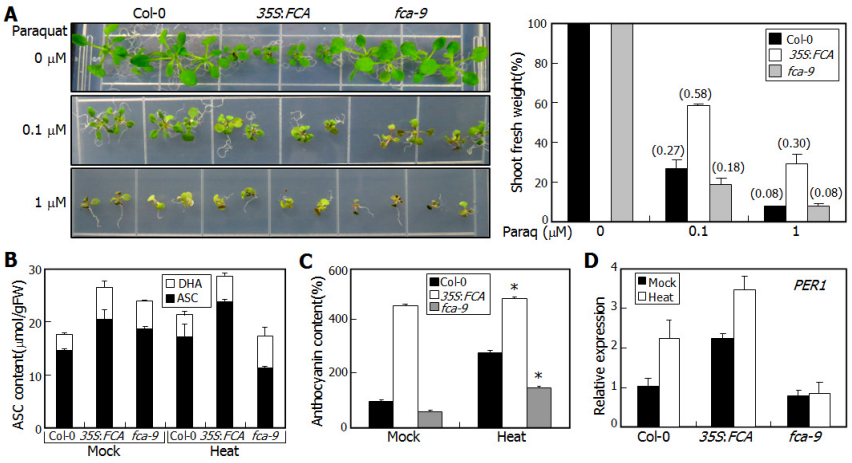

Figure 30. FCA modulates ROS detoxification.

In (B) and (D), one-week-old whole plants grown on MS-agar plates were used for the measurements or heat treatments.

(A) Oxidative stress response of 35S:*FCA* transgenic plants and *fca-9* mutant. Three-day-old plants grown on MS-agar plates were transferred to MS-agar plates containing various concentrations of paraquat and further grown for 2 weeks (left panel). The shoot fresh weights of 30 plants were averaged (right panel). The numbers in parentheses represent the ratio of the shoot fresh weights.

(B) Measurements of ascorbate contents. Three measurements of ascorbate (ASC) and dehydroascorbate (DHA) contents after heat treatments (45^{o}C, 90 min) were averaged.

(C) Measurements of anthocyanin contents. One-week-old plants grown on MS-agar plates were exposed to heat and allowed to recover at 23^{o}C for 2 days. Three

measurements were averaged and statistically treated using Student's *t*-test (**P*<0.01).

(D) Expression of *PER1* gene. The transcript levels were determined by qRT-PCR.

I found that the FCA-ABI5 interaction occurs in the nucleus, as verified by bimolecular fluorescence complementation (BiFC) assays using *Arabidopsis* protoplasts (Fig. 31B).

To study how FCA is functionally linked with ABI5, I generated *abi5-3* 35S:*FCA* plants and examined the expression patterns of the genes functioning downstream of ABI5. All the genes examined, such as *EARLY METHIONINE-LABELLED 6* (*EM6*), *RESPONSIVE TO DESSICATION 29B* (*RD29B*), *RESPONSIVE TO ABA 18* (*RAB18*), and *PER1*, were induced by heat (Fig. 32). However, the heat induction of *EM6*, *RD29D*, and *PER1* disappeared in both the *fca-9* and *abi5-3* mutants. These genes were highly expressed in the 35S:*FCA* transgenic plants under both normal and heat stress conditions, but the high-level expression was largely compromised in the *abi5-3* 35S:*FCA* plants. These observations support that the FCA-mediated heat response requires ABI5.

My data showed that FCA physically interacts with ABI5, a basic leucine zipper transcription factor that mediates ABA signaling. I performed transcriptional activation activity assays in *Arabidopsis* protoplasts to investigate whether FCA influences the transcriptional activation activity of ABI5. The *ABI5* gene sequence was fused in-frame to the 3' end of the GAL4 DNA-binding domain-coding sequence and cotransformed into *Arabidopsis* protoplasts with the reporter plasmid containing the GUS (β-glucuronidase) gene (Fig. 33A). The plasmid containing the

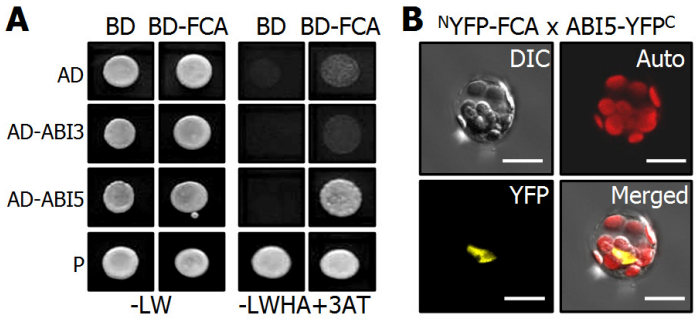

Figure 31. FCA interacts with ABI5.

(A) Interaction of FCA with ABI5 in yeast cells. Yeast cell growth on selective media without Leu, Trp, His, and Ade (-LWHA) but with 14 mM 3-AT (3-amino-1,2,4-triazole) represents positive interactions. P, positive control.

(B) BiFC assays. Partial YFP constructs fused with FCA or ABI5 were transiently coexpressed in *Arabidopsis* protoplasts and visualized by differential interference contrast microscopy (DIC) and fluorescence microscopy. Scale bars, 10 μm.

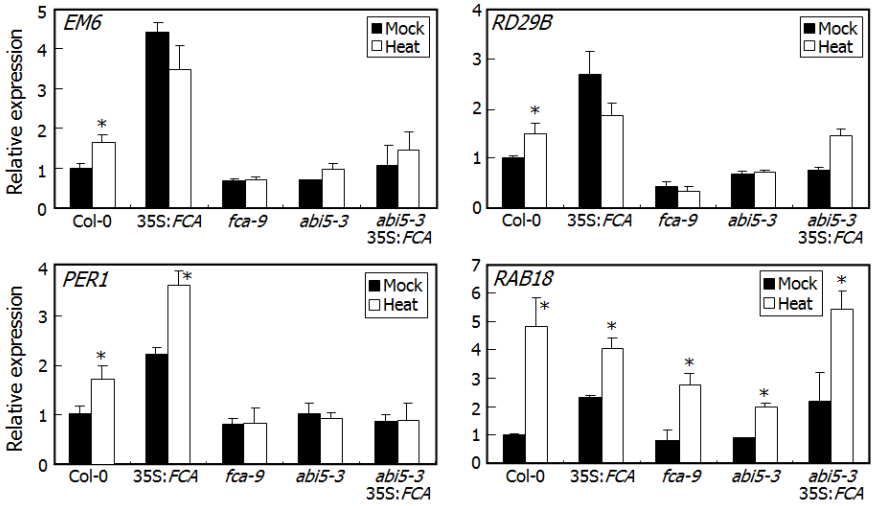

Figure 32. Expression of ABI5 target genes.

One-week-old plants grown on MS-agar plates were exposed to heat before harvesting whole plant materials for the extraction of total RNA. Transcript levels were determined by qRT-PCR. Biological triplicates were averaged. Statistical significance of the measurements was determined using Student *t*-test (**P*<0.01). The bars indicate standard error of the mean.

Renilla luciferase gene was also included in the assays to normalize the assays (Yoo et al., 2007). The transcriptional activation activities of ABI5 were similar in the Col-0 and *fca-9* protoplasts (Fig. 33B and 33C). In addition, the coexpression of the *FCA* gene did not influenced the activity of ABI5 in both the genetic backgrounds, indicating that FCA does not affect the transcriptional activation activity of ABI5.

I next performed transient expression assays in *Arabidopsis* protoplasts to verify whether FCA affect the DNA-binding activity of ABI5. The ABI5-binding sequences (BS's) within the *EM6* and *PER1* gene promoters were used in assays. The potential core binding sequences (ACGT) were mutated, resulting in EM6-mBS and PER1-mBS, to confirm the binding specificities (Fig. 33D). The BS and mBS were fused to the Cauliflower Mosaic Virus (CaMV) 35S minimal promoter (pMin35S), resulting in pEM6/PER1-P or pEM6/PER1-mP constructs (Fig. 33E). The reporter vectors and the effector vectors, such as p35S-ABI5 and p35S-FCA, were transiently coexpressed in *Arabidopsis* protoplasts. The coexpression of p35S-ABI5 with both the pEM6-P and pPER1-P reporters elevated the GUS activity by approximately 2-fold. In contrast, the coexpression of p35S-ABI5 with the pEM6-mP and PER1-mP reporters did not influence the reporter gene expression (Fig. 33F), consistent with the role of ABI5 as a transcriptional activator of the *EM6* and *PER1* genes (Lopez-Molina et al., 2002; Haslekås et al., 2003).

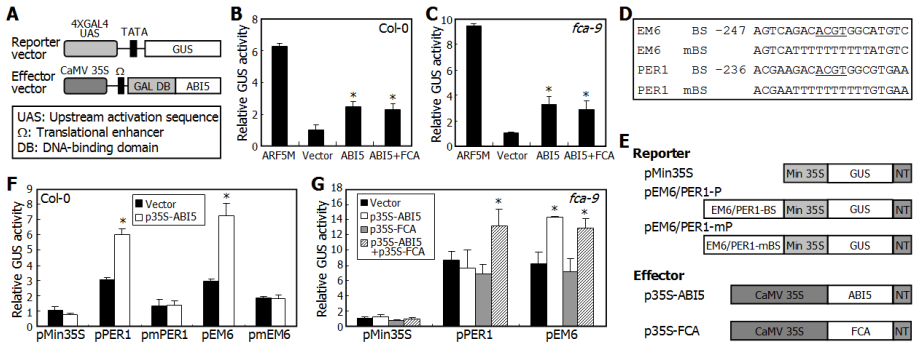

Figure 33. FCA facilitates the binding of ABI5 to DNA.

In (B), (C), (F), and (G,) statistical significance of the measurements was determined using Student *t*-test (**P*<0.01). The bars indicate standard error of the mean.

(A to C) Transcriptional activation activity assays in *Arabidopsis* protoplasts. The reporter and effector vectors used were illustrated (A). The GAL4 transient expression assays were carried out using Col-0 protoplasts (B) and *fca-9* protoplasts (C). ARF5M, transformation with the effector vector containing the *ARF5M* gene (activator control) (Tiwari et al., 2003); Vector, transformation with the effector vector without gene inserts. Three measurements were averaged.

(D) ABI5-binding sequences in the proximal promoter regions of *EM6* and *PER1* genes. Putative core ABRE elements were underlined.

(E) Expression constructs used. The promoter sequence elements shown in (D) were fused to minimal 35S promoter and used as reporters. In the effector vector, *ABI5* and *FCA* genes were transcriptionally fused to the CaMV 35S promoter. NT,

Nos terminator.

(F and G) Transient expression assays in *Arabidopsis* protoplasts. Col-0 protoplasts (F) and *fca-9* protoplasts (G) were used for the assays. GUS activity was determined fluorimetrically. A luciferase vector was cotransformed in each assay for normalization of the transformation efficiency. Three measurements were averaged.

Notably, the inductive effects of the coexpression of p35S-ABI5 with the pPER1-P reporter on the *GUS* expression disappeared in the *fca-9* protoplasts but was recovered when p35S-FCA was coexpressed (Fig. 33G). In contrast, the coexpression of p35S-ABI5 with the pEM6-P reporter still activated the reporter gene expression in the *fca-9* protoplasts. These observations demonstrate that FCA regulates the DNA-binding of ABI5 to the *PER1* gene promoter, but does not affect the ABI5 binding to the *EM6* gene promoter.

FCA regulates ABA-mediated ROS metabolism under heat stress

My data indicate that FCA induces thermotolerance by promoting antioxidant accumulation via the ABI5-mediated ABA signaling. One uncertainty was whether the FCA-ABI5 signaling module is the sole determinant of the thermotolerance response or not. I observed that both the *fca-9* and *abi5-3* mutants exhibited reduced thermotolerance. However, the *fca-9* mutant is more sensitive to heat than the *abi5-3* mutant. In addition, the contents of antocyanins were higher in the *fca-9* mutant than in the *abi5-3* mutant. These observations promoted us to consider that there would be other pathways that mediate the FCA-mediated thermotolerance.

To clarify this uncertainty, I performed RNA-Seq analysis. By comparing the RNA-Seq reads of Col-0 plants versus heat-treated Col-0 plants and heat-treated Col-0 plants versus heat-treated *fca-9* mutant, I obtained 6062 genes and

1054 genes, respectively, after screening out with >two-fold changes and $P<0.05$ parameters (Fig. 34A). Among the heat-regulated 6062 genes, 455 genes were also regulated by FCA, and the expression patterns of the 455 genes were shown by heatmap in Fig. 34B. Using the gene ontology (GO) annotation search tool in TAIR (http://www.arabidopsis.org), I categorized the 455 genes into two parameters: subcellular localization (Fig. 34C) and biological process (Fig. 34D).

The GO analysis in terms of subcellular localization revealed that the largest portion (14%) of the genes was predicted to be localized in mitochondria. Mitochondria are a major organelle for respiratory process (Hatle et al., 2013). Therefore, a large amount of ROS is produced in mitochondria, and eukaryotes have developed diverse ROS scavenging systems functioning in the mitochondria (Wu et al., 2009), suggesting that FCA may enhance thermotolerance by regulating ROS metabolism in the mitochondria. The GO analysis in terms of biological processes predicted that the highest percentage of the genes are involved in various metabolic processes (Fig. 34D), among which ROS metabolic process is most prominent (Fig. 34E), supporting the close relationship between FCA function and ROS metabolism.

I also analyzed the hormone responses of the 455 genes using the GENEVESTIGATOR tool (https://www.genevestigator.com/gv/). It was found that more than 12% of the genes were ABA-responsive (Fig. 34F). Interestingly, the

percentage of the SA-responsive genes were also high, suggesting that SA would also be involved in the FCA-mediated heat stress response.

Since most of the 455 genes have not been functionally characterized in terms of ABA signaling and ROS metabolism, I expanded the criteria to 1.5-fold and identified the *NITRATE REDUCTASE 1* (*NIA1*) and *GALACTINOL SYNTHASE 4* (*GolS4*) genes, which are known to regulate ROS metabolism. The NIA1 enzyme is involved in nitric oxide (NO) biogenesis and oxidative stress response (Neill et al., 2008). The GolS4 enzyme acts as a generator of galactinol that contributes to the promotion of oxidative stress tolerance (Nishizawa et al., 2008). Gene expression analysis showed that the two genes are induced by heat (Fig. 34G). Whereas the inductive effects of heat on the *NIA1* gene was compromised in the *aba3-1* and *abi5-3* mutants, the *GolS4* gene was still induced by heat in the mutants. Meanwhile, the effects of heat on the *NIA1* and *GolS4* genes were detectably reduced in the *fca-9* mutant. These results suggest that the *NIA1* gene is another target of the FCA-ABI5 module in heat stress response.

Altogether, I conclude that FCA induces thermotolerance by promoting antioxidant accumulation via the ABA-mediated heat stress signaling pathway. In this signaling scheme, FCA promotes the binding of ABI5 to the promoter of the *PER1* gene that is involved in ROS scavenging (Fig. 35). My data provide a novel role of FCA, which otherwise plays a major role in flowering time control

(Macknight et al., 1997), in ABA-mediated thermotolerance response in plants.

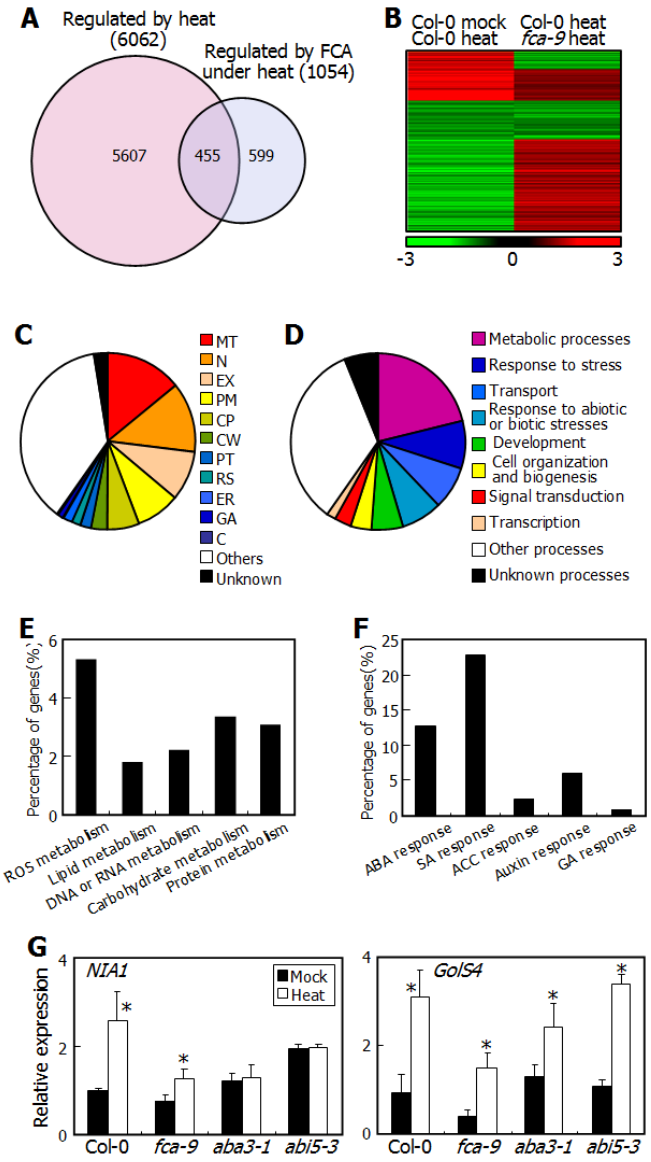

Figure 34. FCA regulates ABA-mediated ROS metabolism under heat stress.

(A) The Venn diagram of the overlap between heat-regulated genes and FCA-regulated genes under heat stress identified by transcriptome sequencing.

(B) Heatmap of 455 genes coregulated by heat and FCA. The scale bar indicates the fold changes in log-2 value.

(C and D) Gene ontology (GO) analyses. GO analyses were classified with subcellular localization (C) and metabolic processes (D) in the pie charts. MT, mitochondria; N, nucleus; EX, extracellular matrix; PM, plasma membrane; CP, chloroplast; CW, cell wall; PT, plastid; RS, ribosome; ER, endoplasmic reticulum; GA, golgi apparatus; C, cytoplasm.

(E) GO analysis of the coregulated genes in different metabolic processes.

(F) Hormone responses of the coregulated genes as analyzed by the GENEVESTIGATOR analysis tool (https://www.genevestigator.com/gv/). ACC, 1-aminocyclopropane-1-carboxylic acid; GA, gibberellic acid.

(G) qRT-PCR analysis of representative FCA-regulated genes. One-week-old plants were exposed to heat (45^{o}C, 90 min). Transcript levels were determined by qRT-PCR. Biological triplicates were averaged and statistically treated (*t*-test, **P*<0.01).

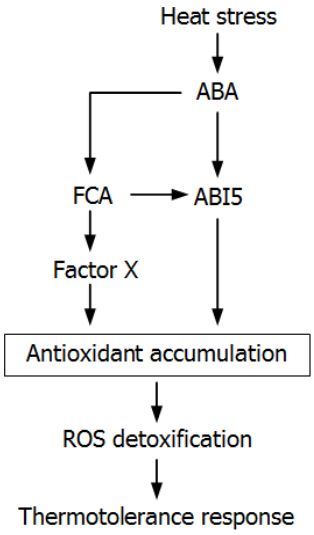

Figure 35. Working scheme of FCA in thermotolerance response.

Under heat stress, FCA interacts with ABI5 that regulates the genes encoding ROS detoxifying enzymes, resulting in antioxidant accumulation. In this working scenario, FCA facilitates the DNA binding of ABI5. It is likely that FCA also modulates additional factors (factor X) in the induction of antioxidant accumulation.

DISCUSSION

FCA in the induction of thermotolerance

Controlled RNA metabolism and editing, which is mediated by a group of RNA-binding proteins and a variety of riboregulators, constitutes a critical part of the gene regulatory networks that govern plant responses to developmental cues and environmental constraints. One of the most extensively studied is the roles of the plant-specific RNA-binding proteins FCA and FPA in the alternative cleavage and polyadenylation of the *FLC* RNAs in flowering time control (Hornyik et al., 2010; Liu et al, 2010; Sonmez et al., 2011), which also requires FY, a critical component of the RNA cleavage and polyadenylation complexes (Simpson et al., 2003). FCA also mediates chromatin silencing at the *FLC* locus in concert with the lysine-specific demethylase FLD (Liu et al., 2007).

In this work, I report s novel role of FCA, which is otherwise a central component of the flowering genetic pathway, in the induction of thermotolerance in *Arabidopsis*. In this process, FCA does not function as regulators of RNA processing and chromatin silencing but potentially acts as a transcriptional coregulator of heat-responsive genes through direct interactions with the ABI5 transcription factor, which is a key component of the ABA-mediated abiotic stress signaling pathways (Brocard et al., 2002; Fujita et al., 2011).

Molecular genetic studies has shown that FCA belongs to the autonomous flowering pathway, in which it accelerates flowering by suppressing the floral repressor *FLC* (Michaels and Amasino, 2001; Liu et al., 2010). It has recently been reported that the FCA activity is regulated at both the gene transcriptional and protein levels by ambient temperature (Jung et al., 2012). I found that the *FCA* gene is induced by high temperatures through the ABA signaling pathway that mediates antioxidant metabolism. While the *FCA*-overexpressing plants exhibited enhanced resistance to heat, the *fca* mutants showed increased heat-susceptibility, indicating that FCA is a component of the ABA-mediated heat stress signaling pathway. The expression of the *FCA* gene is also influenced by cold temperatures, while it is not affected by drought and high salinity conditions. It is therefore postulated that FCA is a constituent of the signaling pathways that mediate plant adaptation responses to a physiological range of high temperatures and temperature extremes. Extensive examination of the effects of varying temperatures on the phenotypic analysis of the *fca* mutants and the *FCA* gene expression and protein stability will help elucidate the physiological relevance of FCA in temperature stress responses.

Ambient temperature changes differentially influence the timing of flowering in different plant species. Whereas *Narcissus tazetta* var. *chinesis* requires high ambient temperature for flowing initiation (Li et al., 2013),

Chrysanthemum morifolium exhibits delayed flowering at high ambient temperature (Nakano et al., 2013). In *Arabidopsis*, ambient temperature regulates flowering time via the thermosensory pathway that includes FCA (Blázquez et al. 2003). Accordingly, the flowering time of the *fca* mutants are insensitive temperature changes (Blázquez et al. 2003; Balasubramanian et al., 2006; Lee et al., 2007). It is currently unclear whether the role of FCA in the thermosensory flowering is related with the FCA function in the thermotolerance response. Heat survival tests on the flowering mutants, such as *ft*, *flc*, and *fy* mutants, would clarify the uncertainty.

Functional mechanism of FCA in antioxidant metabolism

My data show that FCA mediates ABA signaling that triggers antioxidant accumulation, resulting in the induction of thermotolerance under heat stress conditions. Consistent with the signaling scheme, the level of ROS is reduced in the 35S:*FCA* transgenic plants but elevated in the *FCA*-deficient mutants. Notably, FCA physically interacts with the ABI5 transcription factor that activates the expression of the *PER1* gene encoding the antioxidant enzyme, peroxiredoxin. A critical question was how FCA modulates the activity of ABI5 in the regulation of downstream genes involved in antioxidant biosynthesis.

It is well-known that the RNA-binding protein FCA plays a role in RNA

metabolism and chromatin remodeling in *Arabidopsis* (Bäurle et al., 2007; Liu et al., 2010). FCA requires its interacting partner FY, a WD repeat-containing protein homologous to the human CPSF (cleavage and polyadenylation specificity factor) (Simpson et al., 2003), to promote the selection of the proximal polyadenylation site in the *FCA* primary transcripts (Quesada et al., 2003) and to control alternative polyadenylation of the antisense transcripts at the *FLC* locus (Liu et al., 2010; Simpson et al., 2003). Another RNA-binding protein FPA is also required for the full activity of FCA in the RNA processing. In addition, the silencing of the *FLC* chromatin by FCA depends on the lysine-specific demethylase FLD (Liu et al., 2007). I found that FCA physically interacts with the ABI5 transcription factor to facilitate the binding of ABI5 to the *PER1* gene promoter. The previous and my own data suggest that FCA acts as a chaperone for the action of a diverse class of enzymes and regulators in the transcriptional and posttranscriptional control of plant responses to developmental and environmental signals. This view is also consistent with recent findings that a variety of RNA-binding proteins function as RNA chaperones or coregulators by assisting action of transcription factors (Lorković, 2009; McKenna and O'Malley, 2002)

ABI5 binds to the *PER1* gene promoter, and its promoter binding was significantly eliminated in the *fca* mutants, resulting in the downregulation of the *PER1* expression in the mutants. Based on these observations, I propose that FCA

acts as a transcriptional coactivator of the ABI5 transcription factor. Recent accumulating evidence indicates that a variety of proteins functions as transcriptional coregulators. The NONEXPRESSER OF GENES 1 (NPR1) is an ankyrin repeat-containing protein that serves as a salicylic acid receptor (Wu et al., 2012). It regulates the transcriptional activity of the TGA2 transcription factor through protein-protein interactions (Després et al., 2000). Similar to the role of FCA in the regulation of the ABI5 activity, NPR1 enhances the binding of TGA2 to the promoter of the *PATHOGENESIS-RELATED GENE 1* (*PR1*) gene. In humans, the heterogeneous nuclear ribonucleoprotein (hnRNP)-like protein CoAA is a RNA-binding protein containing two RRM's (Iwasaki et al., 2001). CoAA interacts with the transcriptional coactivator, thyroid hormone receptor-binding protein (TRBP) (Iwasaki et al., 2001). Through interaction with transcription factors and transcriptional coactivators, CoAA regulates gene transcription and RNA splicing (Auboeuf et al., 2004; Verreman et al., 2011). It has been found that CoAA negatively regulates the DNA binding of the transcriptional regulators (Li et al., 2009).

My data show that the *PER1* gene, which is one of the ABI5 targets (Haslekås et al., 2003), plays a key role in the FCA-mediated ABA signaling. However, it is likely that the FCA-mediated ABA signals are not soly mediated by ABI5. Although heat sensitivity was elevated in both the *fca* and *abi5* mutants, the

fca mutants were more sensitive to heat stress compared to the *abi5* mutants. In addition, the levels of antioxidants, such as ascorbates and anthocyanins, were significantly reduced in the *fca* mutants but not in the *abi5* mutants, suggesting that FCA modulates the accumulation of these antioxidants via an ABI5-independent pathway. Furthermore, my RNA-Seq analysis showed that the transcript levels of the *NIA1* and *GolS4* genes, which are involved in ROS metabolism (Neill et al., 2008; Nishizawa et al., 2008), were considerably lower in the *fca-9* mutants. Notably, the suppressive effects of heat stress on the *NIA1* gene disappeared in the in the *aba3-1* and *abi5-3* mutants as well as in the *fca-9* mutants, suggesting that whereas the *NIA1* gene is another target of the FCA-ABI5 module under heat stress, the *GolS4* gene is regulated by an ABI5-independent FCA-mediated heat signaling. It is therefore supposed that the thermotolerant phenotype of the *fca* mutants would be a cumulative effect of antioxidant accumulation and altered ROS metabolism.

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ABSTRACT IN KOREAN (국문 초록)

고온, 가뭄 등과 같은 환경적인 스트레스는 활성 산소의 생성을 유발시킨다. 또한 활성 산소는 평상시에도 식물의 세포 내에서 광합성과 호흡에 의한 에너지 대사 작용의 부산물로써 필연적으로 생성되기도 한다. 식물은 이러한 활성 산소의 축적으로부터 야기된 단백질, 핵산, 지질 등에 가해지는 산화적 스트레스에 대항하기 위해 다양한 항산화적 기작을 발달시켜 왔다. 그러나 환경 스트레스 하에서 활성 산소의 생성과 그 조절 메커니즘에 대해서는 아직도 많은 부분이 의문으로 남아 있다.

살리실산은 최근 들어서 병 저항 반응뿐만 아니라 식물의 각종 생장 단계 및 고온, 염분 등과 같은 비생물학적 스트레스에 대한 반응에 있어서도 중요한 역할을 한다고 보고되어져 왔다. 염지에서의 발아에 있어 살리실산의 역할을 정확히 규명하기 위해 야생종인 Col-0와 더불어 살리실산의 생합성이 결여된 *sid2* 돌연변이체로 실험을 수행한 결과, 50 μM 이하의 생리적인 농도에서 활성 산소량의 감소를 통해 고염분으로 인해 늦어진 발아가 어느 정도 회복된 것을 확인할 수 있었다. 염분 스트레스는 활성 산소의 축적을 유발하는데 살리실산은 항산화 기작 조절을 통해 활성 산소량을 낮춤으로써 발아를 촉진시킨다는 결론을 내릴 수 있었다.

식물의 환경 변화에 대한 반응은 전사인자들에 의해 조절되어지는 다양한 신호 전달 경로를 통해 이루어진다. 식물 체내에서 스트레스 반응을 매개하는 대표적인 NAC 전사인자 중 하나인 AtNTL4는 가뭄 하에서 전사 단계뿐만 아니라 단백질 수준에서 막으로부터의 분리가 증가하는 것을 관찰하였다. 막결합 부분이 제거 된 *NTL4ΔC*의 과량발현체는 가뭄 스트레스에 대해 민감하게 반응하였고, 활성 산소량의 증가와 잎의 노화현상이 빨라져 있는 것을 확인하였다. 이와는 대조적으로 *NTL4* 유전자의 기능이 저해된 *ntl4* 돌연변이체에서는 가뭄에 대한 저항성이 강해져 있었고 잎의 노화 현상이 지연되는 표현형을 관찰할 수 있었다. 가뭄 스트레스 하에서의 잎의 노화는 전체적인 식물 체내의 수분 함량의 균형을 유지시켜주고, 영양분에 있어 어린 잎이나 씨앗으로의 재분배를 유도하게 되어 가뭄 하에서 생존율을 높여 번식을 유지할 수 있는 식물 고유의 전략 중 하나로 해석되어 진다. 이러한 결과들을 토대로 본 연구에서는 NTL4가 활성 산소량의 조절을 통해 가뭄 스트레스 하에서의 잎의 노화 현상에 있어서 중요한 역할을 지님을 밝혀냈다.

고온 스트레스는 활성 산소량의 증가를 야기시켜 식물의 생장이 지연되고, 세포 내에 산화적 스트레스가 발생한다. 그러나 활성 산소의 생성이 어떻게 고온에 대한 식물의 반응과 연결되어 있는지에 대해선 많은 부분이 아직 밝혀지지 않았다. 본 연구를 통해 식물의 개화시기를

조절하는 유전자로써 잘 알려진 애기장대의 RNA 결합 단백질인 FCA이 활성 산소량의 조절을 통한 고온 스트레스 반응에 있어서도 관여되어져 있음을 제시하였다. *FCA* 과량 발현체는 고온에 대해 높은 저항성을 가지고 있는 반면, *fca* knockout 돌연변이체는 고온 하에서 야생종 Col-0에 비해 훨씬 민감하게 반응하였다. 또한 FCA 과량 발현체에서는 고온 하에서 비타민 C와 같은 항산화 물질의 함유량은 높고 활성 산소량은 낮아져 있는 반면, *fca* 돌연변이체에서는 그와 반대로 항산화 물질의 함유량은 낮고 활성 산소량은 높아져 있었다. 이러한 결과들을 토대로 FCA는 고온 하에서 증가된 활성 산소량을 낮춤으로 해서 저항성을 부여하는데 기여한다는 사실을 증명하였다.

주요어 : 활성 산소, 비생물학적 스트레스, 애기장대, 식물 호르몬, NTL4, FCA

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