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이학박사 학위논문

Studies on the control of transcriptional regulators in adaptive responses to environmental changes in *Arabidopsis*

외부 환경 변화에 대한 적응 반응에서 나타나는 애기장대 전사 인자의 조절에 대한 연구

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화학부

이 효 준

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ABSTRACT

Since the sessile nature of the plants, they have developed various adaptive systems in response to environmental changes. Transcriptional control is one of the most important regulatory systems in adaptive responses. Transcriptional regulators such as transcription factors and transcription co-factors directly or indirectly control transcription of their downstream genes. Thus, modulating the activity of transcriptional regulators is a key issue in studies on transcription. By molecular and genetic studies, transcriptional regulators that play a key role in plant adaptive responses are identified. However, how plants control their activity in response to environmental changes is still largely unknown. In this study, regulatory mechanisms of two transcriptional regulators which are critical for temperature and immune responses are identified.

In Chapter 1, a study on the modulation of PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) activity under high ambient temperature conditions is described. In *Arabidopsis*, the basic helix-loop-helix transcription factor PIF4 regulates high temperature-induced adaptive responses by modulating auxin biosynthesis. At high temperature, PIF4 directly activates expression of *YUCCA8* (*YUC8*), a gene encoding an auxin biosynthetic enzyme, resulting in auxin accumulation. Here, I demonstrate that the RNA-binding protein FCA attenuates the PIF4 activity by inducing

its dissociation from the *YUC8* promoter at high temperature. At 28ºC, the auxin content is elevated in *FCA*-deficient mutants that exhibit elongated stems but reduced in *FCA*-overexpressing plants that exhibit reduced stem growth. Therefore, the proposed mechanism is that the FCA-mediated regulation of *YUC8* expression tunes down the PIF4-induced architectural changes to achieve thermal adaptation of stem growth at high ambient temperature.

In Chapter 2, regulatory mechanism of NONEXPRESSER OF PR GENES 1 (NPR1) localization in response to pathogen infection is identified. In plants, necrotic lesions occur at the pathogen infection site through hypersensitive response (HR), which is followed by the activation of systemic acquired resistance (SAR) in distal tissues during defense responses. Salicylic acid (SA) plays a key role in inducing SAR by activating NPR1, but a high level of SA causes toxic effects to plant growth and development. During HR, SA biosynthesis increases in both local and systemic tissues. However, the SA level is only slightly elevated in systemic tissues, obscuring the role of SA in SAR. Here, I demonstrate that SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8) phosphorylates NPR1 to facilitate its nuclear transport in systemic tissues which are under low SA conditions. Therefore, *Arabidopsis* requires the SnRK2.8-mediated activation system to induce immune responses while reducing growth defects by maintaining a low level of SA in systemic tissues.

key words: Transcriptional regulators, auxin, high ambient temperature, immune response, systemic acquired resistance, subcellular localization

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ABBREVIATIONS

ABI5	ABA INSENSITIVE 5
ACS5	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 5
AXR1	AUXIN RESISTANT 1
BiFC	Bimolecular fluorescence complementation
BR	Brassinosteroid
BZR1	BRASSINAZOLE-RESISTANT 1
CaMV	Cauliflower mosaic virus
ChIP	Chromatin immunoprecipitation
CPD	CONSTITUTIVE PHOTOMORPHOGENIC DWARF
CYP79B2	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2
DIC	Differential interference contrast
DIR1	DEFECTIVE IN INDUCED RESISTANCE 1
DWF4	DWARF4
ETI	Effector-triggered immunity
FLC	FLOWERING LOCUS C
FLD	FLOWERING LOCUS D
FRI	FRIGIDA

FT	FLOWERING LOCUS T
GA	Gibberellic acid
GA3OX1	GIBBERELLIN 3-BETA-DIOXYGENASE 1
GA20OX1	GIBBERELLIN 20-OXIDASE 1
GFP	Green fluorescence protein
GUS	β-glucuronidase
H3K4me2	Histone-3 lysine-4 dimethylation
HOS1	HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1
HR	Hypersensitive response
IAA	Indole acetic acid
IAA29	INDOLE-3-ACETIC ACID INDUCIBLE 29
ICE1	INDUCER OF CBF EXPRESSION 1
IP	Immunoprecipitation
JA	Jasmonic acid
LD	Long day
MBP	Maltose binding protein
miR172	MicroRNA 172
MS	Murashige and Skoog
NPA	1-N-naphthylphthalamic acid
NPR1	NONEXPRESSER OF PR GENES 1
NTL6	NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 6
PAMP	Pathogen-associated molecular pattern

PER1	1-CYSTEINE PEROXIREDOXIN 1
PHYB	PHYTOCHROME B
PIF4	PHYTOCHROME-INTERACTING FACTOR 4
PKS5	SOS2-LIKE PROTEIN KINASE 5
PR	Pathogenesis-related genes
PRR	Pattern-recognition receptor
Pst DC3000	Pseudomonas syringae pv. tomato DC3000
PTI	PAMP-triggered immunity
RRM	RNA-recognition motif
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Quantitative real-time RT-PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
SAUR	SMALL AUXIN UPREGULATED
SE	Standard error of the mean
Serine 11/15	S11/15
SID2	SALICYLIC ACID INDUCTION DEFICIENT 2
SnRK2.8	SNF1-RELATED PROTEIN KINASE 2.8
TAA1	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1
TGA	TGACG SEQUENCE-SPECIFIC BINDING PROTEIN
TIR1	TRANSPORT INHIBITOR RESPONSE 1

YUC8 YUCCA8

BACKGROUNDS

1. PIF4 and FCA functions in plant development

1.1 High ambient temperature responses in plants

Temperature is a critical factor to the life of the plant since it affects all kinds of chemical reactions occurring in plants. When high or low temperature is applied, plants induce stress responses to survive under such harsh conditions (Chinnusamy *et al*, 2007; Nover *et al*, 2001). However, temperature change within ambient range does not cause stress responses. Plants recognize it as an environmental signal, thus they modulate growth and development to adapt to surrounding conditions. Under low ambient temperature conditions, short hypocotyl and delayed flowering phenotype are observed (Lee *et al*., 2007; Wigge, 2013). In contrast, under high ambient temperature conditions, plants show elongated hypocotyl and leaf stem and increase of leaf hyponasty, and decrease of stomata density (Crawford *et al*., 2012; Franklin *et al*., 2011; Koini *et al*., 2009; Sun *et al*., 2012).

These architectural adaptations help plants minimize the chance of heat damage and enhance evaporative leaf cooling for optimized plant growth (Crawford *et al*., 2012). Elongated hypocotyl and leaf stem and increase of

leaf hyponasty would separate each leaf to increase diffusion of water vapor from the stomata. The reduced stomata density may also facilitate water evaporation from each stoma to reduce leaf temperature (Crawford *et al*., 2012). However, a trade-off occurs when plants obtain the fitness benefits during long-term exposure to high temperature: plants having abnormally elongated stems tend to fall down and suffer from physiological imbalance (Mooney *et al*., 2010). This necessitates that there should be a fine-tuning mechanism that attenuates the effects of high temperature on stem growth.

High temperature-induced hypocotyl elongation is closely related with plant hormones such as auxin, gibberellin (GA), and brassinosteroid (BR) (Bai *et al*., 2012; Oh *et al*., 2012; Stavang *et al*., 2009). Auxin is a first hormone which is involved in growth at high temperature (Gray *et al*., 1998). When the high temperature is applied, *AUXIN RESISTANT 1* (*AXR1*)-deficient *axr1* and *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*)-deficient *tir1* mutants show highly reduced responses than wild type plants. AXR1 is known to be involved in auxin responses through activation of E3 ligase enzymes (Nakasone *et al*., 2012), and TIR1 is an auxin receptor (Dharmasiri *et al*., 2005), indicating that auxin-mediated signaling induces high temperature-induced hypocotyl elongation. Further studies have found that auxin biosynthesis is promoted by the induction of key auxin biosynthetic genes including *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*), *CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2* (*CYP79B2*), and *YUCCA 8* (*YUC8*) (Franklin *et al*.,

2011; Sun *et al*., 2012).

GA pathway is also important in the elongation of hypocotyl under high temperature conditions. The expressions of GA biosynthetic genes, *GIBBERELLIN 20-OXIDASE 1* (*GA20OX1*) and *GIBBERELLIN 3 BETA-HYDROXYLASE 1* (*GA3OX1*) are induced at the early time point by high temperature treatment while GA-inactivating enzyme *GIBBERELLIN 2-OXIDASE 1* is reduced significantly (Stavang *et al*., 2009). These expression changes suggest that biosynthesis of GAs is increased under high temperature conditions. Consistently, treatment of GA biosynthetic inhibitor paclobutrazol to the wild type plants and mutation of GA-biosynthetic gene *GA REQUIRING 1* both cause reduced elongation response at high temperature, indicating that GA pathway is also important in temperature-induced hypocotyl growth.

BR also affects hypocotyl growth at high temperature. The expressions of BR biosynthetic genes, *CONSTITUTIVE PHOTOMORPHOGENIC DWARF* (*CPD*) and *DWARF 4* (*DWF4*) are moderately induced by high temperature treatment (Stavang *et al*., 2009). The phosphorylation of BR signaling proteins BRASSINAZOLE-RESISTANT 1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 are important in transducing BR signaling (Li and Deng, 2005). Their phosphorylation statuses are increased under high ambient temperature conditions (Stavang *et al*., 2009), indicating that BR signaling is activated in these conditions.

1.2 PIF4 is a key transcription factor in high ambient temperature responses

The *Arabidopsis* basic helix-loop-helix transcription factor PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) plays a major role in high temperature-induced plant responses. However, the first identified function of PIF4 is signal transduction in light responses. The mutant screening by T-DNA mutagenesis found that *pif4* mutants show short hypocotyl under red light conditions (Huq and Quail, 2002). PIF4 directly interacts with red light receptor *PHYTOCHROME B* (*PHYB*) and negatively regulates red light-induced photomorphogenesis (Lucas *et al*., 2008). It is demonstrated that red light activates PHYB which is located in the cytoplasm, and active PHYB is translocated to the nucleus to interact with PIFs for their inactivation through protein degradation (Chen and Chory, 2011). Since PIFs including PIF4 induce growth-promoting genes, degradation of PIFs enable plants to suppress skotomorphogenesis and promote photomorphogenesis (Lucas *et al*., 2008).

Recently, the role of PIF4 in high ambient temperature responses is identified. In the thermosensory flowering pathway, PIF4 accelerates flowering under high temperature conditions (Kumar *et al*., 2012). High temperature-induced acceleration of flowering is largely suppressed in *pif4* mutant. High temperature promotes protein stabilization of PIF4 and activates DNA binding activity of it to the promoter regions of *FLOWERING LOCUS T* (*FT*), which is well-known floral integrator for the

induction of flowering. DNA binding activity of PIF4 is regulated by H2A.Z nucleosomes, which are temperature signaling mediator (Kumar and Wigge, 2010). H2A.Z preoccupied *FT* promoter to block PIF4 binding at low temperature. However, when high temperature is applied, H2A.Z is dissociated from the *FT* promoter, enabling PIF4 to bind to the G-box motifs located within promoter region of *FT* gene (Kumar and Wigge, 2010).

PIF4 also regulates high temperature-induced architectural changes. Elongation of hypocotyl, increase of hyponasty, and induction of flowering are suppressed by *pif4* mutations under high temperature conditions (Franklin *et al*., 2011; Koini *et al*., 2009; Sun *et al*., 2012). At high temperature, plants induce biosynthesis of growth hormones including auxin, GA, and BR to promote growth for the leaf cooling (Bai *et al*., 2012; Crawford *et al*., 2012; Oh *et al*., 2012; Stavang *et al*., 2009). PIF4 is involved in signaling pathways of all these hormones, suggesting that PIF4 is a master regulator of high ambient temperature responses. In the auxin signaling, PIF4 promotes auxin biosynthesis by inducing auxin biosynthetic genes, *TAA1*, *CYP79B2*, and *YUC8* (Franklin *et al*., 2011; Sun *et al*., 2012). The high temperature-triggered induction of auxin responsive genes including *SMALL AUXIN UPREGULATED* (*SAUR*) genes and *INDOLE-3-ACETIC ACID INDUCIBLE 29* (*IAA29*) are also highly suppressed in *pif4* mutants (Franklin *et al*., 2011; Koini *et al*., 2009). Also, thermal induction of auxin content is largely inhibited by *pif4* mutation, indicating that PIF4 is a key regulator of high temperature-induced auxin biosynthesis (Sun *et al*.,

2012). The PIF4 function in high temperature is independent from light signaling pathway, because the hypocotyl of *phyb phyd phye* triple mutant, which lacks red light-triggered light signaling, is also elongated by high temperature treatment (Koini *et al*., 2009).

The relationship among PIF4, BR, and GA is recently identified. PIF4 directly interacts with BZR1, which is a key transcription factor in BR signaling, and their functions in growth promotion are highly interdependent (Oh *et al*., 2012). At high temperature, both the protein stability of PIF4 and biosynthesis of BRs are increased (Oh *et al*., 2012; Stavang *et al*., 2009). The increased BRs activate BZR1 proteins and they induce growth-promoting genes including *PACLOBUTRAZOL RESISTANCE 1* and *5*.

GA promotion of hypocotyl growth is a well-known phenomenon. In light responses, GA plays an important role in dark-induced hypocotyl growth. GA promotes degradation of DELLA proteins which directly interact with PIF3 and 4 and inhibit their DNA binding activities (Lucas *et al*., 2008). Recently, it is demonstrated that DELLA proteins also inhibit DNA binding of BZR1 by direct interaction. Since BZR1 and PIF4 act interdependently, it is suggested that GA would affect BZR1-PIF4 module for the induction of hypocotyl growth. The genome-wide transcriptome analysis showed that the genes regulated by BZR1-PIF4 module are also affected by GA, indicating that GA function is largely related with BZR1-PIF4 complex (Bai *et al*., 2012). Because high temperature induces GA and BR biosynthetic genes and protein stability of PIF4, the GA-BR-PIF4

regulatory module would also be applied to the high temperature-induced hypocotyl growth.

1.3 FCA functions in flowering time control

FCA is a plant-specific RNA-binding protein that constitutes the autonomous flowering pathway in *Arabidopsis*. This protein promotes flowering by suppressing the floral repressor *FLOWERING LOCUS C* (*FLC*) through the alternative cleavage and polyadenylation of embedded antisense *FLC* RNAs (Manzano *et al*., 2009; Liu *et al*., 2010), which correlate with transcriptional control of the *FLC* gene (Liu *et al*., 2007). FCA also autoregulates its own expression by modulating the alternative polyadenylation of the *FCA* pre-mRNA (Quesada *et al*., 2003). FCA interacts with FY, a central component of the RNA cleavage and polyadenylation complex (Simpson *et al*., 2003), and this interaction is necessary for selection of the proximal polyadenylation site in the *FCA* pre-mRNA and in antisense *FLC* RNAs (Simpson *et al*., 2003). FCA is also involved in chromatin silencing in concert with FLOWERING LOCUS D (FLD), a lysine-specific demethylase (Liu *et al*., 2007).

Recently, it is found that FCA is involved in thermosensory flowering by regulating microRNA172 (miR172), which promotes flowering by inducing *FT* gene expression (Jung *et al*., 2012). Under low ambient temperature conditions, miR172 processing is suppressed, thus flowering time is delayed. However, the processing of miR172 is induced to activate

flowering under higher temperatures. FCA directly binds to pri-miR172 strands and promotes its processing to the mature miR172 when the temperature is increased (Jung *et al*., 2012). Therefore, FCA promotes flowering by regulating miR172 processing at higher temperatures.

1.4 The broader role of FCA in transcriptional and post-transcriptional regulation

There are several reports that FCA plays a broader role in transcriptional regulation of genes which are not directly related to flowering time. FCA is involved in small interfering RNA-mediated chromatin silencing pathway in concert with FPA (Bäurle *et al*., 2007). In the *fca fpa* double mutant, DNA methylation levels in some retroelements and transposons are reduced, thus their expressions are reactivated, indicating that FCA and FPA are important in transcriptional silencing. Since both FCA and FPA have RNA-recognition motif (RRM) RNA binding domain, it is suggested that they would recognize aberrant RNAs and trigger silencing of their expressions (Bäurle *et al*., 2007).

FCA and FPA are also involved in RNA 3' processing and transcriptional termination (Sonmez *et al*., 2011). The genome-wide analysis revealed that transcriptional read-through and altered polyadenylation are increased, thus considerable intergenic transcription occurs in *fca fpa* mutant. Also, DNA methylation levels are increased in the read-through transcripts in *fca fpa* mutant, suggesting that the connection between transcriptional

termination and DNA methylation (Sonmez *et al*., 2011). It is currently unclear how FCA and FPA are involved in these processes, but it is clear that they have genome-wide functions other than *FLC* regulation.

FCA also interacts with transcription factor to regulate specific gene expressions under stress conditions. Under heat stress conditions, FCA is required to reduce reactive oxygen species by increasing antioxidant capacity (Lee *et al*., 2014). Notably, FCA directly interacts with ABA INSENSITIVE 5 (ABI5) transcription factor to induce *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*) under heat stress conditions. PER1 acts as an antioxidant, thus induction of *PER1* reduces oxidative damage caused by heat stresses. FCA promotes DNA binding activity of ABI5 to the promoter sequence of *PER1* gene, suggesting its role of transcriptional coactivator (Lee *et al*., 2014).

In this work, it is found that FCA mediates the dissociation of PIF4 from its target gene chromatin, such as *YUC8*, at high ambient temperature. FCA is recruited to *YUC8* promoter through interaction with PIF4, resulting in the dissociation of PIF4 from *YUC8* promoter and the suppression of *YUC8* transcription. These observations illustrate that FCA attenuates PIF4 action in auxin accumulation occurring during high temperature-induced stem growth to maintain architectural and physiological balance.

2. NPR1-mediated defense responses in plants

2.1 SA is required for inducing systemic acquired resistance

Unlike animal, it is inevitable that plants are attacked by pathogen including bacteria, fungi, oomycetes, nematodes, and viruses because of its sessile nature (Gururani *et al*., 2012). To survive under these conditions, plant developed various protection systems. When the bacterial pathogen is attached to the plant leaves, plants recognize conserved pathogen-associated molecular pattern (PAMP) on the bacterial cell surface by pattern-recognition receptor (PRR) in plant cells (Fu and Dong, 2013). PRR then induces PAMP-triggered immunity (PTI) as an active defense system (Zipfel, 2009). However, bacteria developed inhibitors of PTI which is called effectors, to suppress PTI and establish successful infection. When the effectors are injected to the plant cells, plants activate effector-triggered immunity (ETI) using effector as a signaling molecule (Nomura *et al*., 2011). ETI induces programmed cell death around the infection site to suppress spreading of bacterial invasion (Fu and Dong, 2013; Nomura *et al*., 2011; Zipfel, 2009).

By recognition of bacterial effectors, plants trigger another wave of immune response to the distal leaves, called systemic acquired resistance (SAR) (Fu and Dong, 2013). SAR does not trigger cell death, but rather promotes salicylic acid (SA) biosynthesis to induce genes which are related

with antimicrobial functions including pathogenesis-related genes (*PR*s) (Cameron *et al*., 1999). Pathogen infection triggers SA biosynthesis. A critical player in pathogen-induced SA biosynthesis SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2). The *SID2* expression is highly increased by pathogen infection (Shah, 2003). SA is essential for inducing SAR (Delaney *et al*., 1994). In the SA-deficient *sid2* mutant and *nahG* transgenic plants, SAR-induced pathogen resistance is largely reduced and pathogen-triggered induction of *PR1* gene is suppressed (Delaney *et al*., 1994; Fu and Dong, 2013). However, SA causes toxic effects to the plant growth and development. Under the cold conditions, the growth of the wild type plants is inhibited while that of SA-deficient *nahG* transgenic plants is largely unaffected (Scott *et al*., 2004). Together with the fact that SA biosynthesis is increased under cold conditions, these results suggest that SA accumulation causes growth retardation at cold temperature. Also, it is reported that 100 μM SA causes reduced shoot biomass and leaf epidermal cell size in tobacco plants (Dat *et al*., 2000). Furthermore, 1 mM SA treatment causes DNA damage responses (Yan *et al*., 2013). Therefore, SA level should be regulated to reduce its toxic effects while triggering SAR responses in the pathogen-infected plants.

2.2 NPR1 is regulated by SA

The master regulator of SA signaling in SAR is NONEXPRESSER OF PR GENES 1 (NPR1). The NPR1 was first identified as a regulatory protein of

the SAR pathway through mutagenesis (Cao *et al*., 1997). Since the *npr1* mutants are impaired in SA-induced *PR*s expressions and SAR responses, it has been extensively studied as a critical regulator of plant immune responses. When the plants are infected with the bacterial pathogen such as *pseudomonas syringae* pv. *tomato* DC 3000, NPR1 acts as a coactivator of TGACG SEQUENCE-SPECIFIC BINDING PROTEIN (TGA) transcription factors to induce *PR*s expressions by activating their transcriptional activation activity and increasing their DNA binding activity (Després *et al*., 2003; Rochon *et al*., 2006).

Since its importance in SAR, the regulatory mechanisms of NPR1 have been studied in detail. NPR1 proteins form oligomers through disulfide bonds at cytoplasm, thus their function is silenced in normal cells (Mou *et al*., 2003). When SA content is increased after pathogen infection, it causes changes of redox status by inducing thioredoxins, which reduce disulfide bonds to free thiol groups (Tada *et al*., 2008). The monomerized NPR1 is then transferred to nucleus to activate downstream target genes such as *PR1*. After the induction of transcriptions, NPR1 is subjected to protein degradation for exchanging used NPR1 into fresh NPR1 (Spoel *et al*., 2009). These processes reinitiate the transcription cycle, thus they enable full activation of the transcription of defense-related genes.

The phosphorylation of NPR1 is important in regulating its activity. NPR1 is phosphorylated by as yet unidentified kinases which would be activated by SA (Spoel *et al*., 2009). In the regulation of NPR1 protein

stability, SA-induced phosphorylation at serine 11/15 (S11/15) plays an important role. The site-specific mutation of S11/15 to alanine causes defects in protein turnover after pathogen infection (Spoel *et al*., 2009). It is recently identified that SOS2-LIKE PROTEIN KINASE 5 (PKS5) interacts with and phosphorylates NPR1, but the molecular function of PKS5-mediated phosphorylation is still unclear (Xie *et al*., 2010).

3. The purpose of this study

Since the sessile nature of plants, they developed rapid and delicate adaptive systems to survive against environmental changes including photoperiodic change, temperature fluctuation, changes of nutrient supply, and pathogen attack (Cahill and McNickle, 2011; Dempsey and Klessig, 2012; Johansson and Staiger, 2014; Wigge, 2013). Plants have various kinds of transcription factors to establish successful expression of genes involved in those responses. Extensive studies identified the function of transcription factors and their physiological roles, but how plants control their activity is still largely unknown. In this study, regulatory mechanisms of two transcription factors that play a critical role in ambient temperature sensing and immune response, respectively, are described.

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 continuous light and long day (LD; 16 h light and 8 h dark) conditions, unless specified otherwise, with white light illumination (120 μmol photons/m^2s) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). The *fca-9*, *fca-11*, and *pif4-101* mutants and *35S:FCA* transgenic plants have been described previously (Bäurle *et al*., 2007; Jung *et al*., 2012; Koini *et al*., 2009). To generate *35S:MYC-FCA* transgenic plants, a full-size *FCA*γ cDNA was fused in-frame to the 3′ end of the MYC-coding sequence in the myc-pBA vector (Seo *et al*., 2010). The *SnRK2.8*-deficient *snrk2.8-1* and *snrk2.8-2* mutants (SALK-073395 and SALK-053423, respectively) were isolated from a T-DNA insertional mutant pool deposited in the *Arabidopsis* Information Resource (TAIR, Ohio State University, OH). The *NPR1*-deficient *npr1-2* mutant, which has been described previously (Cao *et al*., 1997), was also obtained from Xinnian Dong. Homozygotic mutants were obtained by selection for three or more generations and analysis of segregation ratios, and lack of gene expression was verified by reverse transcription polymerase chain reaction (RT-PCR) in the mutants. A full-size *SnRK2.8* cDNA was overexpressed driven by the

Cauliflower Mosaic Virus (CaMV) 35S promoter in Col-0 plants, resulting in *35S:2.8* plants. Multiple independent transgenic lines were obtained, and two representative lines were assayed in parallel. *Agrobacterium tumefaciens*-mediated *Arabidopsis* transformation was performed by a modified floral dip method, as described previously (Zhang *et al*., 2006). Overexpression of transgenes in the transgenic plants was verified by quantitative real-time RT-PCR (RT-qPCR).

2. Analysis of gene transcript levels

RT-qPCR was employed to examine relative levels of gene transcripts. Total RNA sample preparation, reverse transcription, and quantitative polymerase chain reaction were carried out following the proposed rules to ensure reproducible measurements (Udvardi *et al*., 2008). RT-qPCR reactions were performed using 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. PCR primers were listed in Table 1. The two-step thermal cycling profile was 15 s at 95^{o}C for denaturation and 1 min at 60^{o}C for annealing and polymerization. An *eIF4A* gene (At3g13920) was included in the reactions as internal control in order to normalize the variations in the amounts of cDNA used.

RT-qPCR reactions were carried out in biological triplicates using total RNA samples extracted from three independent plant materials grown under identical conditions. The comparative $\Delta\Delta C_T$ method was employed to

evaluate relative quantities of each product amplified from the samples. The threshold cycle (C_T) was automatically determined for each reaction by using the default parameters of the system.

3. Measurements of free indole acetic acid (IAA) content

Plants grown on MS-agar plates at 23ºC for 6 days were incubated at 28ºC for 24 h. Aerial plants parts were harvested for the extraction of free IAA. Plant materials were finely ground in liquid nitrogen, and 0.1 g of the powder was extracted in 1 ml of ethyl acetate-formic acid solution (99.5:0.5) containing 20 ng of D_5-IAA as a standard. The samples were then vortex-mixed for 10 min and centrifuged at 16,000 X *g* for 20 min at 4ºC. The supernatants were then evaporated at room temperature in SpeedVac (Eppendorf, Hamburg, Germany). The pellets were resuspended in 50 μl of methanol-water solution (70:30) and dissolved for 5 min using an ultrasonic bath (Branson, Danbury, CT). IAA content was measured using liquid chromatography (Agilent technologies, Santa Clara, CA), which was coupled to a mass spectrometer (Applied biosystems, Foster City, CA), as described previously (Woldemariam *et al*., 2012). Separation was performed on a Zorbax Eclipse column (Agilent technologies). Mobile phases A (0.05% acetic acid in water) and B (0.05% acetic acid in acetonitrile) were used for the separation. The mass spectrometer was operated in the positive ionization mode with an ion spray voltage of 5,500 V and a turbo gas temperature of 700ºC.

4. Bimolecular fluorescence complementation (BiFC) assay

BiFC assays were carried out as described previously (Seo *et al*., 2011). A full-size *FCA* gene was fused in-frame to the 3′ end of a DNA sequence encoding the C-terminal half of YFP in the pSATN-cYFP-C1 vector (E3082). A *PIF4* cDNA was fused in-frame to the 3′ end of a DNA sequence encoding the N-terminal half of YFP in the pSATN-nYFP-C1 vector (E3081). A full-size *SnRK2.8* cDNA sequence was fused in-frame to the 3′ end of a DNA sequence encoding the N-terminal half of EYFP in the pSATN-cEYFP-C1 vector (E3082). A full-size *NPR1* cDNA sequence was fused in-frame to the 3′ end of a DNA sequence encoding the C-terminal half of EYFP in the pSATN-nEYFP-C1 vector (E3081). The expression constructs were co-transformed into *Arabidopsis* protoplasts. Expression of the fusion constructs was visualized by differential interference contrast microscopy (DIC) and fluorescence microscopy using a Zeiss LSM510 confocal microscope (Carl Zeiss, Yena, Germany). Reconstitution of YFP fluorescence was observed using a confocal microscope with the following YFP filter set up: excitation 515 nm, 458/514 dichroic, and emission 560-615 nm BP filter.

5. Preparation of recombinant proteins

Full-size *FCA*, *SnRK2.8* and *NPR1* cDNA sequences was subcloned into the pMAL-c2X *Escherichia coli* expression vector (NEB, Ipswich, MA)

containing a maltose binding protein (MBP)-coding sequence. Recombinant MBP, MBP-FCA, MBP-SnRK2.8, and MBP-NPR1 fusion proteins were produced in *E. coli* Rosetta2 (DE3) pLysS strain (Novagen, Madison, WI) and purified as described previously (Seo *et al*., 2011). One-tenth volume of precultured cells (5 ml) was added to 500 ml of Luria-Bertani medium and cultured at 37ºC until OD_{600} = 0.3 - 0.6. Then, isopropyl-β-D-thiogalactopyranoside was added to the culture at a final concentration of 0.5 mM, and the mixture was incubated for 16 h at 22ºC to induce protein production. Cells were harvested and resuspended in MBP buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO; Cat No. P9599)). Cell lysates were prepared by three cycles of freezing and thawing followed by centrifugation. The supernatants were stored at -80 ºC until use.

6. *in vitro* pull-down assay

To examine the interactions of FCA with PIF4, the *PIF4* cDNA was amplified by RT-PCR and subcloned into the pGBKT7 vector, which contains the T7 RNA polymerase promoter upstream of a multiple cloning sequence. The [^{35}S]methionine-labeled PIF4 protein was synthesized by *in vitro* translation using the TNT coupled reticulocyte lysate system (Promega, Madison, WI). Five μl of the ^{35}S-labeled protein solution was incubated with 1 μg of purified MBP or MBP-FCA fusion protein bound to MBP-beads in 1

ml of binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich)) containing 5% skim milk for 12 h at 4ºC. The beads were washed 10 times with TN buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl). The bound proteins were eluted with 1 X SDS-PAGE loading buffer by heating for 10 min at 70ºC and subject to SDS-PAGE and autoradiography.

7. Yeast two-hybrid assay

Yeast two-hybrid assays were employed to examine whether SnRK2.8 interacts with NPR1 using the BD Matchmaker system (Clontech, Mountain View, CA). The pGADT7 vector was used for GAL4 activation domain (AD) fusion, and the pGBKT7 vector was used for GAL4 DNA binding domain (BD) fusion. PCR products of full-size *SnRK2.8* and *NPR1* cDNAs were digested with *Nde*I and *Eco*RI and subcloned into the pGBKT7 and pGADT7 vectors. The yeast strain AH109, which harbors chromosomally integrated reporter genes *lacZ* encoding β-galactosidase and *HIS* mediating histidine biosynthesis under the control of the GAL1 promoter, was used for transformation of the expression constructs. Transformation of AH109 cells was performed according to the manufacturer's instruction. Colonies obtained were streaked on medium without His, Ade, Leu, and Trp.

8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously (Lee *et al*., 2012). The assays were performed in biological triplicates using three independent plant materials grown under identical growth conditions. Plants were grown on MS-agar plates for 6 days and further grown for appropriate time periods at either 23^{o}C or 28^{o}C. Whole plants were harvested for the analysis. Plant materials were vacuum-infiltrated with 1% (v/v) formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. The ground powder was resuspended in nuclear extraction buffer [1.7 M sucrose, 10 mM Tris-Cl, pH 7.5, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets (Sigma-Aldrich)] and centrifuged at 16,000 X g for 1 h at 4^{o}C to obtain nuclear fraction. The nuclear fraction was lysed with lysis buffer [50 mM Tris-Cl, pH 8.0, 0.5 M EDTA, 1% SDS, and protease inhibitor cocktail tablets (Sigma-Aldrich)] to extract chromatin. Chromatin preparations were sonicated into 0.4- to 0.7-kb fragments. Five μg of anti-MYC (Millipore, Billerica, MA; Cat No. 05-724), anti-FLAG (Sigma-Aldrich; Cat No. F1804), or anti-H3K4me2 (Millipore; Cat No. 05-1338) antibody was added to the chromatin solutions, which were precleared with salmon sperm DNA/ Protein G 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 Promega spin column (Promega). Quantitative PCR was used to determine the amounts of

genomic DNA enriched in the chromatin preparations, and the values were normalized to the amount of input in each sample. The primers used are listed in Table 1.

9. Coimmunoprecipitation assay

Coimmunoprecipitation assays were performed as described previously (Song *et al*., 2012). Plants were grown for 6 days at 23ºC and further grown for 24 h at either 23ºC or 28ºC. Whole plants were harvested for the assays. Plant materials were ground in liquid nitrogen, and proteins were extracted in coimmunoprecipitation buffer [50 mM Tris-Cl pH 7.4, 500 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40, and protease inhibitor cocktail tablets (Sigma-Aldrich)]. Five % of the extract was used as input control. Five μg of anti-FLAG antibody (Sigma-Aldrich; Cat No. F1804) was added to the extract and incubated for 2 h. After the incubation, protein G agarose beads were added and further incubated for 2 h. The beads were then washed 5 times with coimmunoprecipitation buffer lacking protease inhibitor cocktail. To elute proteins, 50 μl of 2 X SDS loading buffer (100 mM Tris-Cl pH 6.8, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) was added to the beads and incubated at 100ºC for 10 min. Twenty % of the eluted proteins was used for IP control. Anti-FLAG antibody (Sigma-Aldrich; Cat No. F1804) and anti-MYC antibody (Millipore; Cat No. 05-724) were used for the detection of FLAG-PIF4 and MYC-FCA, respectively (dilution = 1:4000).

Coimmunoprecipitation assays using *Nicotiana benthamiana* cells was performed as described previously (Song *et al*., 2012). The *MYC-SnRK2.8* and *FLAG-NPR1* fusion sequences subcloned into the pBA002 and pEarleyGate202 vector (ABRC, Columbus, Ohio), respectively, and the expression constructs were transiently coexpressed driven by the CaMV 35S promoter in tobacco leaves. Plant materials were ground in liquid nitrogen, and proteins were extracted in coimmunoprecipitation buffer (50 mM Tris-Cl pH 7.4, 500 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 1% Nonidet P-40) containing protease inhibitor cocktail tablets (Sigma-Aldrich). Ten % of the extract was used as input control. An anti-FLAG antibody (Sigma-Aldrich) was added to the extract and incubated for 2 h. After the incubation, protein G agarose beads were added and further incubated for 2 h. The beads were then washed 5 times with coimmunoprecipitation buffer lacking protease inhibitor cocktail. To elute bound proteins, 50 μl of 2 X SDS loading buffer (100 mM Tris-Cl pH 6.8, 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT) was added and boiled for 5 min. Twenty % of the eluted proteins was used for IP control. Anti-FLAG and anti-MYC (Millipore, Billerica, MA) antibodies were used for the immunological detection of FLAG-NPR1 and MYC-SnRK2.8 fusion proteins, respectively.

10. Cell fractionation for subcellular localization of NPR1

A *MYC-NPR1* fusion construct, in which a MYC-coding sequence was

fused in-frame to the 5' end of a full-size *NPR1* cDNA, was overexpressed driven by the CaMV 35S promoter in Col-0 or *snrk2.8-1* background. The 4th or 5th leaves of four-week-old plants grown in soil under LDs were pressure-infiltrated with *Pst* DC3000/*avrRpt2* cells for 2 days. Two upper leaves were harvested before cell fractionation. Preparation of nuclear and cytoplasmic fractions was performed as described previously (Wang *et al*., 2011). The fractions were analysed on 10% SDS-PAGE and blotted onto a Millipore Immobilon-P membrane (Billerica, MA). The NPR1 proteins were immunologically detected using an anti-MYC antibody. The α-tubulin protein, a marker for cytoplasmic localization (Saslowsky *et al*., 2005), was detected using an anti-tubulin antibody (Sigma-Aldrich). Blots were quantitated using the ImageJ software (http://rsbweb.nih.gov/ij/) (right panel).

11. Pathogen infection assay and SA treatment

Four-week-old *Arabidopsis* plants grown in soil under LDs at 23ºC were used for pathogen infection and gene expression assay. Bacterial cells of a virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) were cultured for 2 days at 28°C in Luria-Bertani medium supplemented with rifampicin (50 mg/l). A bacterial cell suspension was prepared at 10^7 colony forming unit (cfu)/ml in 10 mM $MgCl_2$ supplemented with 250 ppm TWEEN 80 and sprayed directly onto rosette leaf surface. After incubation

for 16 h at 25°C and 100% relative humidity in complete darkness, the inoculated plants were transferred to a growth chamber set at 23°C and 80% relative humidity and further grown for 2 days under LDs. Inoculated leaves were used for the extraction of total RNA. For measuring bacterial cell growth, the spray-inoculated plants were incubated for 3 - 6 days under LDs, and bacterial cells were counted as previously described (Park *et al*., 2007).

To examine the induction of SAR in systemic leaves, the 4th or 5th rosette leaves were pressure-infiltrated with an avirulent *Pst* DC3000/*avrRpt2* cells resuspended in 10 mM $MgCl_2$ solution and incubated for 2 days under LDs. Two upper leaves were then infiltrated with *Pst* DC3000 cells and incubated for 3 additional days before taking photographs and counting bacterial cells, as described above.

For SA treatments, two-week-old plants grown on MS-agar plates were sprayed with 0.5 mM SA and incubated for appropriate time periods before harvesting whole plant materials.

12. *in vitro* phosphorylation assay

The *in vitro* phosphorylation assays were performed as described previously (Kim *et al*., 2012). The 5 μg of purified recombinant NPR1 proteins and 1.5 μg of SnRK2.8 proteins were added to reaction buffer [20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 1mM Na_3VO_4, 2mM DTT, 0.5mM PMSF, 2 mM EDTA, and 200 nM dATP] including 1 μCi of [γ-^{32}P] ATP. The reaction

mixtures were incubated at 30°C for 30 min, and then terminated by adding 4 μl of 6 X SDS loading buffer. The mixtures were boiled at 100°C for 5 min and loaded on 10% SDS PAGE gels. The gels were stained with Coomassie Blue and dried under vacuum for the detection of radioactive signals.

Primers	Sequences	Usage	Primers	Sequences	Usage
eIF4A-F	5′-TGACCACACAGTCTCTGCAA	RT-qPCR	YUC4-F	5′-GCGAGCTTCCTCTTATGCCT	"
eIF4A-R	5′-ACCAGGGAGACTTGTTGGAC	"	YUC4-R	5′-CGAACTCTGCTTTCTCGACG	"
ACS5-F	5′-CGGGTTGGTTTAGGGTTTGT	"	YUC5-F	5′-TATGGCCTAAAGAGGCCCAC	"
ACS5-R	5′-CCCGGAAAACCCAGTTAGAG	"	YUC5-R	5′-TTAATTCCCGGGACGATTTC	"
CPD-F	5′-AGGGAGGAGGAGGAAGAAGG	"	YUC6-F	5′-CTACGCTCGGAGGTTTGACA	"
CPD-R	5′-GCGACAAGTAAAGCCACCAA	"	YUC6-R	5′-ACTCCGTCGTGCCTTCTTCT	"
DWF4-F	5'-CGGTGATCTCAGCCGTACAT	"	YUC8-F	5′-GACTGCTCGGTTCGATGAGA	"
DWF4-R	5'-CCCACGTCGAAAAACTACCA	"	YUC8-R	5′-TGAATCACCTCACCGGAAAA	"
GA20OX1-F	5'-TAGTGACGCCACCGAGAGAG	"	YUC9-F	5′-TCAACGGTCCGGTTATCGTA	"
GA20OX1-R	5'-TAGATGGGTTTGGTGAGCCA	"	YUC9-R	5′-GTTTTTGCCACAGTGACGCT	"
GA3OX1-F	5'-GGGTTAACCAAACCAGAGCC	"	YUC10-F	5′-CCAACACTCAATCCCAAACG	"
GA3OX1-R	5'-CGATTCAACGGGACTAACCA	"	YUC10-R	5′-CATGAAAGGAAGCTGGCAAA	"
IAA2-F	5'-GAAGAATCTACACCTCCTACCAAAA	"	SAUR20-F	5′-AACTTGAATCTTTTCATACATCTTCAGAAGA	"
IAA2-R	5'-CACGTAGCTCACACTGTTGTTG	"	SAUR20-R	5′-TAACTAGGAAGAAAAATGTTGGCTCATC	"
IAA3-F	5'-CAAAGATGGTGATTGGATGCT	"	SAUR22-F	5′-GACAAATAGAGAATTATAAATGGCTCTG	"
IAA3-R	5'-TGATCCTTAGTCTCTTGCACGTA	"	SAUR22-R	5′-ATGAATTAAGTCTATATCTAACTCGGAAA	"
IAA5-F	5′-TGAAGACAAAGATGGAGATTGG	"	PIF4-F	5′-AGATCATCTCCGACCGGTTT	"
IAA5-R	5′-GCACGATCCAAGGAACATTT	"	PIF4-R	5′-CGCCGGTGAACTAAATCTCA	"
IAA7-F	5'-GAAGGTCTCCATGGACGGTG	"	FCA-F	5′-GCTCTTGTCGCAGCAAACTC	"
IAA7-R	5'-ATTTTGGCCAATGCATCAGA	"	FCA-R	5′-GATCCAGCCCACTGTTGTTTAC	"
IAA11-F	5'-ATTGCTGGGATCAAGAGGAC	"	SnRK2.8-F	5′-CCTGAAGTGCTCTCCACGAA	"
IAA11-R	5'-GTGGCCATCCCACAACTT	"	SnRK2.8-R	5′-GCATTCATCCGAAACTCGAA	"
IAA13-F	5'-CATCTCCTCCTCGTTCAAGC	"	PR1-F	5′-TGATCCTCGTGGGAATTATGT	"
IAA13-R	5'-CTGTTCATCCTGTGTGACCCTA	"	PR1-R	5′-TGCATGATCACATCATTACTTCAT	"
IAA19-F	5′-TTGTATCAAATTGTGAGAGGAAAAA	"	PR5-F	5'-AGTTCCTCCCGTCACTCTGG	"
IAA19-R	5′-CGTTATCTCAAGCCCGAGTC	"	PR5-R	5'- TCCTCCGGATGGTCTTATCC	"
IAA27-F	5′-GATGTCCCTTGGGAAATGTTTA	"	SID2-F	5'-TCTGCAGTGAAGCTTTGGCT	"
IAA27-R	5′-GCTTCTGCACTTCTCCATCA	"	SID2-R	5'-GGTCGTCTTTCGGACTGGTT	"
IAA29-F	5′-TCCTCTGGAATCCGAGTCTTC	"	YUC8-P1-F	5′-CAACTCCCAATAAAAGACCATCA	ChIP-qPCR
IAA29-R	5′-GGTGGCCATCCAACAACTT	"	YUC8-P1-R	5′-TGGTTTCCTCAATCAATTTTCAA	"
IAA30-F	5'-TTCAATGCTTCAATCCTTTGG	"	YUC8-P2-F	5′-GGGAATGGGTTTGATGTGGAATT	"
IAA30-R	5'-AGCACGTGACTCTTCTCACTACA	"	YUC8-P2-R	5′-GAGAAGGGAAGTGATGGAATTAG	"
YUC1-F	5'-TCCTAACGGCTGGAGAGGAG	"	YUC8-P3-F	5′-TCCATTGATTCTCTCTCTCTCTCT	"
YUC1-R	5'-GTGGACCCCTTGATTTCGTC	"	YUC8-P3-R	5′-TCTTGATCCATCAAACGAAACA	"
YUC2-F	5′-GAGAATGCCGAGGAGGTGAT	"	YUC8-P4-F	5′-GGGGTTCTCTTCGAAGGTGT	"
YUC2-R	5′-CAAACTTCCATCCCGGAGTT	"	YUC8-P4-R	5′-TTTGGGTCTTCAGGAGAGGG	"
YUC3-F	5′-GTTTCAAAACCTCATCCCCG	"	YUC8-P5-F	5′-GTGGAGTTGCGGATTCGAG	"
YUC3-R	5′-GCAATGCAGTTAGCTCGCTC	"	YUC8-P5-R	5′-TTTTGCAATTCCTTAAGCACC	"

Table 1. Primers used in this work

The primers used were designed using the Primer3 software (version 0.4.0, http://primer3.sourceforge.net/releases.php) in a way that they have calculated melting temperatures in a range of 50-60ºC. F, forward primer; R, reverse primer.

CHAPTER 1

FCA mediates thermal adaptation of stem growth by attenuating auxin action in *Arabidopsis*

INTRODUCTION

Plants adapt to high ambient temperature by adjusting plant architecture, such as elongation of hypocotyl and leaf stem and increase of leaf hyponasty (Crawford *et al*., 2012; Franklin *et al*., 2011; Koini *et al*., 2009; Sun *et al*., 2012). These architectural adaptations help plants minimize the chance of heat damage and enhance evaporative leaf cooling for optimized plant growth (Crawford *et al*., 2012). However, a trade-off occurs when plants obtain the fitness benefits during long-term exposure to high temperature: plants having abnormally elongated stems tend to fall down and suffer from physiological imbalance (Mooney *et al*., 2010). This necessitates that there should be a fine-tuning mechanism that attenuates the effects of high temperature on stem growth.

The *Arabidopsis* basic helix-loop-helix transcription factor PIF4 plays a major role in high temperature-induced plant responses. Elongation of hypocotyl, increase of hyponasty, and induction of flowering are suppressed by *pif4* mutations under high temperature conditions (Franklin *et al*., 2011; Koini *et al*., 2009; Sun *et al*., 2012). It has been reported that PIF4 directly regulates transcription of auxin biosynthesis enzyme genes, such as *YUC8*, resulting in accumulation of auxin content (Sun *et al*., 2012). PIF4 function is enhanced at high temperature. The *PIF4* transcription is induced by exposure to high temperature (Kumar *et al*.,

2012; Sun *et al*., 2012). In addition, protein stability and DNA-binding affinity of PIF4 are elevated at high ambient temperatures (Kumar *et al*., 2012; Oh *et al*., 2012). While the role of PIF4 in thermal regulation of stem growth is evident, it is not fully understood how PIF4 function is modulated at the molecular level.

The *Arabidopsis* RNA-binding protein FCA is a critical component of the autonomous flowering pathway, which induces flowering by suppressing the floral repressor *FLC* (Liu *et al*., 2007; Liu *et al*., 2010). FCA plays a role in alternative polyadenylation of its own pre-mRNA and antisense *FLC* RNA (Liu *et al*., 2010; Quesada *et al*., 2003). FCA also acts as a chromatin remodeling factor: it mediates histone demethylation of *FLC* chromatin, causing suppression of *FLC* expression (Liu *et al*., 2007). Notably, it has been reported that FCA incorporates ambient temperature signals into the thermosensory flowering pathway (Blázquez *et al*., 2003), raising a possibility that FCA would participate in plant responses to changes in ambient temperature.

In this work, I demonstrated that FCA mediates the dissociation of PIF4 from its target gene chromatin, such as *YUC8*, at high ambient temperature. FCA is recruited to *YUC8* promoter through interaction with PIF4, resulting in the dissociation of PIF4 from *YUC8* promoter and the suppression of *YUC8* transcription. These observations illustrate that FCA attenuates PIF4 action in auxin accumulation occurring during high temperature-induced stem growth to maintain architectural and

physiological balance.

RESULTS

FCA mediates thermal acceleration of stem growth

While working on the role of FCA in thermosensory flowering, it was found that *FCA*-deficient mutants *fca-9* and *fca-11* exhibited elongated hypocotyls at 28ºC compared to those of wild-type (Col-0) plants (Fig. 1). In contrast, *FCA*-overexpressing transgenic plants (*35S:FCA*) had shorter hypocotyls under identical conditions, suggesting that FCA is associated with ambient temperature regulation of hypocotyl growth.

The effects of *fca* mutation on leaf hyponasty and leaf stem growth, which are also affected by high temperature were also examined (Gray *et al*., 1998; Koini *et al*., 2009). Leaf hyponasty markedly increased in *fca-9* when grown at 28ºC, but it was reduced in *35S:FCA* transgenic plants (Fig. 2). In addition, leaf stem (petiole) was elongated in *fca-9* compared to that of Col-0 plants, but the high temperature sensitivity disappeared in *35S:FCA* transgenic plants (Fig. 3). These observations support the notion that FCA mediates the high ambient temperature-induced architectural modifications.

FCA-mediated ambient temperature signals regulate flowering time via the floral repressor *FLC* (Blázquez *et al*., 2003; Liu *et al*., 2007; Liu *et al*., 2010). To examine whether FLC is involved in the enhanced stem growth in *fca-9* at high temperature, hypocotyl growth was measured in *FLC*-deficient *flc-3* mutant and *FLC*-accumulating plants, such as *flk-1* and *fld-6* mutants

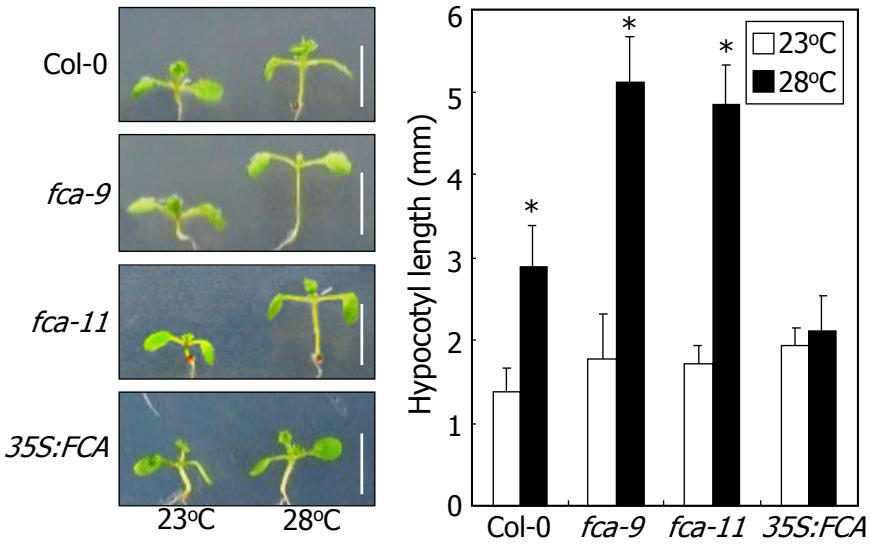

Figure 1. *fca* mutants showed elongated hypocotyl at 28°C

Plants were grown on Murashige and Skoog-agar plates (MS-agar plates) at 23°C under continuous light conditions for 4 days and further grown at either 23°C or 28°C for 3 days. Measurements of 15-20 seedlings were averaged and statistically treated using Student *t*-test (*P<0.01). Bars indicate standard error of the mean (SE). Scale bars, 1 cm.

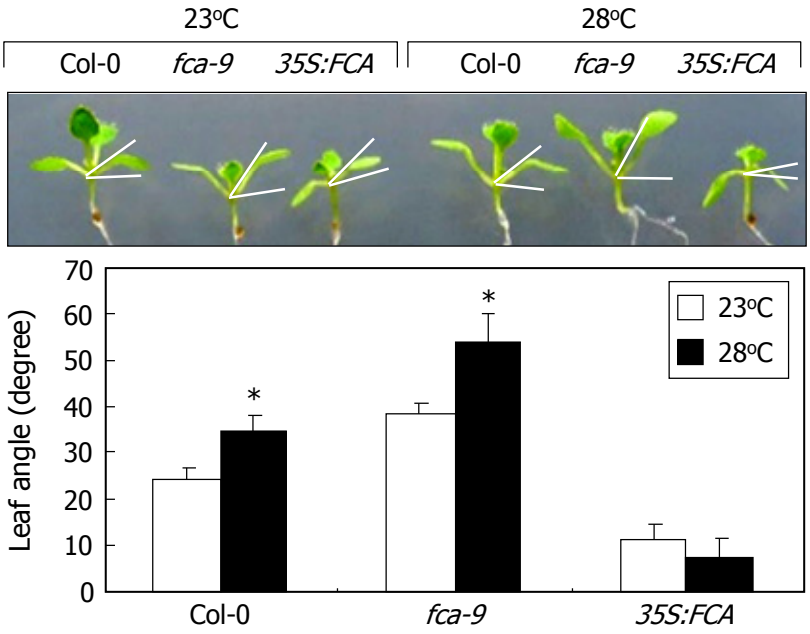

Figure 2. *fca* mutants showed increased leaf hyponasty at 28°C

Plants were treated as described in Figure 1. Measurements of 15-20 seedlings were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE. Scale bars, 1 cm.

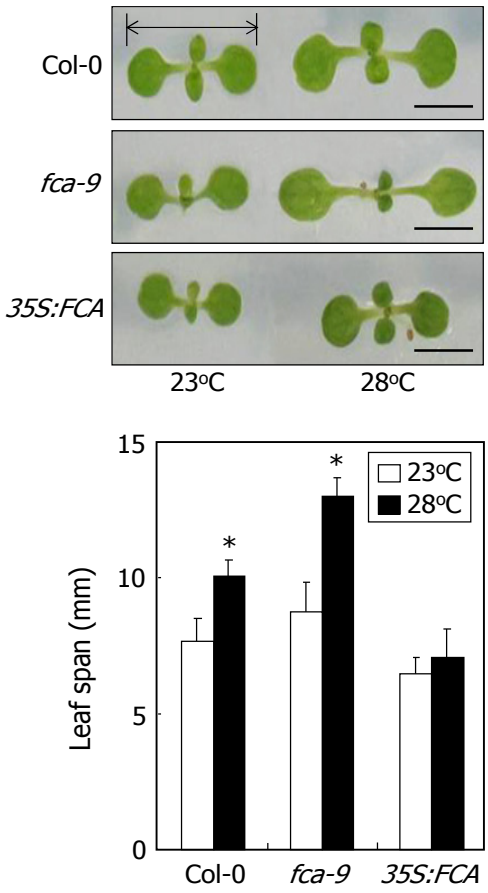

Figure 3. *fca* mutants showed increased petiole growth at 28°C

Plants were treated as described in Figure 1. Measurements of 15-20 seedlings were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE. Scale bars, 1 cm.

(Lim *et al*., 2004; Yu *et al*., 2011), active *FRIGIDA* (*FRI*) allele-containing *FRI*-Col plants (Choi *et al*., 2011), and *FLC*-overexpressing plants. Their hypocotyl growth was similar to that of Col-0 plants at 28ºC (Figs. 4A and B), showing that the accelerated hypocotyl elongation in *fca-9* at 28ºC is not related with *FLC*.

IAA content is elevated in *fca-9* at 28ºC

To obtain clues as to how FCA regulates stem growth at high temperature, the expressions of genes encoding biosynthetic enzymes of ethylene, gibberellic acid, brassinosteriod, and auxin, which mediate stem growth, were investigated (Bai *et al*., 2012; Muday *et al*., 2012). Among those, only auxin biosynthetic enzyme genes were significantly induced in *fca-9* when grown at 28ºC (Fig. 5). Among the *IAA* and *YUC* genes tested, *IAA29* and *YUC8*, which have been shown to mediate high temperature-induced stem elongation (Koini *et al*., 2009; Sun *et al*., 2012), were induced by more than 6-fold in the high temperature-treated *fca-9* mutant (Fig. 6). Auxin-responsive *SAUR20* and *SAUR22* genes are also involved in high temperature regulation of hypocotyl growth (Franklin *et al*., 2011). It was found that they were expressed at higher levels in *fca-9* compared to that in Col-0 plants at 23ºC (Fig. 7), and their transcripts levels were further elevated in the high temperature-treated *fca-9* mutant.

The up-regulation of *IAA29*, *YUC8*, and *SAUR* genes in *fca-9* at 28ºC

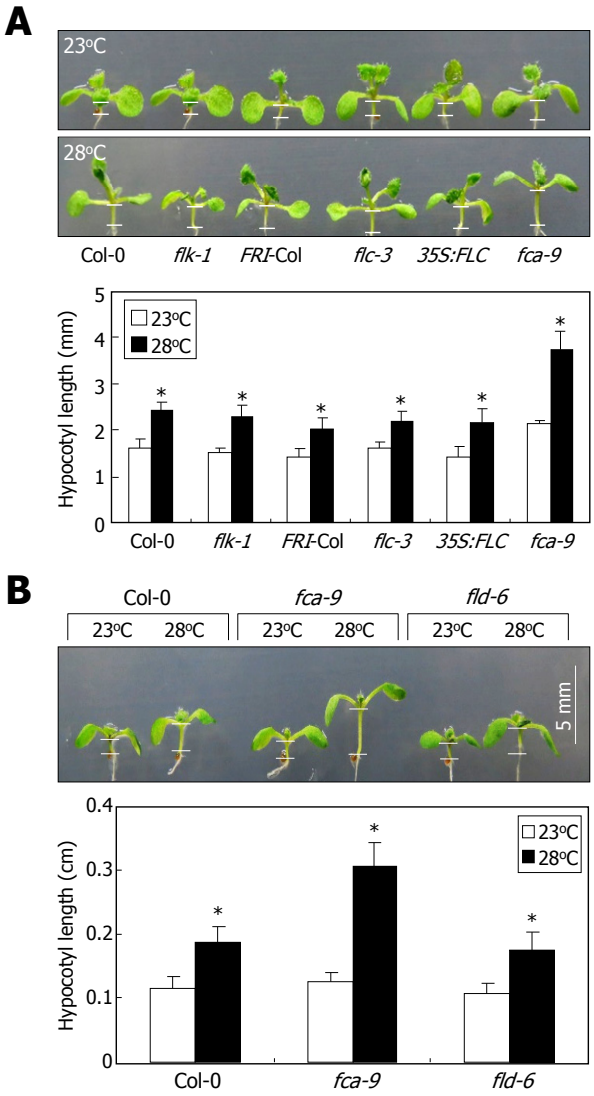

Figure 4. Elongated hypocotyl of *fca* mutants was not related with FLC

Plants were treated as described in Figure 1. Measurements of 20 seedlings were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(A) High temperature-induced hypocotyl elongation of *flk-1*, *FRI*-Col, *flc-3*, *35S:FLC* plants.

(B) High temperature-induced hypocotyl elongation of *fld-6* mutant.

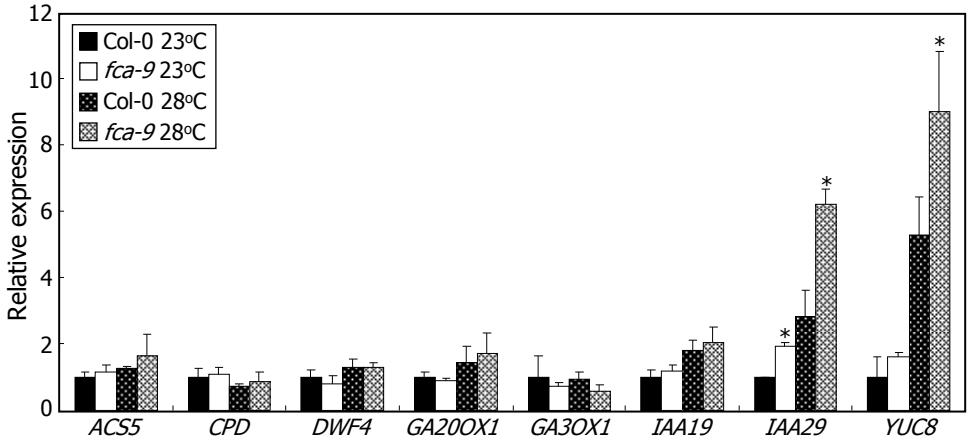

Figure 5. Genes encoding biosynthetic enzymes of ethylene, gibberellic acid, and brassinosteroid are not influenced by high temperature

Six-day-old plants grown on MS-agar plates at 23°C under continuous light conditions were subsequently exposed to 28°C for 24 h. Whole plants were used for the extraction of total RNA and protein. Gene transcript levels were measured by RT-qPCR. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE. *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 5* (*ACS5*) is involved in ethylene biosynthesis (Vogel *et al*., 1998). *CPD* and *DWF4*, are involved in brassinosteroid biosynthesis (Choe *et al*., 1998). *GA20OX1* and *GA3OX1* are involved in gibberellic acid biosynthesis (Hedden and Phillips, 2000).

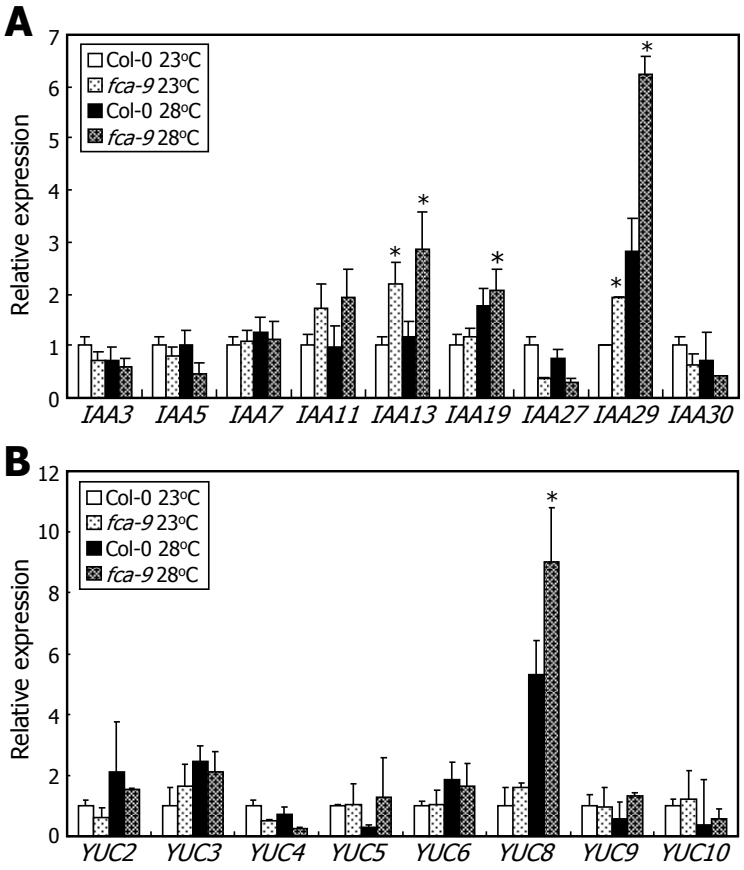

Figure 6. *IAA29* and *YUC8* are highly expressed in *fca-9* mutant at 28°C

Transcript levels were measured by RT-qPCR. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(A) Transcript levels of *IAA* genes in *fca-9*. Plants were grown on MS-agar plates for 6 days at 23°C and exposed to 28°C for 24 h before harvesting whole plant materials.

(B) Transcript levels of *YUC* genes in *fca-9*. Total RNA samples prepared in **(A)** were used for RT-qPCR.

suggested that endogenous auxin content is elevated in *fca-9*. To examine whether the elongated hypocotyl of *fca-9* at 28ºC is caused by elevated auxin content, the mutant was grown at either 23ºC or 28ºC in the presence of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) (Fernández-Marcos *et al*., 2011). The high temperature-induced hypocotyl elongation of the mutant was completely suppressed by NPA (Fig. 8). Direct measurements of IAA content revealed that at 28ºC, the level of free IAA was higher by 36% in *fca-9* but lower by 28% in *35S:FCA* transgenic plants compared to those in Col-0 plant (Fig. 9), indicating that the high temperature-induced hypocotyl elongation is due to elevated IAA content in *fca-9*.

FCA interacts with PIF4 in thermal induction of stem growth

IAA29, *SAUR20, SAUR22*, and *YUC8* genes are regulated by PIF4 at high temperature (Franklin *et al*., 2011; Koini *et al*., 2009; Sun *et al*., 2012), suggesting that PIF4 plays a role in the induction of the auxin-responsive genes in *fca-9*. Thus, it was first examined whether FCA interacts with PIF4. BiFC assays showed that they interact with each other in the nucleus (Fig. 10A). *in vitro* pull-down assays also supported the physical association between FCA and PIF4 (Fig. 10B).

Next, the functional relationship between FCA and PIF4 was explored. A *PIF4*-deficient *pif4-101* mutant was crossed with *fca-9*, resulting *fca-9 pif4-101*. The high temperature-induced hypocotyl elongation

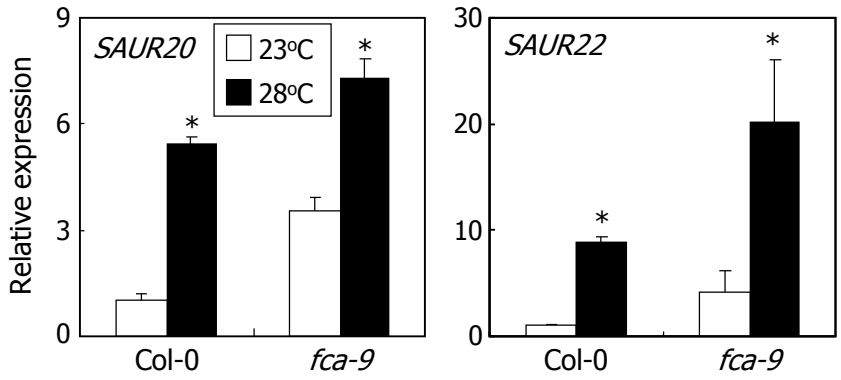

Figure 7. Transcript levels of *SAUR* genes in *fca-9*

Total RNA samples prepared in Figure 6A were used for RT-qPCR. Biological triplicates were averaged and statistically treated using Student *t*-test (*P<0.01). Bars indicate SE.

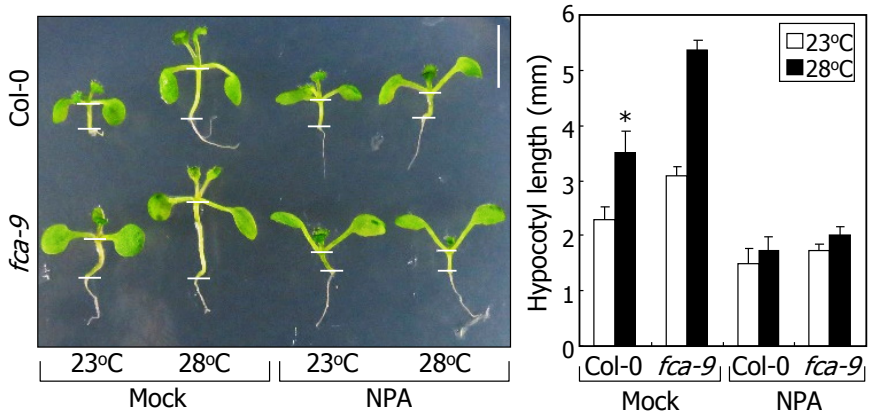

Figure 8. Elongated hypocotyls of *fca-9* is compromised in the presence of 100 μM NPA

Plants were grown as described in Figure 1 but in the presence of the auxin transport inhibitor NPA (left panel). Measurements of 20 hypocotyls were averaged and statistically treated using Student *t*-test (**P*<0.01) (right panel). Bars indicate SE.

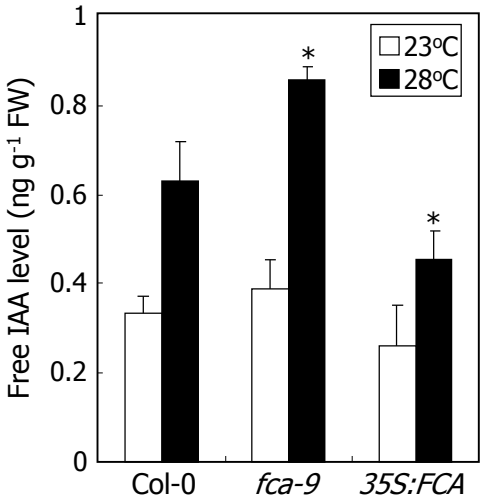

Figure 9. Measurements of free IAA content in *fca-9*

Plants were grown on MS-agar plates at 23ºC under continuous light conditions for 4 days and further grown at either 23ºC or 28ºC for 1 day. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

phenotype of *fca-9* was suppressed in *fca-9 pif4-101*, which is similar to the hypocotyl phenotype of *pif4-101* (Fig. 11A). In addition, the inductive effects of high temperature on *YUC8* transcription were not observed in *fca-9 pif4-101* and *pif4-101* (Fig. 11B). Together with the elevated IAA content in *fca-9*, these observations indicate that FCA is associated with the PIF4-regulated auxin biosynthesis.

FCA regulates PIF4 binding to *YUC8* chromatin

A question was how FCA regulates PIF4 function. *PIF4* gene is induced when plants grown at 16ºC are exposed to 28ºC (Kumar *et al*., 2012). It was examined whether the high temperature induction of *PIF4* gene is regulated by FCA. The inductive effects of high temperature on *PIF4* transcription were still evident in *fca-9* (Figs. 12A and B). In addition, *fca* mutation did not affect the protein stability of PIF4 (Fig. 12C). Next, it was examined whether *fca* mutation influences the transcriptional activity of PIF4 using a β-glucuronidase (GUS) transient expression system in *Arabidopsis* protoplasts (Yoo *et al*., 2007), which were prepared from *fca-9* mutant. It was found that the transcriptional activity of PIF4 was not altered in *fca-9*, and coexpression of FCA did not affect the PIF4 activity (Fig. 13), indicating that FCA does not affect the transcriptional activity of PIF4. Instead, it was hypothesized that FCA might influence the DNA binding of PIF4.

It has been known that PIF4 binds to G-box elements in *YUC8* promoter,

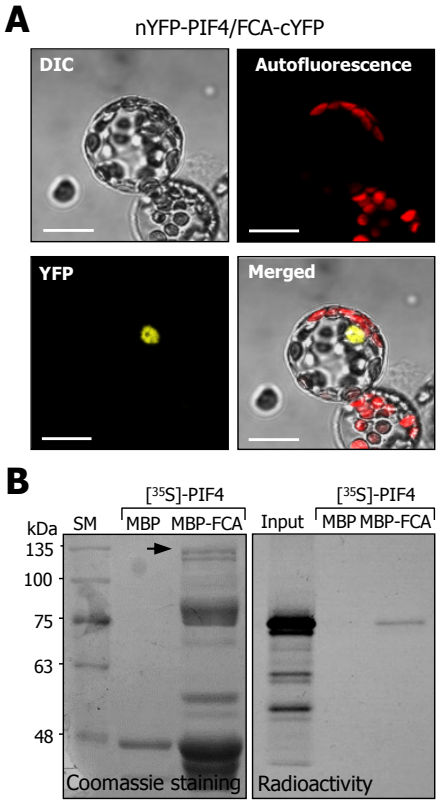

Figure 10. FCA interacts with PIF4

(A) BiFC assays on FCA-PIF4 interaction. nYFP-PIF4 and cYFP-FCA gene fusions were coexpressed transiently in *Arabidopsis* protoplasts. FCA-PIF4 interactions were visualized by differential interference contrast microscopy (DIC). Scale bars, 10 μm.

(B) *in vitro* pull-down assays on FCA-PIF4 interaction. Recombinant MBP-FCA fusion protein was produced in *E. coli* cells. ^{35}S-labeled PIF4 protein was prepared by *in vitro* translation. MBP alone was used as negative control (right panel). Part of Coomassie blue-stained gel was shown as loading control (left panel). kDa, kilodalton.

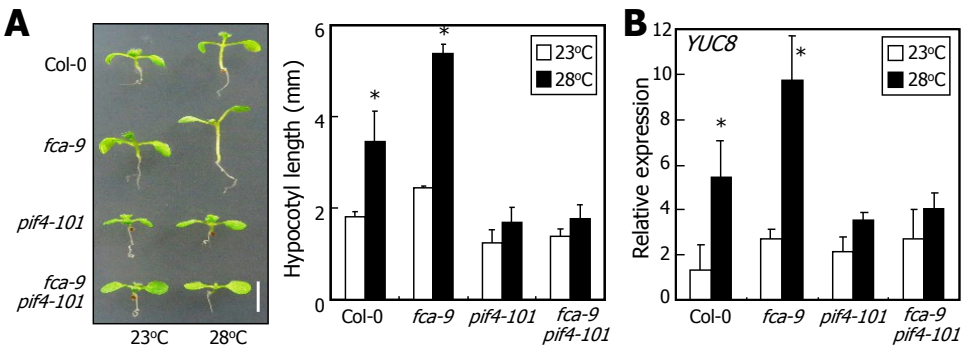

Figure 11. FCA-mediated regulation of hypocotyl length at 28°C is associated with PIF4

(A) Hypocotyl growth of *fca-9 pif4-101*. Plants grown on MS-agar plates for 4 days at 23°C were further grown at 28°C for 3 days (upper panel). Scale bar, 0.5 cm. Measurements of 15 hypocotyls were averaged and statistically treated using Student *t*-test (**P*<0.01) (lower panel). Bars indicate SE.

(B) Transcript levels of *YUC8* gene in *fca-9 pif4-101*. Transcript levels were measured as described in Figure 6. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

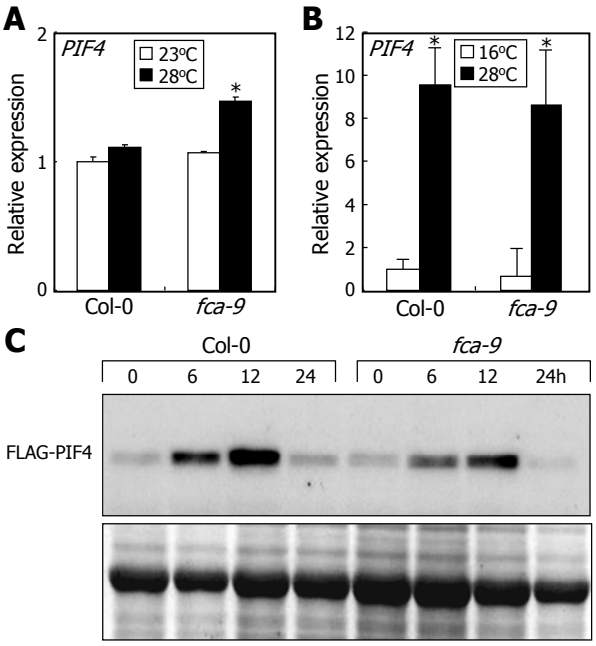

Figure 12. FCA is not related with high temperature-mediated regulation of *PIF4* expression and protein stability

(A, B) Effects of high temperature on *PIF4* transcription in *fca-9* mutant. Six-day-old plants grown on MS-agar plates at either 23ºC **(A)** or 16ºC **(B)** under continuous light conditions were subsequently exposed to 28ºC for 24 h before harvesting whole plant materials for the extraction of total RNA. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(C) Effects of *fca* mutation on PIF4 protein stability.

Six-day-old p*PIF4*:*FLAG-PIF4* transgenic plants grown on MS-agar plates at 23ºC under continuous light conditions were subsequently exposed to 28ºC for up to 24 h.

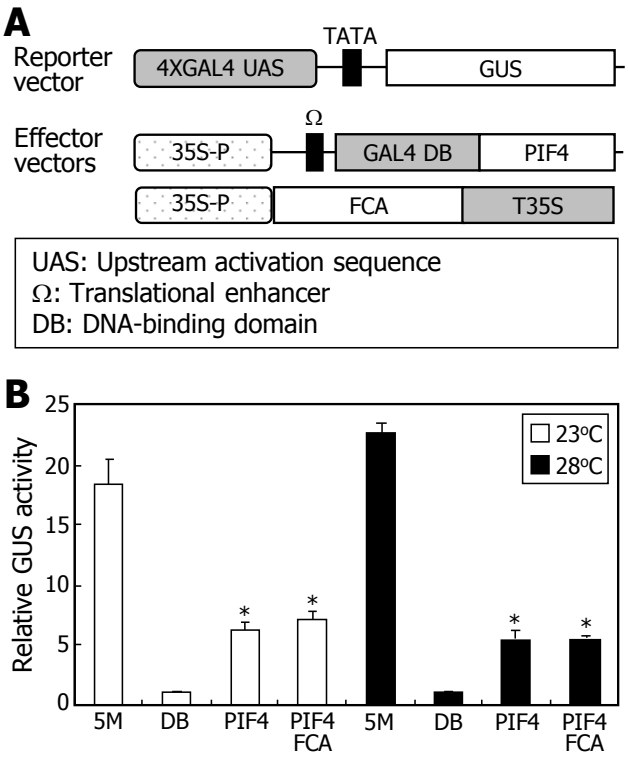

Figure 13. FCA does not affect the transcriptional activation activity of PIF4

A series of reporter and effector constructs was constructed for transient expression assays in *Arabidopsis* protoplasts **(A)**. The reporter and effector vectors were cotransformed into *Arabidopsis* protoplasts, and relative β-glucuronidase (GUS) activity was measured **(B)**. ARF5M (5M) was used as activator control. Three measurements were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

although it is unclear whether high temperature influences the DNA binding of PIF4 (Sun *et al*., 2012). Notably, it has recently been reported that PIF4 binds to *FT* promoter and its DNA binding is enhanced at high temperature (Kumar *et al*., 2012). Therefore it was hypothesized that FCA might influence the binding of PIF4 to *YUC8* promoter in response to high temperature. ChIP assays revealed that the binding of PIF4 to a sequence region covering G-box elements in *YUC8* promoter, which was designated P2 region in this work (Fig. 14A), was elevated in both Col-0 and *fca-9* plants exposed to 28ºC for 6 h (Fig. 14B). Next, ChIP assays were performed using plants that were exposed to 28ºC for longer time (24 h). Notably, while the binding of PIF4 to P2 was reduced to a basal level in Col-0 plants, the high-level binding of PIF4 to P2 was maintained in *fca-9* (Fig. 14C). Consistent with these observations, the transcript levels of *YUC8* were similar in Col-0 and *fca-9* that were exposed to 28ºC for 6 h but was higher in *fca-9* than in Col-0 when the plants were exposed to 28ºC for 24 h (Fig. 14D). It was therefore concluded that FCA does not affect the binding step of PIF4 to *YUC8* promoter but trigger the dissociation of PIF4 from *YUC8* promoter, especially under long-term exposure to high temperature.

FCA induces chromatin modification at *YUC8* locus

FCA requires a histone demethylase, which targets the active marker histone-3 lysine-4 dimethylation (H3K4me2), to down-regulate *FLC*

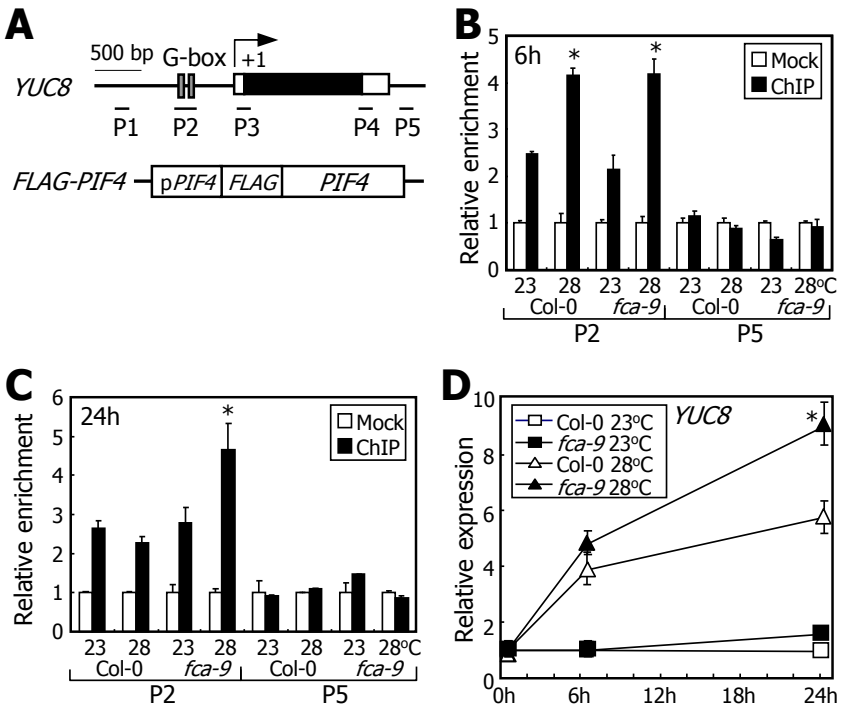

Figure 14. FCA mediates dissociation of DNA binding of PIF4 at 28°C

(A) Genomic structure of *YUC8* gene. P1 to P5 sequence regions were used in ChIP assays. Black boxes are exons, and white boxes are untranslated regions. bp, base pair. The FLAG-PIF4 expression construct, in which a FLAG octapolypeptide-coding sequence is fused in-frame to the 5' end of *PIF4* gene under the control of the endogenous *PIF4* promoter consisting of approximately 2-kbp sequence upstream of the translation start site, is shown at the bottom.

(B, C) ChIP assays on the effects of FCA on PIF4 binding to *YUC8* promoter. The FLAG-PIF4 gene fusion was transformed into Col-0 and *fca-9* plants. Plants grown on MS-agar plates for 6 days at 23°C were further grown at either 23°C or 28°C for 6 h **(B)** and 24 h **(C)** before extracting

chromatin from whole plants. ChIP assays were performed with P2 and P5 sequences using an anti-FLAG antibody. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(D) Accumulation kinetics of *YUC8* transcripts in *fca-9* after 28ºC treatment. Transcript levels were measured as described in Figure 6. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

expression (Liu *et al*., 2007). Therefore, it was suspected that FCA influences the dissociation of PIF4 from *YUC8* promoter via chromatin remodeling. Chip assays showed that FCA binds to P2 region in *YUC8* chromatin at 23ºC and its DNA binding was considerably elevated at 28ºC (Fig. 15A). FCA also bound to P1 region, which is approximately 500 bases upstream of P2 region, at 28ºC. In addition, the levels of H3K4me2 was elevated by more than 2-fold in P2 region in *fca-9* at 28ºC (Fig. 15B). The H3K4me2 level was also elevated in P3 region covering the transcription start site. These data indicate that FCA modifies *YUC8* chromatin, thus promoting the dissociation of PIF4 from *YUC8* promoter at 28ºC.

Next, the kinetics of H3K4me2 accumulation in *fca-9* after exposure to 28ºC was monitored. In Col-0 plant, the H3K4me2 level in P2 region was elevated by approximately 2-fold at 6 h but reduced to a basal level at 24 h (Fig. 15C). In *fca-9*, it was largely unchanged at 6 h but significantly elevated by more than 2-fold at 24 h. The kinetics patterns of H3K4me2 accumulation in P3 regions were similar to those observed in P2 regions of both Col-0 and *fca-9* plants (Fig. 15D). These results indicate that FCA-mediated demethylation events are induced by long-period exposure to high temperature in *YUC8* chromatin.

FCA modulates dissociation of PIF4 from *YUC8* chromatin

A critical question was how high temperature signals are incorporated into the FCA-PIF4-YUC8 module. *FCA* transcription was only moderately

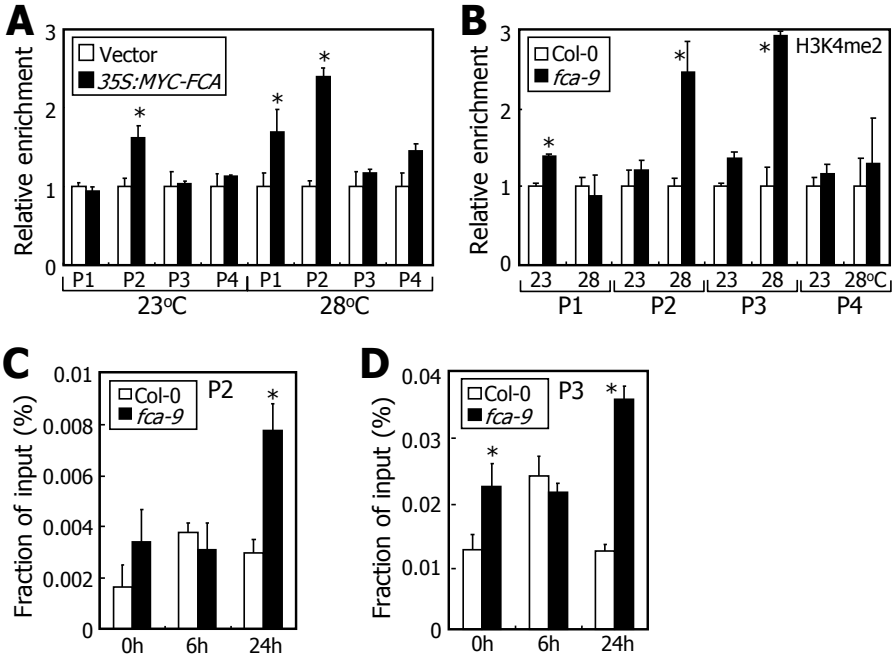

Figure 15. FCA mediates histone demethylation at *YUC8* promoter

Chromatin was prepared as described in Figure 14. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(A) ChIP assays on FCA binding to *YUC8* chromatin. A MYC-FCA fusion was overexpressed in Col-0 plants. ChIP assays were performed with P1 to P4 sequences using an anti-MYC antibody.

(B) Histone modifications at *YUC8* chromatin in *fca-9*. ChIP assays were performed using an anti-H3K4me2 antibody.

(C, D) Kinetics of histone modifications at *YUC8* chromatin in *fca-9*. Chromatin was prepared from plants at different time points after exposure to 28ºC. ChIP assays were performed with either P2 **(C)** or P3 **(D)** sequence using an anti-H3K4me2 antibody.

induced by approximately 2-fold at 28ºC (Fig. 16A). In addition, high temperature did not discernibly affect the FCA protein stability (Fig. 16B). Next, coimmunoprecipitation assays were performed on Col-0 plants coexpressing FLAG-PIF4 and MYC-FCA fusions using anti-FLAG and anti-MYC antibodies for the pull-down of the fusion proteins. FCA interacted with PIF4 *in planta* primarily at 28ºC (Fig. 16C). Furthermore, ChIP assays on *pif4-101* mutant overexpressing MYC-FCA showed that FCA binding to *YUC8* chromatin did not occur in the mutant at 28ºC (Fig. 16D). Together with the increased binding of PIF4 to *YUC8* chromatin (Fig. 14B), these observations indicate that at high temperatures, PIF4 recruits FCA to the chromatin, where FCA induces chromatin modifications, causing PIF4 dissociation (Fig. 17). All together, it is concluded that FCA tunes down the high temperature-induced stem elongation by attenuating PIF4 action probably through remodeling of *YUC8* chromatin (Fig. 18).

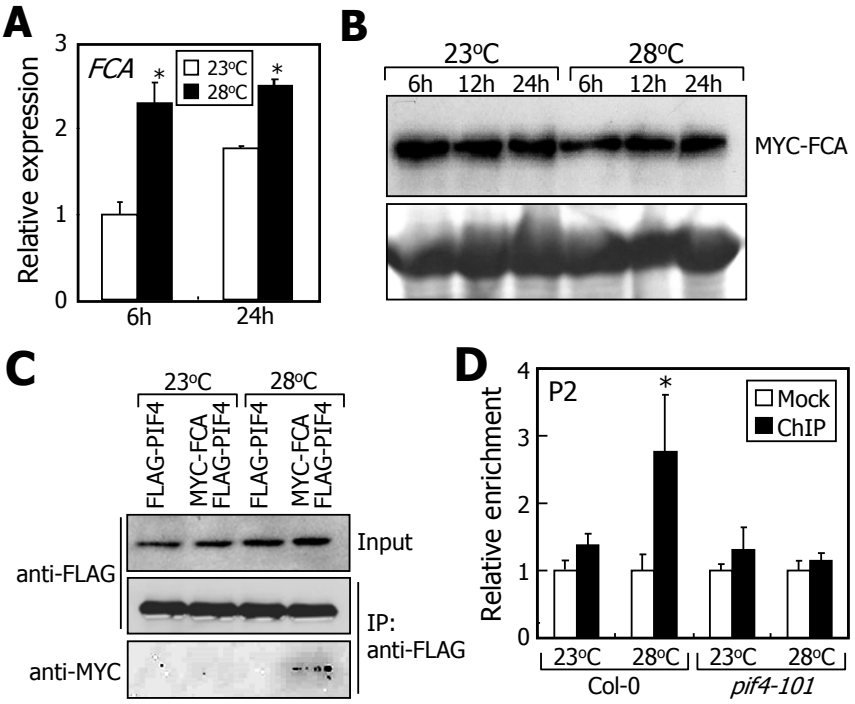

Figure 16. High temperature induces FCA-PIF4 interaction

Six-day-old Col-0 plants **(A)** and *35S:MYC-FCA* transgenic plants **(B)** grown on MS-agar plates at 23°C under continuous light conditions were subsequently exposed to 28°C for up to 24 h. Whole plants were used for the extraction of total RNA and protein. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(A) Effects of high temperature on *FCA* transcription. Gene transcript levels were measured by RT-qPCR.

(B) Effects of high temperature on FCA protein stability. Part of Coomassie blue-stained gel is shown at the bottom.

(C) Coimmunoprecipitation assays on FCA-PIF4 interaction. The FLAG-PIF4 expression construct was transformed into *35S*:*MYC-FCA* transgenic

plants. Plants were treated with 28ºC, as described in Figure 14C. Imunoprecipitation (IP) was performed using an anti-FLAG antibody. Input represents 5% of the IP reaction.

(D) ChIP assays on FCA binding to *YUC8* chromatin in *pif4-101*. The assays were performed as described in Figure 14C using *pif4-101* mutant overexpressing MYC-FCA driven by the CaMV 35S promoter. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

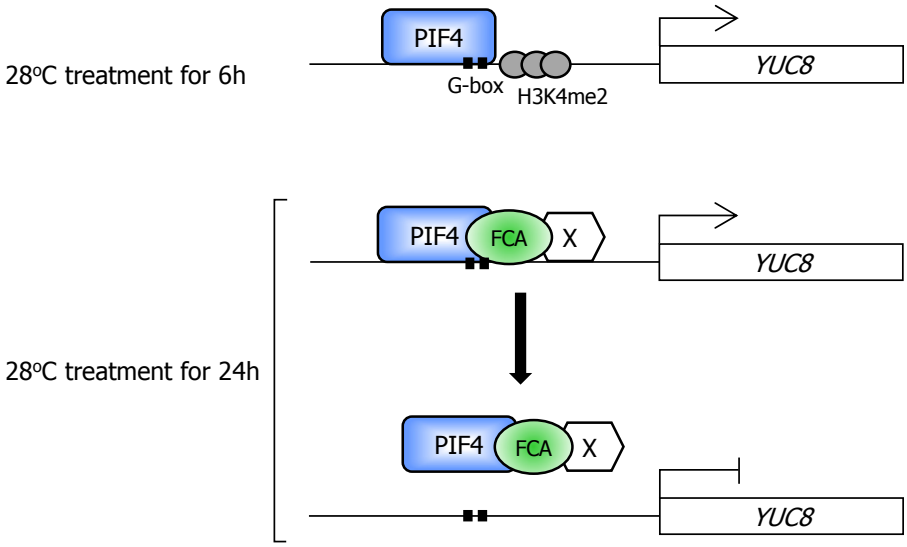

Figure 17. Schematic diagram for FCA function in transcriptional regulation of *YUC8* at high temperatures

High ambient temperature induces PIF4 binding to *YUC8* promoter (upper panel). H3K4me2 level is also elevated at high temperatures. Under long-period exposure to high temperatures, PIF4 recruits FCA to *YUC8* chromatin (lower panel). The chromatin-bound FCA induces histone demethylation possibly in concert with an as yet unidentified histone demethylase, designated factor X. The FCA-triggered chromatin modification induces the dissociation of PIF4, resulting in suppression of *YUC8* transcription.

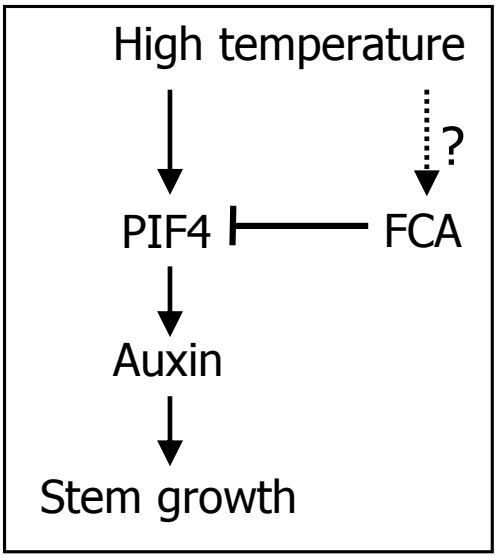

Figure 18. FCA attenuation of PIF4 action in thermal acceleration of stem growth

FCA regulates the binding of PIF4 to *YUC8* promoter, providing an adaptation strategy at high temperature.

DISCUSSION

FCA in attenuation mechanism of high ambient temperature responses

It is found that FCA, which is otherwise a well-characterized RNA-binding protein functioning in flowering time control (Liu *et al*., 2007; Liu *et al*., 2010), plays a role in modulation of *YUC8* chromatin through an interaction with PIF4 in a response to high temperatures. Based on these data, the proposed scheme is that the FCA-mediated counteracting mechanism is required to offset the accelerated stem elongation at high temperatures within the ambient temperature range, contributing to thermal adaptation of stem growth. The physiological role of FCA in this high temperature signaling is similar to that proposed for HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) E3 ubiquitin ligase. In cold acclimation signaling, HOS1 triggers protein degradation of INDUCER OF CBF EXPRESSION 1 (ICE1), which induces transcription of *C-REPEAT BINDING FACTOR 1* and *3* (Chinnusamy *et al*., 2003; Dong *et al*., 2006). The HOS1-mediated negative regulation of cold signaling is important for maintaining a physiological balance under prolonged cold temperature conditions, because over-induction of cold response causes developmental defects and growth retardation (Gilmour *et al*, 2000).

Histone demethylation mediated by FCA

It is notable that FLD, which acts together with FCA to modify *FLC* chromatin (Liu *et al*., 2007), is not functionally related with FCA in thermal regulation of hypocotyl growth. While these data strongly support the role of FCA in *YUC8* chromatin modification at high temperatures, the observed modification of *YUC8* chromatin in *fca*-9 background would simply reflect the thermal induction of *YUC8* in the mutant. More works are required to clarify this uncertainty concerning the molecular mechanism of FCA in the transcriptional control of *YUC8* and its functional relevance in thermal regulation of hypocotyl growth.

High temperature-mediated regulation of FCA

In this report, high temperature promotes protein interaction between FCA and PIF4. It is reported that transcription of *PIF4* and protein stability of this protein are increased under high ambient temperature conditions (Oh *et al*., 2012; Sun *et al*., 2012). However, nor the expression of *FCA* and its protein stability were unchanged at high temperature. Therefore, FCA would be regulated by protein modification such as phosphorylation. There are many reports that the level of temperature-activated proteins does not changed by changing temperatures. For example, HOS1 acts at cold temperature to promote protein degradation of ICE1, but the expression of *HOS1* and the protein stability of it do not show significant different under cold conditions (Jung *et al*., 2012).

Additional role of FCA at high temperature

Plants show various growth and developmental traits when they are exposed to high ambient temperatures. The hypocotyl length is elongated and flowering is activated (Kumar *et al*., 2012; Sun *et al*., 2012). Also, stomata development and circadian rhythms are affected by increased temperature (Amanda *et al*., 2012; Wigge, 2013). PIF4 is involved in high temperature-induced growth and flowering. Because PIF4 is also regulated by circadian rhythm (Nozue *et al*., 2007), it is possible that temperature regulation of circadian rhythm is related with PIF4. In this report, I demonstrated that FCA directly interacts with PIF4. Therefore, FCA may be involved in all the PIF4-mediated temperature responses. In the previous reports, it is shown that FCA is related with flowering delay at low ambient temperature (Jung *et al*., 2012). However, it is currently unknown whether FCA is involved in thermal activation of flowering at high temperature. Further works focused on the other functions which are related with PIF4 will be important for identifying the broader role of FCA in temperature responses.

FCA as a transcriptional coregulator

Since FCA has RRM domains, it is predicted that FCA plays a role in RNA regulation, such as regulation of RNA stability, 3' polyadenylation, and transcriptional termination (Bäurle *et al*., 2007; Somez *et al*., 2012). However, recent study revealed the novel function of FCA as a transcriptional coactivator (Lee *et al*., 2014). Under heat stress conditions,

FCA directly interacts with ABI5 to increase its activity of DNA binding (Lee *et al*., 2014). ABI5 then induces expression of *PER1* to reduce oxidative damage caused by heat stress (Lee *et al*., 2014). In this study, FCA interacts with another transcription factor, PIF4, to regulate its activity. Although FCA negatively regulates PIF4 functions, this is another example that supports the role of FCA as a transcriptional coregulator. Since protein interaction is important for regulating the activities of transcription factors, it would be interesting to screen which transcription factor interacts with FCA.

CHAPTER 2

Feedforward activation of systemic acquired resistance via NPR1 phosphorylation in *Arabidopsis*

INTRODUCTION

When bacterial pathogens invade plant tissues, an oxidative burst is triggered in infected plants, which is often called HR, to induce cell death in the infected area (Fu and Dong, 2013; Torres *et al*., 2005). HR-induced cell death inhibits the invasion of pathogens beyond the infection site (Spoel and Dong, 2012). HR triggers the biosynthesis of SA in both infected (local) and distal (systemic) tissues to activate SAR in order to prevent secondary infections (Alvarez, 2000; Fu and Dong, 2013; Leon *et al*., 1995). NPR1 is a master regulator of SAR. It acts as a coactivator of TGA transcription factors that regulate the expression of *PR* genes (Fu and Dong, 2013). SA regulates NPR1 protein stability (Spoel *et al*., 2009) and mediates the dynamic NPR1 oligomer-to-monomer reaction, which is a prerequisite for its nuclear import (Mou *et al*., 2003). While NPR1 activation requires SA during SAR, SA level is only slightly elevated in distal tissues (Nandi *et al*., 2004), entailing that additional signals would be necessary for NPR1 nuclear import and SAR induction.

It has been recently reported that SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8) phosphorylates the membrane-associated NAC transcription factor NAC WITH TRANSMEMBRANE MOTIF 1-LIKE 6 (NTL6) to induce its nuclear localization (Kim *et al*., 2012). NTL6 plays a role in cold-induced disease resistance (Seo *et al*., 2010), suggesting that SnRK2.8, and

perhaps other SnRK2 members as well, is associated with pathogen resistance. In this study, the regulatory mechanism of NPR1 by SnRK2.8-mediated phosphorylation is investigated. SnRK2.8 interacts with NPR1 and phosphorylates at threonine 373 to promote its nuclear import. The role of SnRK2.8 is important in distal tissue, while its role in local tissue is only marginal. Together with the fact that SA-independent signal induces *SnRK2.8* transcriptions, it is proposed that SnRK2.8 activates NPR1 in concert with SA to obtain pathogen resistance while maintaining low SA level at distal tissue. Since high level of SA causes toxic effects on plant growth and development, it seems that plants modulate SA biosynthesis at distal tissue to reduce its harmful effects. This study will provide explanation how plants induce immune response under low SA conditions.

RESULTS

SnRK2.8 mediates systemic acquired resistance

It was recently reported that SnRK2.8 phosphorylates the membrane-associated NAC transcription factor NTL6 to induce its nuclear localization (Kim *et al*., 2012). NTL6 plays a role in cold-induced disease resistance (Seo *et al*., 2010), suggesting that SnRK2.8, and possibly other SnRK2 members as well, is associated with pathogen resistance. It was found that *SnRK2.8* transcription was induced by more than 20-fold in plants that are infected with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Fig. 19A), supporting the notion that SnRK2.8 is involved in pathogen resistance. Several other *SnRK2* genes, such as *SnRK2.3*, *2.6*, *2.7*, and *2.9*, were also moderately induced after pathogen infection. In contrast, flg22, a pathogen-mimic peptide flagellin (Zipfel *et al*., 2004), did not significantly affect *SnRK2.8* transcription (Fig. 19B).

Next, It was examined whether *SnRK2.8* transcription was affected by systemic signals produced by the primary infection. *SnRK2.8* transcription was rapidly induced in systemic leaves within 6 h following local infection by *Pst* DC3000/*avrRpt2* cells (Mudgett *et al*., 1999) (Fig. 19C). In contrast, systemic expression of *PR1* was initiated 6 h after local infection. These sequential expression patterns suggest that SnRK2.8 plays a role in SAR

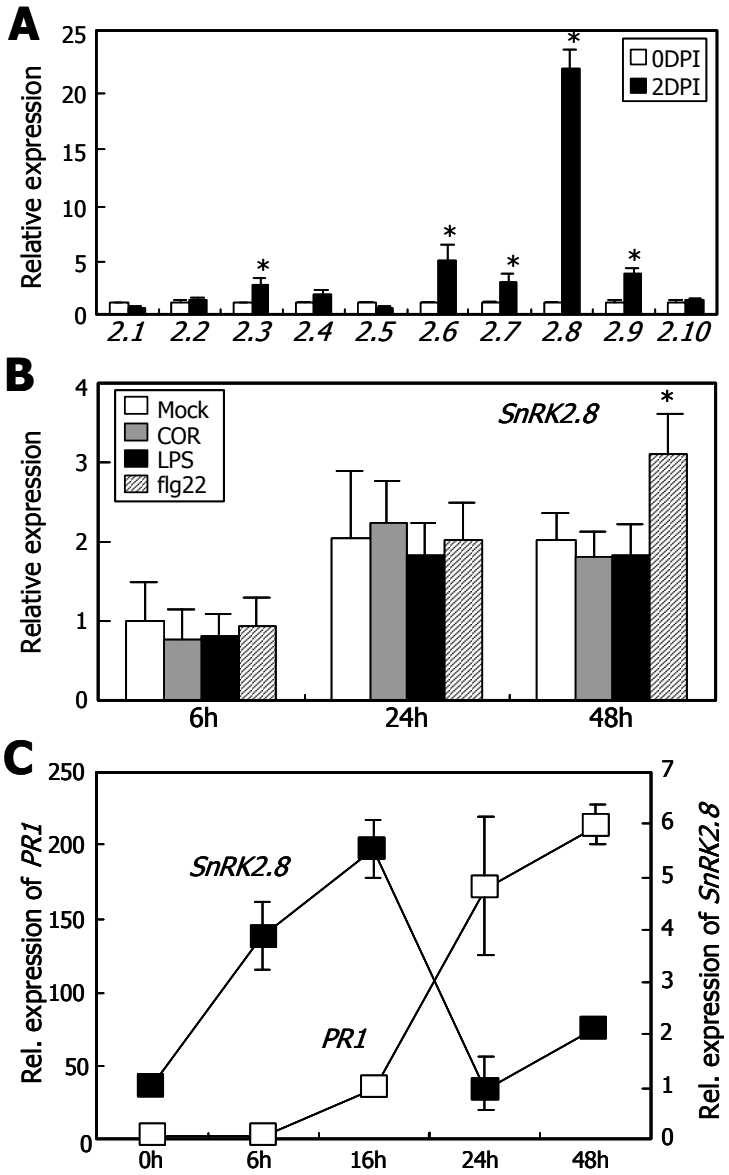

Figure 19. Pathogen infection induces *SnRK2.8* expression

Transcript levels were examined by RT-qPCR. Biological triplicates were averaged and statistically analyzed using Student *t*-test (*$P < 0.01$, **$P < 0.05$). Bars indicate SE.

(A) Effects of pathogen infection on *SnRK2* expression. Four-week-old plants grown under LDs were used. Plants were spray-inoculated with *Pst*

DC3000 cells and incubated for 2 d. Rosette leaves were assayed. DPI, days post-infection.

(B) Effects of flg22 on *SnRK2.8* transcription. Two-week-old plants grown on MS-agar plates under LD conditions were sprayed with 1 μM solution of flg22, which is a 22-amino acid sequence of the conserved N-terminal region of flagellin and known to induce plant defense response. Aerial plant part was harvested at the indicated time points after flg22 application for the extraction of total RNA. Transcript levels of *SnRK2.8* were examined by RT-qPCR.

(C) *SnRK2.8* and *PR1* expression kinetics in systemic leaves. Four-week-old plants were used for the analysis. The 4^{th} or 5^{th} leaves were pressure-infiltrated with *Pst* DC3000/*avrRpt2* cells, and two upper leaves were harvested at each time point.

by acting upstream of *PR1* regulation by TGA.

Consistent with *SnRK2.8* induction by pathogen infection, *SnRK2.8*-overexpressing plants (*35S:2.8*) exhibited improved resistance to *Pst* DC3000 infection, while the *SnRK2.8*-defective mutant (*snrk2.8-1*) exhibited reduced resistance compared to Col-0 plants (Figs. 20A and B). Accordingly, *PR1* transcription was markedly elevated in *35S:2.8* plants, although it was not distinctly altered in the *snrk2.8-1* mutant (Fig. 20C).

Next, the potential role of SnRK2.8 in SAR was investigated by counting bacterial cells in systemic leaves, in which SAR was induced by *Pst* DC3000/*avrRpt2* infection. Notably, *snrk2.8-1* and *snrk2.8-2* mutants did not exhibit improved resistance when compared to Col-0 plants (Figs. 21A and B). The reduced SAR phenotype of *snrk2.8*-defective mutants was similar to that of SA-deficient mutant *sid2* and *NPR1*-defective *npr1-2* mutant, supporting the notion that SnRK2.8 plays a role in SAR induction, as previously reported for SA and NPR1 (Delaney *et al*., 1994; Fu *et al*., 2013). Examination of *PR1* transcription in SAR-activated plants revealed that *PR1* induction was slightly influenced in local leaves but largely diminished in systemic leaves (Fig. 22). In contrast, the induction of *PR1* transcription was significantly reduced in both local and systemic leaves of the *npr1-2* mutant, as reported previously (Cao *et al*., 1997). These observations demonstrate that SnRK2.8 plays a role primarily in systemic tissues rather than in local tissues.

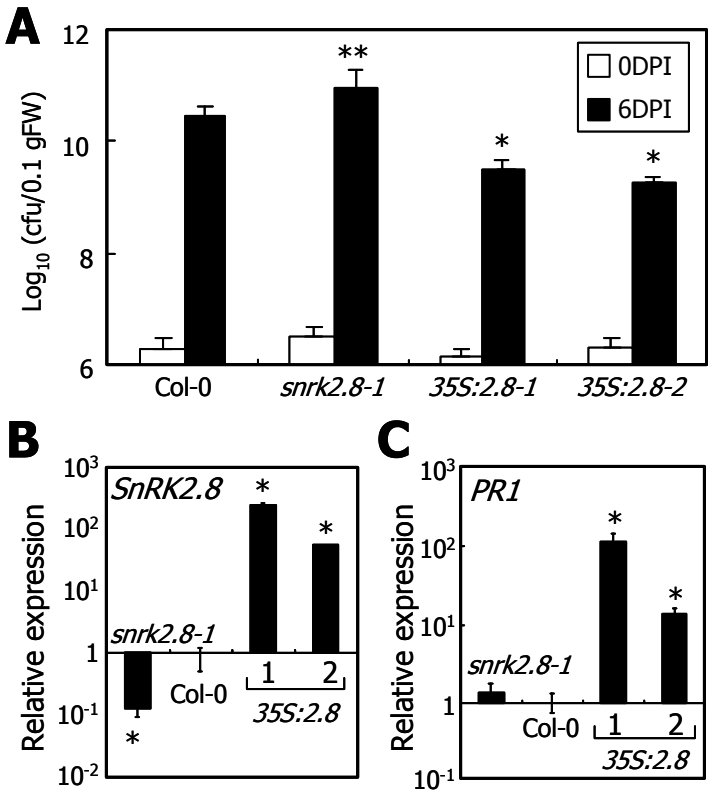

Figure 20. *SnRK2.8* overexpression confers pathogen resistance

Biological triplicates were averaged and statistically analyzed using Student *t*-test (*$P < 0.01$). Bars indicate SE.

(A) *Pst* DC3000 cell growth in *snrk2.8-1* mutant and *35S:2.8* plants. Plants were spray-inoculated with *Pst* DC3000 cells and incubated for 6 d. cfu, colony forming unit.

(B) Construction of *SnRK2.8*-overexpressing plants.

(C) *PR1* expression in *snrk2.8-1* mutant and *35S:2.8* plants. The 4th or 5th leaves were assayed.

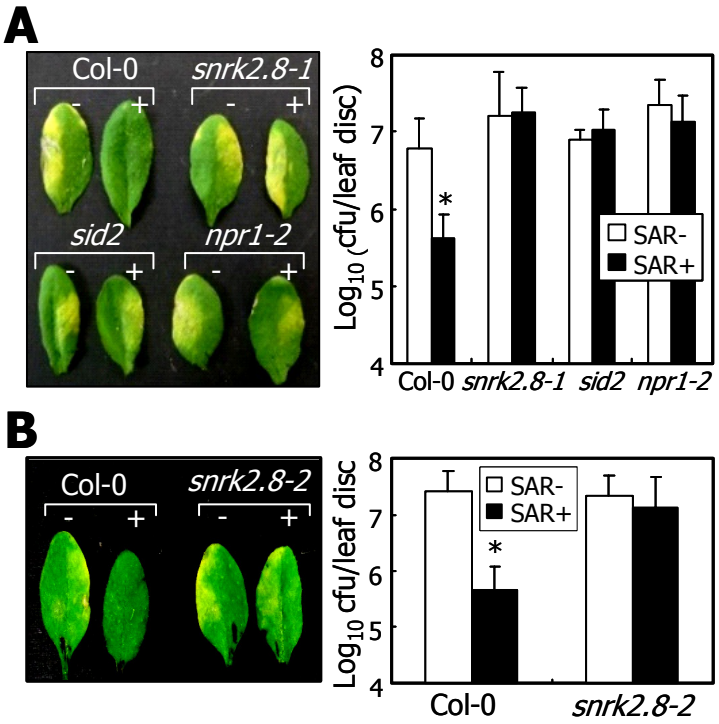

Figure 21. SAR is impaired in *snrk2.8* mutants

Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE. cfu, colony forming unit.

(A) SAR induction in *snrk2.8-1* mutant. Four-week-old plants grown in soil under LDs were used for pathogen infection. The 4th or 5th leaves were pressure-infiltrated with 10 mM $MgCl_2$ (-) or *Pst* DC3000/*avrRpt2* cells (+) and incubated for 2 d. Two upper leaves were infiltrated with *Pst* DC3000 cells and incubated for 3 d before taking photographs (left panel) and counting bacterial cells (right panel).

(B) Impaired SAR in *snrk2.8-2* mutant. Plants were treated as described in **(A)** before taking photograph (upper panel) and counting bacterial cells (lower panel).

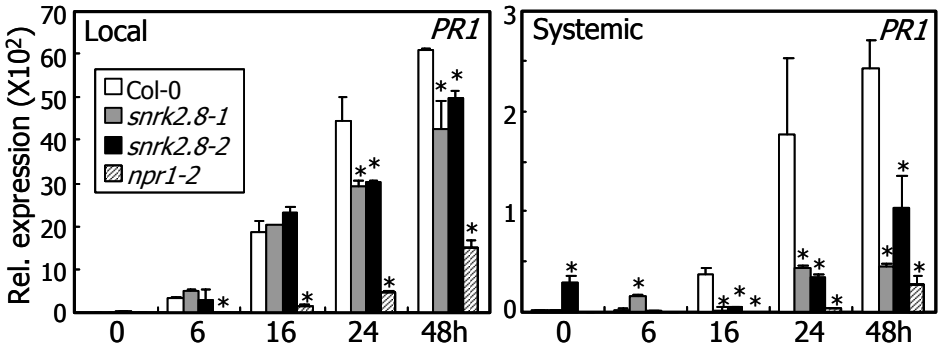

Figure 22. *PR1* expression in local and systemic leaves of *snrk2.8* mutants

Pathogen infection was conducted as described in Figure 19C. Infected leaf (local) and two upper leaves (systemic) were assayed. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

SnRK2.8 regulation of SAR requires SA

SA is essential for the induction of SAR (Delaney *et al*., 1994; Fu *et al*., 2013). Thus, it was investigated whether SnRK2.8 was related to SA. The effects of SA on *SnRK2.8* transcription were examined. It was found that *SnRK2.8* transcription was not affected by SA (Fig. 23A). In addition, the *sid2* mutation did not affect SAR-induced *SnRK2.8* expression in systemic leaves (Fig. 23B). Pathogen-mediated induction of *SID2* was also unaltered by the *snrk2.8* mutation (Figs. 24A and B). Meanwhile, whereas SA induction of *PR1* and *PR5* was not observed in the *npr1-2* mutant, it normally occurred in the *snrk2.8-1* mutant (Figs. 24C and D), indicating that SA-mediated induction of *PR*s requires NPR1 but does not depend on SnRK2.8. However, the high-level induction of *PR1* by *SnRK2.8* overexpression was not observed in *sid2* and *npr1-2* backgrounds (Figs. 25A and B). Together, these observations suggest that SnRK2.8 and SA signaling are independent but converge at the step of *PR* expression, possibly via NPR1.

SnRK2.8 phosphorylates NPR1

NPR1, as a coactivator, acts together with TGA transcription factors to regulate *PR* gene expression (Després *et al*., 2000; Després *et al*., 2003). Thus, it was examined whether SnRK2.8 interacts with upstream regulators. Yeast two-hybrid assay showed that SnRK2.8 interacted only with NPR1 among the upstream regulators tested (Fig. 26A). The SnRK2.8-NPR1

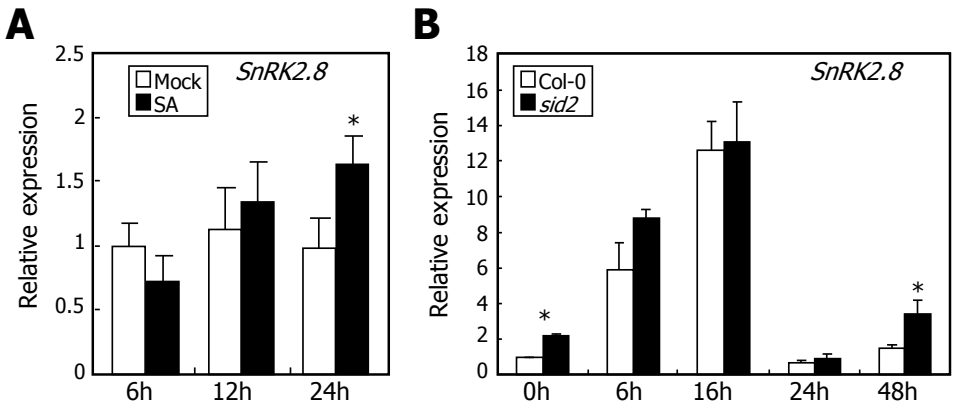

Figure 23. SA does not induce *SnRK2.8*

Transcript levels were examined by RT-qPCR. Biological triplicates were averaged and statistically analyzed (*t*-test, **P* < 0.01). Bars indicate SE.

(A) Effects of SA on *SnRK2.8* expression. Two-week-old plants grown on MS-agar plates were used for gene expression assays. Plants were treated with 0.5 mM SA for the indicated periods before harvesting whole plant materials. h, hour

(B) Kinetics of *SnRK2.8* expression in systemic leaves of *sid2* mutant. The 4th or 5th leaves of four-week-old plants grown in soil were pressure-infiltrated with *Pst* DC3000/*avrRpt2* cells, and two upper leaves were harvested at the indicated time points for total RNA extraction.

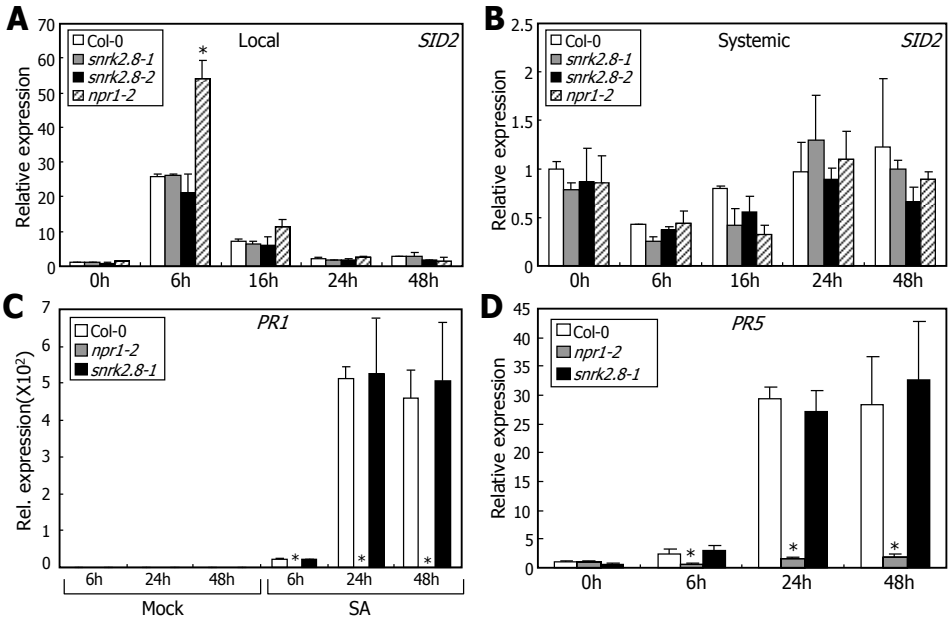

Figure 24. SA biosynthesis and SA-induced *PR* gene expressions do not require SnRK2.8

Transcript levels were examined by RT-qPCR. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(A, B) *SID2* expression in *snrk2.8* mutants after pathogen infection. Plants were treated as described in Figure 19C. Local leaves **(A)** and systemic leaves **(B)** were harvested for gene expression assay.

(C, D) *PR*s expression in *snrk2.8-1* mutant after SA treatment. SA-induced expression of *PR1* **(C)** and *PR5* **(D)** were analyzed. Plants were treated as described in Figure 23A.

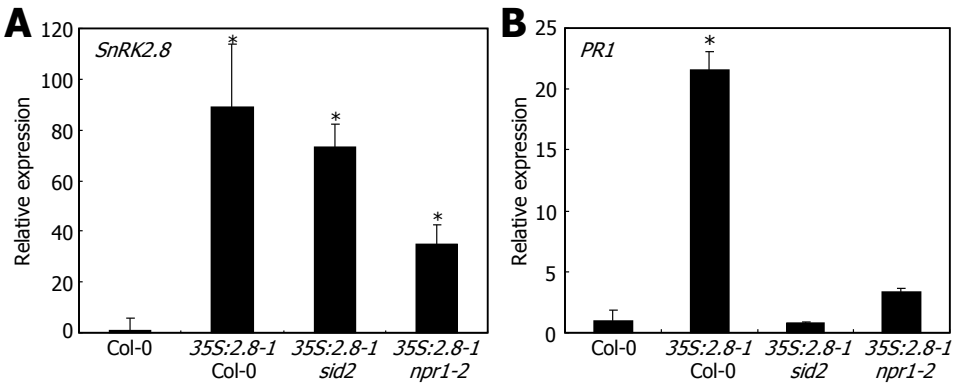

Figure 25. SnRK2.8-mediated *PR1* induction requires SA

Transcript levels were examined by RT-qPCR. Biological triplicates were averaged and statistically treated using Student *t*-test (**P*<0.01). Bars indicate SE.

(A) Construction of *SnRK2.8*-overexpressing Col-0, *sid2* and *npr1-2* plants. A full-size *SnRK2.8* cDNA was overexpressed driven by the CaMV 35S promoter in Col-0, *sid2*, and *npr1-2* plants. Total RNA was extracted from two-week-old whole plants grown on MS-agar plates.

(B) Effects of *SnRK2.8* overexpression on *PR1* expression in *sid2* and *npr1-2* mutants. The 4th or 5th leaves of four-week-old plants were harvested for gene expression assay.

interaction occurred in both the nucleus and the cytoplasm, as examined by BiFC assay (Fig. 26B). The *in planta* interaction of SnRK2.8 and NPR1 was verified by coimmunoprecipitation assay using tobacco cells transiently coexpressing FLAG-NPR1 and MYC-SnRK2.8 fusion proteins (Fig. 26C).

The SnRK2 family members are serine/threonine (S/T) protein kinases that mediate plant development and environmental stress responses (Kulik *et al*., 2011). Thus, it was investigated whether SnRK2.8 phosphorylates NPR1. Recombinant proteins were prepared in *Escherichia coli* cells, and *in vitro* kinase assays were performed. The results showed that SnRK2.8 phosphorylated NPR1 (Fig. 27A). It was also autophosphorylated. In contrast, SnRK2.6, which is a close homolog of SnRK2.8 (Boudsocq *et al*., 2004), was autophosphorylated but did not phosphorylate NPR1 (Fig. 27B). Basophilic kinases, such as SnRK2 members, phosphorylate preferentially S and T residues in the sequence context of K/RXXS/T, in which X represents any amino acid (Kim *et al*., 2012). The potential S/T residues were replaced with alanine (A) in NPR1, resulting in M1–M5 proteins (Fig. 28A). Phosphorylation assays *in vitro* showed that SnRK2.8-mediated phosphorylation was reduced by approximately 70% in M4 (Fig. 28B), indicating that T373 is the primary target residue of SnRK2.8. Sequence analysis revealed that T373 is absolutely conserved in NPR1 proteins from different plant species (Fig. 28C). Meanwhile, SnRK2.8 did not phosphorylate NPR1 S11/15 (Fig. 28D), which are known to be phosphorylated in SA-treated plants (Spoel *et al*., 2009).

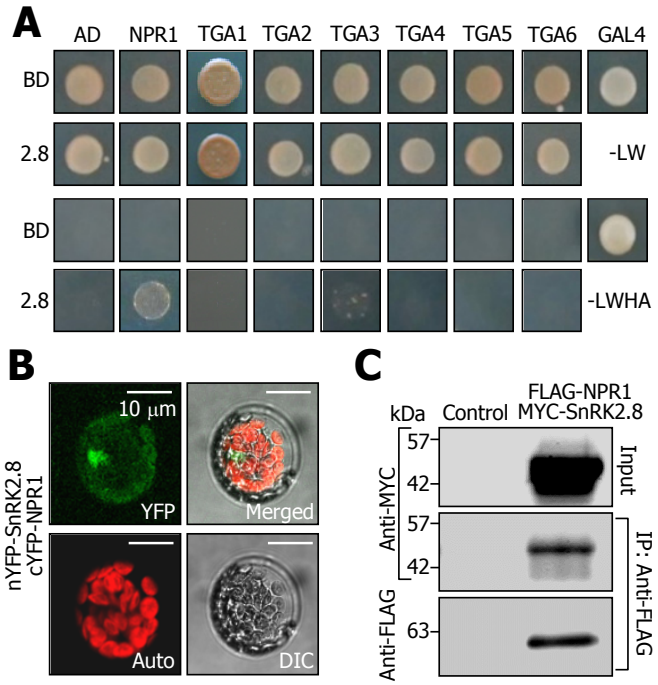

Figure 26. SnRK2.8 interacts with NPR1

(A) Interaction of SnRK2.8 with NPR1 in yeast cells. Yeast cell growth on selective media lacking Leu, Trp, His, and Ade (-LWHA) represents positive interaction.

(B) BiFC. Partial YFP constructs were fused to SnRK2.8 or NPR1 and transiently coexpressed in *Arabidopsis* protoplasts. YFP signals were visualized by differential interference contrast (DIC) and fluorescence microscopy.

(C) Coimmunoprecipitation. MYC-SnRK2.8 and FLAG-NPR1 fusion constructs were transiently coexpressed in tobacco leaves. The input represents 10% of the protein extract. Control, protein extract without transient expression; kDa, kilodalton.

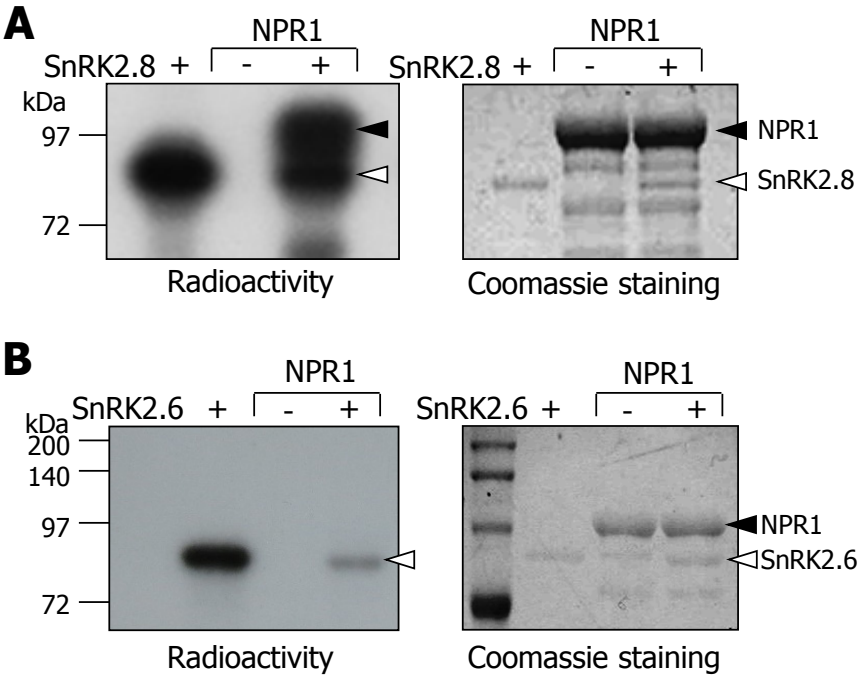

Figure 27. SnRK2.8 phosphorylates NPR1

(A) *in vitro* kinase assay of SnRK2.8. Recombinant SnRK2.8 and NPR1 proteins were prepared as MBP fusions in *E. coli* cells. One half μg of MBP-SnRK2.8 and 5 μg of MBP-NPR1 were used. Black arrowheads indicate MBP-NPR1, and white arrowheads indicate MBP-SnRK2.8.

(B) SnRK2.6 does not phosphorylate NPR1. Recombinant SnRK2.8 and NPR1 were prepared and reaction was performed as described in **(A)**. Black arrowheads indicate MBP-NPR1, and white arrowheads indicate MBP-SnRK2.6.

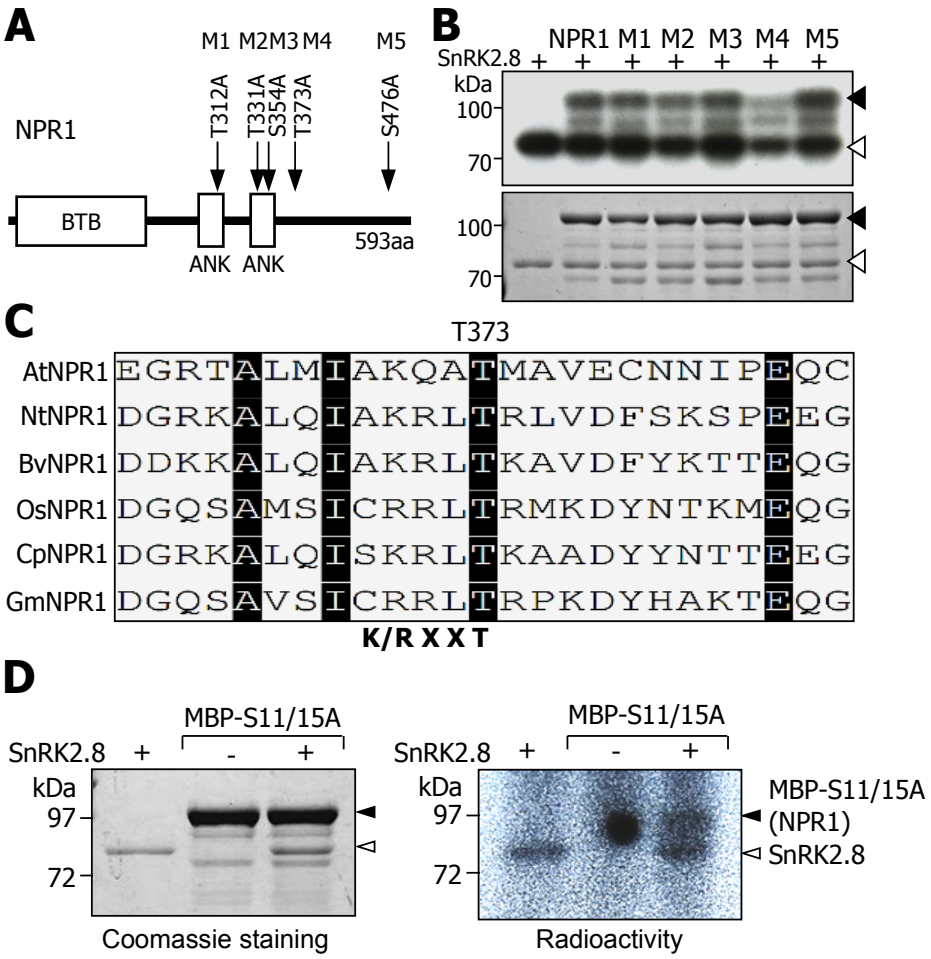

Figure 28. SnRK2.8 phosphorylates T373 of NPR1

(A) Protein structure of NPR1. M1 to M5 indicate mutations of putative SnRK2.8 target sites. BTB, BTB/POZ core motif. ANK, ankyrin-repeat motif.

(B) *in vitro* kinase assay on mutated NPR1 proteins. Preparation of NPR1 proteins and *in vitro* kinase assays were performed as described in Figure 27. Black and white arrowheads indicate NPR1 and SnRK2.8, respectively.

(C) Sequence alignment of NPR1 proteins around T373. A SnRK2-mediated phosphorylation motif (K/RXXS/T) is marked. At, *Arabidopsis thaliana*; Nt,

Nicotiana tabacum; Bv, *Beta vulgaris*; Os, *Oryza sativa*; Cp, *Carica papaya*; Gm, *Glycine max*.

(D) SnRK2.8 does not phosphorylate S11 and S15 of NPR1. Both S11 and S15 were mutated to alanine, and recombinant S11/15A protein was produced and *in vitro* phosphorylation was performed as described in Figure 27. Black arrowheads indicate MBP-S11/15A, and white arrowheads indicate MBP-SnRK2.8. kDa, kilodalton.

SnRK2.8 promotes NPR1 nuclear import

SA triggers NPR1 monomerization, which is required for its nuclear localization (Mou *et al*., 2003). It has been reported that low SA concentrations still induce the oligomer-to-monomer reaction of NPR1 but are insufficient for its nuclear import (Kinkema *et al*., 2000; Mou *et al*., 2003). NPR1 is efficiently imported into the nucleus in systemic tissues, where SA level is only slightly elevated after pathogen infection (Nandi *et al*., 2004). Thus, it was assessed whether SnRK2.8 is required for the NPR1 nuclear import during SAR. After primary infection with *Pst* DC3000/*avrRpt2* cells, nuclear and cytoplasmic fractions of systemic leaves were isolated and relative distribution of NPR1 was examined in each fraction. In mock-treated Col-0 plants, 47% of NPR1 was localized in the nucleus (Fig. 29A). After SAR induction, the portion of nuclear NPR1 increased up to 66%. In contrast, 16% of NPR1 was localized in the nucleus of mock-treated *snrk2.8-1* mutant and the portion of nuclear NPR1 was not significantly affected by SAR induction, indicating that SnRK2.8 mediates NPR1 nuclear localization during SAR.

It was also examined whether SnRK2.8 affected the NPR1 oligomer-to-monomer reaction using the *snrk2.8-1* mutant that overexpresses MYC-NPR1 fusion protein driven by the CaMV 35S promoter. The level of monomers was equally elevated in both Col-0 and *snrk2.8-1* backgrounds (Figs. 29B and C), indicating that SnRK2.8 does not affect the NPR1 monomerization in pathogen-infected plants.

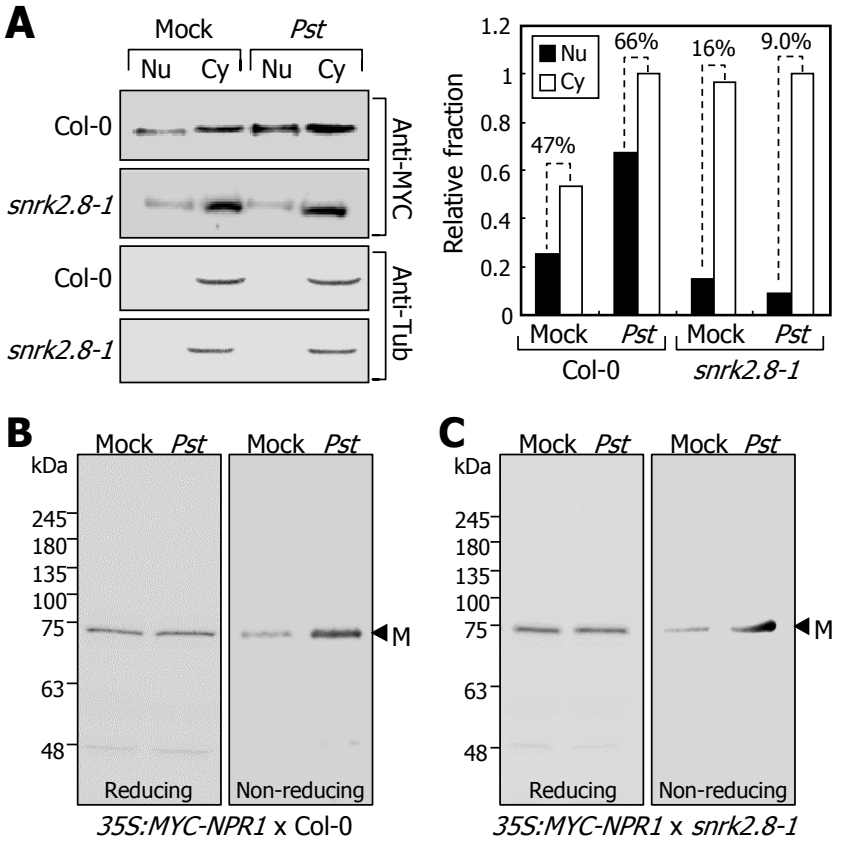

Figure 29. SnRK2.8 promotes NPR1 nuclear import

(A) Effects of pathogen infection on nucleo-cytoplasmic localization of NPR1. A *MYC-NPR1* fusion construct was overexpressed driven by the CaMV 35S promoter in Col-0 or *snrk2.8-1* background. The 4th or 5th leaves of four-week-old plants were pressure-infiltrated with *Pst* DC3000/*avrRpt2* cells, and two upper leaves were harvested after 24 h before cell fractionation. Nu, nuclear fraction. Cy, cytoplasmic fraction. The NPR1 proteins were immunologically detected using an anti-MYC antibody (left panel). Blots were quantitated using the ImageJ software (http://rsbweb.nih.gov/ij/) (right panel). Percentage indicates the ratio of

nuclear to cytoplasmic distribution.

(B, C) SnRK2.8 does not affect the pathogen-induced monomerization of NPR1. The MYC-NPR1 fusion was overexpressed driven by the CaMV 35S promoter in either Col-0 plants **(B)** or *snrk2.8-1* mutant **(C)**. Four-week-old plants grown in soil under LDs were used for pathogen infection and immunological assay. The 4th or 5th leaves were pressure-infiltrated with *Pst* DC3000/*avrRpt2* cells and incubated for 3 days. Two upper leaves were harvested for the preparation of total protein extract. Protein extracts were resuspended in 2 X SDS loading buffer with or without β-mercaptoethanol, which was annotated as either reducing (left panel) or non-reducing (right panel), respectively. MYC-NPR1 proteins were immunologically detected using an anti-MYC antibody.

Impaired NPR1 nuclear import in the *snrk2.8-1* mutant was further investigated using systemic leaves of *35S:NPR1-GFP* transgenic plants in the *snrk2.8-1* background. After SAR induction, green fluorescence protein (GFP) signal was detected predominantly in the nucleus of Col-0 background (Fig. 30A). In contrast, GFP signal was relatively weaker in the nucleus, but stronger in the cytoplasm of *snrk2-8-1* background compared to those detected in Col-0 background. In contrast, NPR1 nuclear localization patterns in local tissues were similar in both Col-0 and *snrk2.8* backgrounds (Fig. 30B), supporting the role of SnRK2.8 in NPR1 nuclear localization in systemic leaves.

It was finally examined whether SnRK2.8-mediated NPR1 nuclear localization was associated with SA. In mock-treated Col-0 plants, 6.4% of NPR1 was localized in the nucleus, and the nuclear portion of NPR1 significantly increased up to 39% after SA treatment (Fig. 31). Notably, a similar nucleo-cytoplasmic distribution pattern was observed in the *snrk2.8-1* mutant, indicating that SA is not directly linked with SnRK2.8-mediated NPR1 nuclear localization.

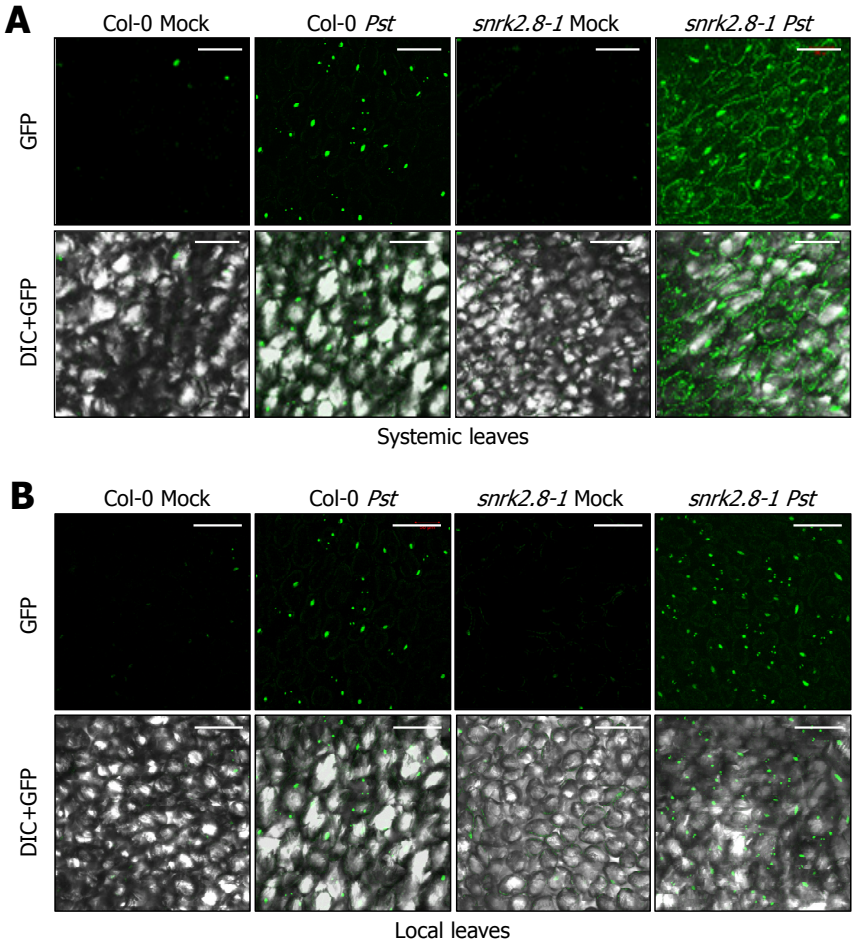

Figure 30. Nucleo-cytoplasmic localization of NPR1 in *snrk2.8-1* mutant

(A) Subcellular distribution of NPR1 in systemic leaves of pathogen-infected *snrk2.8-1* mutant. The *NPR1-GFP* fusion construct was overexpressed driven by the CaMV 35S promoter in Col-0 and *snrk2.8-1* plants. Plants were treated as described in Figure 29A. DIC, differential interference contrast microscopy. Scale bars, 10 μm.

(B) Subcellular distribution of NPR1 in local leaves of pathogen-infected *snrk2.8-1* mutant. Plants were treated as described in **(A)**. Scale bars, 10 μm.

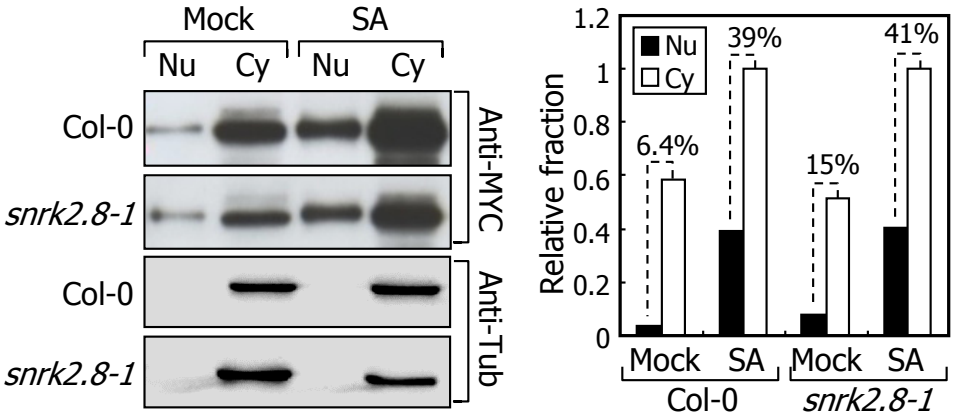

Figure 31. Nucleo-cytoplasmic localization of NPR1 in SA-treated plant cells

Two-week-old plants grown on MS-agar plates were treated with 0.5 mM SA for 24 h. Whole plants were used for cell fractionation, and NPR1 detection was performed as described in Figure 29A.

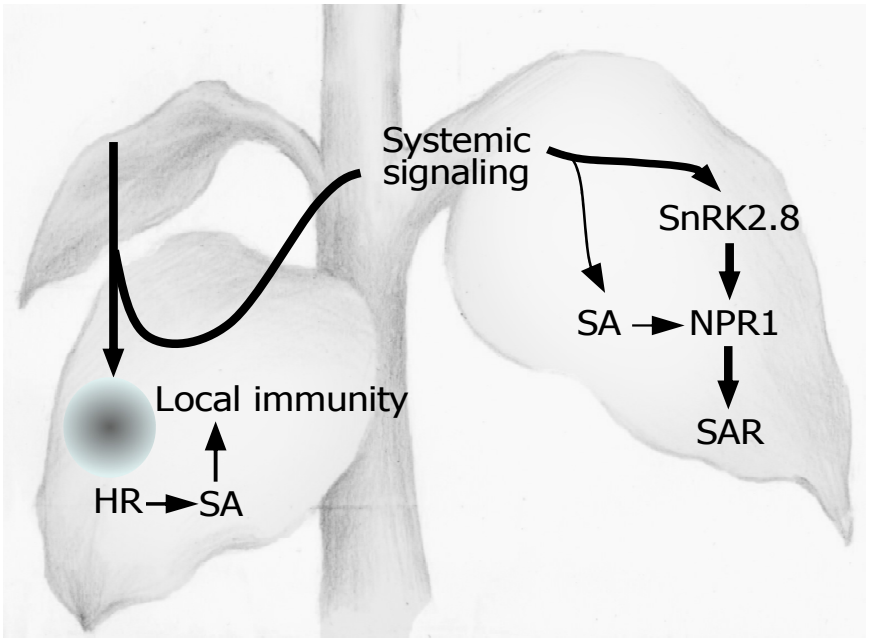

Figure 32. SnRK2.8-mediated feedforward induction of SAR

SA signal is integrated with the SnRK2.8 signal to activate NPR1, resulting in the induction of SAR.

DISCUSSION

Regulation of NPR1 function in systemic leaves

After the NPR1 protein was found, most of the studies have been focused on the control mechanisms of NPR1 by SA, since SA-induced SAR is largely dependent on NPR1 (Cao *et al*., 1997; Fu *et al*., 2013). SA triggers NPR1 monomerization by changing its redox status (Mou *et al*., 2003). It also regulates protein turnover of NPR1 in the nucleus by binding to NPR3 and NPR4 (Fu *et al*., 2012). However, in these studies, high concentrations of SA were used for the analysis of NPR1 function. In plants, pathogen infection triggers accumulation of a large amount of SA in local leaves, while only slight induction of it occurs in systemic leaves (Nandi *et al*., 2004). These slight increases of SA could trigger monomerization and protein turnover of NPR1 (Mou *et al*., 2003; Spoel *et al*., 2009), but whether NPR1 was imported to the nucleus under low SA conditions was still unclear (Kinkema *et al*., 2000). In this work, it was found that SnRK2.8, which plays a major role in systemic leaves of pathogen-infected plants triggers nuclear import of monomerized NPR1. These results answer the question on how NPR1 is translocated from the cytoplasm to the nucleus under low SA conditions. The regulation of subcellular localization by SnRK2.8 is necessary for SAR, because *snrk2.8* mutants did not show SAR (Fig. 21). These results indicate that NPR1 is regulated at various steps

including oligomer-monomer transition, subcellular localization, and protein stability, for tight regulation of SAR in plants.

Convergence of SnRK2.8- and SA-mediated immune signals into NPR1

While SA is essential for SAR development, it should be maintained at low levels in parts of the plant other than the site of HR to avoid toxic effects, such as growth retardation and physiological disturbance (Rivas-San Vicente *et al*., 2011; Scott *et al*., 2004). Therefore, it is likely that SA serves as an initiating signal that links local infection with the induction of SAR in systemic tissues and, therefore, high levels of SA are not required. Once NPR1 is potentiated by SA through the oligomer-to-monomer transition, the SnRK2.8-mediated feedforward pathway activates NPR1 through protein phosphorylation, leading to the induction of downstream *PR* genes. It is proposed that the integration of SA signaling and the SnRK2.8-mediated activation system at NPR1 ensures that SAR is developed, while maintaining normal plant growth in systemic tissues (Fig. 32), providing an adaptation strategy under biotic stress conditions. These findings will contribute to the identification of long-distance signaling molecules and schemes during SAR development in the future.

Upstream signals of SnRK2.8

Because SA did not induce expression of *SnRK2.8* after pathogen infection, it is predicted that mobile systemic signals are involved in the induction of

this gene. Several chemical compounds have been suggested as mobile signals. The DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) was found by mutagenesis for identifying inducers of SAR (Maldonado *et al*., 2002). The *dir1* mutant shows normal resistance in pathogen-treated local tissues, but SAR and pathogen-induced gene expressions are abolished in distal tissues, suggesting that DIR1 acts in distal tissues to activate SAR (Maldonado *et al*., 2002). Petiole exudates assays suggest that DIR1 proteins are transferred possibly via vascular tissues (Maldonado *et al*., 2002). These results propose that DIR1 would be a mobile signal which induce SAR. In the other studies, it is found that azelaic acid and glycel-3-phosphate are also putative candidates for mobile immune signals (Fu and Dong, 2013). Examining *SnRK2.8* expression in the mutants which are deficient of these putative mobile signals will provide clues to identify which signal is responsible for *SnRK2.8* induction.

Crosstalk between plant growth and immune responses

SnRK2 family proteins were suggested to act in stress responses via ABA signaling. However, because ABA only activates 5 members of SnRK2 family, additional role of SnRK2 proteins were predicted (Boudsocq *et al*., 2004). Among them, SnRK2.8 is studied to be involved in plant growth (Shin *et al*., 2007). Overexpression of SnRK2.8 enhances overall plant growth, suggesting its role as a growth promoter. The proteome analysis using two-dimensional electrophoresis assays found that SnRK2.8

phosphorylates proteins involved in metabolic processes such as glycolysis and carbon fixation. For example, SnRK2.8 phosphorylates glyoxalase 1, which detoxify byproducts produced by glycolysis (Shin *et al*., 2007).

In this report, the expression *SnRK2.8* is highly induced in distal tissues after pathogen infection in local tissues. Because SA causes harmful effects to plant growth, it might be compensatory strategies that plants use SnRK2.8, a growth promoter, as a component of immune responses.

Post-translational regulation of NPR1

After the identification of NPR1, many studies have been focused on the post-translational regulation, rather than transcriptional or translational regulation. For example, SA induces phosphorylation at S11/15 and the phosphorylated NPR1 is targeted for protein degradation (Spoel *et al*., 2009). In this report, NPR1 was phosphorylated by SnRK2.8 at T373 for its nuclear translocation, which plays an important role in distal tissues. Since nuclear localization of NPR1 in local tissues was not affected by *snrk2.8* mutation, it seems that there would be other protein modifications that promote nuclear import of NPR1. In the previous reports, NPR1 is highly phosphorylated by SA treatment (Spoel *et al*., 2009). Although the phosphorylation of S11/15 is demonstrated that it is important for regulating protein stability of NPR1 in the nucleus, it is still unclear whether the phosphorylation of NPR1 occurs at the other sites. Furthermore, it is reported that cytoplasmic NPR1 has a role in jasmonic acid (JA) signaling (Stein *et al*., 2008). The post-

translational modifications of NPR1 might be a switch that changes the function of NPR1 from SA signaling mediator to JA signaling mediator. It will be interesting to find which site of NPR1 is phosphorylated and identify its function in SA-induced nuclear localization or JA signaling.

REFERENCES

Alvarez, M. E. (2000). Salicylic acid in the machinery of hypersensitive cell death and disease resistance. *Plant molecular biology*, *44*(3), 429-442.

Bai, M. Y., Shang, J. X., Oh, E., Fan, M., Bai, Y., Zentella, R., Sun, T., & Wang, Z. Y. (2012). Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in *Arabidopsis*. *Nature Cell Biology*, *14*(8), 810-817.

Bäurle, I., Smith, L., Baulcombe, D. C., & Dean, C. (2007). Widespread role for the flowering-time regulators FCA and FPA in RNA-mediated chromatin silencing. *Science*, *318*(5847), 109-112.

Blázquez, M. A., Ahn, J. H., & Weigel, D. (2003). A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. *Nature Genetics*, *33*(2), 168-171.

Boudsocq, M., Barbier-Brygoo, H., & Laurière, C. (2004). Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. *Journal of Biological Chemistry*, *279*(40), 41758-41766.

Cahill Jr, J. F., & McNickle, G. G. (2011). The behavioral ecology of nutrient foraging by plants. *Annual Review of Ecology, Evolution, and Systematics*, *42*, 289-311.

Cameron, R. K., Paiva, N. L., Lamb, C. J., & Dixon, R. A. (1999). Accumulation of salicylic acid and *PR-1* gene transcripts in relation to

the systemic acquired resistance response induced by *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. *Physiological and Molecular Plant Pathology*, *55*(2), 121-130.

Cao, H., Glazebrook, J., Clarke, J. D., Volko, S., & Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell*, *88*(1), 57-63.

Chen, M., & Chory, J. (2011). Phytochrome signaling mechanisms and the control of plant development. *Trends in cell biology*, *21*(11), 664-671.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B. H., Hong, X., Agarwal, M., & Zhu, J. K. (2003). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes & Development*, *17*(8), 1043-1054.

Chinnusamy, V., Zhu, J., & Zhu, J. K. (2007). Cold stress regulation of gene expression in plants. *Trends in Plant Science*, *12*(10), 444-451.

Choe, S. Dilkes, B. P., Fujioka, S., Takatsuto S., Sakurai, A., & Feldmann, K. A. (1998) The *DWF4* gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22 alpha-hydroxylation steps in brassinosteroid biosynthesis. *The Plant Cell*, *10*(2) 231-243.

Choi, K., Kim, J., Hwang, H. J., Kim, S., Park, C., Kim, S. Y., & Lee, I. (2011). The FRIGIDA complex activates transcription of *FLC*, a strong flowering repressor in *Arabidopsis*, by recruiting chromatin modification factors. *The Plant Cell*, *23*(1), 289-303.

Crawford, A. J., McLachlan, D. H., Hetherington, A. M., & Franklin, K. A.

(2012). High temperature exposure increases plant cooling capacity. *Current Biology*, *22*(10), R396-R397.

Dat, J. F., Lopez-Delgado, H., Foyer, C. H., & Scott, I. M. (2000). Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. *Journal of Plant Physiology*, *156*(5), 659-665.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., & Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. *Science*, *266*(5188), 1247-1250.

Dempsey, D., & Klessig, D. F. (2012). SOS–too many signals for systemic acquired resistance? *Trends in Plant Science*, *17*(9), 538-545.

Després, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., & Fobert, P. R. (2003). The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. *The Plant Cell*, *15*(9), 2181-2191.

Després, C., DeLong, C., Glaze, S., Liu, E., & Fobert, P. R. (2000). The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. *The Plant Cell*, *12*(2), 279-290.

Dharmasiri, N., Dharmasiri, S., & Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. *Nature*, *435*(7041), 441-445.

Dong, C. H., Agarwal, M., Zhang, Y., Xie, Q., & Zhu, J. K. (2006). The

negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proceedings of the National Academy of Sciences*, *103*(21), 8281-8286.

Fernández-Marcos, M., Sanz, L., Lewis, D. R., Muday, G. K., & Lorenzo, O. (2011). Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. *Proceedings of the National Academy of Sciences*, *108*(45), 18506-18511.

Franklin, K. A., Lee, S. H., Patel, D., Kumar, S. V., Spartz, A. K., Gu, C., Ye, S., Yu, P., Breen, G., Cohen, J. D., Wigge, P. A. & Gray, W. M. (2011). Phytochrome-interacting factor 4 regulates auxin biosynthesis at high temperature. *Proceedings of the National Academy of Sciences*, *108*(50), 20231-20235.

Fu, Z. Q., & Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. *Annual Review of Plant Biology*, *64*, 839-863.

Fu, Z. Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S. H., Tada, Y., Zheng, N., & Dong, X. (2012). NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature*, *486*(7402), 228-232.

Gilmour, S. J., Sebolt, A. M., Salazar, M. P., Everard, J. D., & Thomashow, M. F. (2000). Overexpression of the *Arabidopsis* CBF3 transcriptional activator mimics multiple biochemical changes associated with cold

acclimation. *Plant Physiology*, *124*(4), 1854-1865.

Gray, W. M., Östin, A., Sandberg, G., Romano, C. P., & Estelle, M. (1998). High temperature promotes auxin-mediated hypocotyl elongation in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *95*(12), 7197-7202.

Gururani, M. A., Venkatesh, J., Upadhyaya, C. P., Nookaraju, A., Pandey, S. K., & Park, S. W. (2012). Plant disease resistance genes: Current status and future directions. *Physiological and Molecular Plant Pathology*, *78*, 51-65.

Hedden, P., & Phillips, A. L. (2000). Gibberellin metabolism: new insights revealed by the genes. *Trends in Plant Science*, *5*(12), 523-530.

Huq, E., & Quail, P. H. (2002). PIF4, a phytochrome-interacting bHLH factor, functions as a negative regulator of phytochrome B signaling in *Arabidopsis*. *The EMBO journal*, *21*(10), 2441-2450.

Johansson, M., & Staiger, D. (2014). Time to flower: interplay between photoperiod and the circadian clock. *Journal of Experimental Botany*, eru441.

Johnson, C., Boden, E., & Arias, J. (2003). Salicylic acid and NPR1 induce the recruitment of trans-activating TGA factors to a defense gene promoter in *Arabidopsis*. *The Plant Cell*, *15*(8), 1846-1858.

Jung, J. H., Seo, P. J., Ahn, J. H., & Park, C. M. (2012). *Arabidopsis* RNA-binding protein FCA regulates microRNA172 processing in thermosensory flowering. *Journal of Biological Chemistry*, *287*(19),

16007-16016.

Kim, M. J., Park, M. J., Seo, P. J., Seo, J. S., Kim, H. J., & Park, C. M. (2012). Controlled nuclear import of the transcription factor NTL6 reveals a cytoplasmic role of SnRK2.8 in the drought-stress response. *Biochemical Journal*, *448*(3), 353-363.

Kinkema, M., Fan, W., & Dong, X. (2000). Nuclear localization of NPR1 is required for activation of *PR* gene expression. *The Plant Cell*, *12*(12), 2339-2350.

Koini, M. A., Alvey, L., Allen, T., Tilley, C. A., Harberd, N. P., Whitelam, G. C., & Franklin, K. A. (2009). High temperature-mediated adaptations in plant architecture require the bHLH transcription factor PIF4. *Current Biology*, *19*(5), 408-413.

Kulik, A., Wawer, I., Krzywińska, E., Bucholc, M., & Dobrowolska, G. (2011). SnRK2 protein kinases-key regulators of plant response to abiotic stresses. *Omics: A Journal of Integrative Biology*, *15*(12), 859-872.

Kumar, S. V., Lucyshyn, D., Jaeger, K. E., Alós, E., Alvey, E., Harberd, N. P., & Wigge, P. A. (2012). Transcription factor PIF4 controls the thermosensory activation of flowering. *Nature*, *484*(7393), 242-245.

Kumar, S. V., & Wigge, P. A. (2010). H2A. Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis*. *Cell*, *140*(1), 136-147.

Lee, J. H., Yoo, S. J., Park, S. H., Hwang, I., Lee, J. S., & Ahn, J. H. (2007).

Role of SVP in the control of flowering time by ambient temperature in *Arabidopsis*. *Genes & Development*, *21*(4), 397-402.

Lee, S., Lee, H. J., Jung, J. H., & Park, C. M. (2014). The *Arabidopsis* thaliana RNA-binding protein FCA regulates thermotolerance by modulating the detoxification of reactive oxygen species. *New Phytologist*, doi: 10.1111/nph.13079.

Lee, S., Seo, P. J., Lee, H. J., & Park, C. M. (2012). A NAC transcription factor NTL4 promotes reactive oxygen species production during drought - induced leaf senescence in *Arabidopsis*. *The Plant Journal*, *70*(5), 831-844.

Leon, J., Lawton, M. A., & Raskin, I. (1995). Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. *Plant Physiology*, *108*(4), 1673-1678.

Li, L., & Deng, X. W. (2005). It runs in the family: regulation of brassinosteroid signaling by the BZR1–BES1 class of transcription factors. *Trends in plant science*, *10*(6), 266-268.

Lim, M. H., Kim, J., Kim, Y. S., Chung, K. S., Seo, Y. H., Lee, I., Kim, J., Hong, C. B., Kim, H. J., & Park, C. M. (2004). A new *Arabidopsis* gene, *FLK*, encodes an RNA binding protein with K homology motifs and regulates flowering time via *FLOWERING LOCUS C*. *The Plant Cell*, *16*(3), 731-740.

Liu, F., Marquardt, S., Lister, C., Swiezewski, S., & Dean, C. (2010).

Targeted 3′ processing of antisense transcripts triggers *Arabidopsis FLC* chromatin silencing. *Science*, *327*(5961), 94-97.

Liu, F., Quesada, V., Crevillén, P., Bäurle, I., Swiezewski, S., & Dean, C. (2007). The *Arabidopsis* RNA-binding protein FCA requires a lysine-specific demethylase 1 homolog to downregulate *FLC*. *Molecular Cell*, *28*(3), 398-407.

Lucas, M., Davière, J. M., Rodríguez-Falcón, M., Pontin, M., Iglesias-Pedraz, J. M., Lorrain, S., Fankhauser, C., Blázquez, M. A., Titarenko, E., & Prat, S. (2008). A molecular framework for light and gibberellin control of cell elongation. *Nature*, *451*(7177), 480-484.

Maldonado, A. M., Doerner, P., Dixon, R. A., Lamb, C. J., & Cameron, R. K. (2002). A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature*, *419*(6905), 399-403.

Manzano, D., Marquardt, S., Jones, A.M., Bäurle, I., Liu, F., & Dean, C. (2009). Altered interactions within FY/AtCPSF complexes required for *Arabidopsis* FCA-mediated chromatin silencing. *Proceedings of the National Academy of Sciences, USA 106*(21), 8772-8777.

Mooney, K. A., Halitschke, R., Kessler, A., & Agrawal, A. A. (2010). Evolutionary trade-offs in plants mediate the strength of trophic cascades. *Science*, *327*(5973), 1642-1644.

Mou, Z., Fan, W., & Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell*, *113*(7), 935-944.

Muday, G. K., Rahman, A., & Binder, B. M. (2012). Auxin and ethylene: collaborators or competitors? *Trends in Plant Science*, *17*(4), 181-195.

Mudgett, M. B., & Staskawicz, B. J. (1999). Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. *Molecular Microbiology*, *32*(5), 927-941.

Nakasone, A., Fujiwara, M., Fukao, Y., Biswas, K. K., Rahman, A., Kawai-Yamada, M., Narumi, I., Uchimiya, H., & Oono, Y. (2012). SMALL ACIDIC PROTEIN1 acts with RUB modification components, the COP9 signalosome, and AXR1 to regulate growth and development of *Arabidopsis*. *Plant physiology*, *160*(1), 93-105.

Nandi, A., Welti, R., & Shah, J. (2004). The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene *SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1* is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *The Plant Cell*, *16*(2), 465-477.

Nomura, K., Mecey, C., Lee, Y. N., Imboden, L. A., Chang, J. H., & He, S. Y. (2011). Effector-triggered immunity blocks pathogen degradation of an immunity-associated vesicle traffic regulator in *Arabidopsis*. *Proceedings of the National Academy of Sciences*, *108*(26), 10774-10779.

Nover, L., Bharti, K., Döring, P., Mishra, S. K., Ganguli, A., & Scharf, K. D. (2001). *Arabidopsis* and the heat stress transcription factor world: how

many heat stress transcription factors do we need? *Cell Stress & Chaperones*, *6*(3), 177.

Nozue, K., Covington, M. F., Duek, P. D., Lorrain, S., Fankhauser, C., Harmer, S. L., & Maloof, J. N. (2007). Rhythmic growth explained by coincidence between internal and external cues. *Nature*, *448*(7151), 358-361.

Oh, E., Zhu, J. Y., & Wang, Z. Y. (2012). Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. *Nature Cell Biology*, *14*(8), 802-809.

Park, J. E., Park, J. Y., Kim, Y. S., Staswick, P. E., Jeon, J., Yun, J., Kim, S. Y., Kim. J., Lee, Y. H., & Park, C. M. (2007). GH3-mediated auxin homeostasis links growth regulation with stress adaptation response in *Arabidopsis*. *Journal of Biological Chemistry*, *282*(13), 10036-10046.

Quesada, V., Macknight, R., Dean, C., & Simpson, G. G. (2003). Autoregulation of FCA pre - mRNA processing controls *Arabidopsis* flowering time. *The EMBO Journal*, *22*(12), 3142-3152.

Rivas-San Vicente, M., & Plasencia, J. (2011). Salicylic acid beyond defence: its role in plant growth and development. *Journal of Experimental Botany*, *62*(10), 3321-3338.

Rochon, A., Boyle, P., Wignes, T., Fobert, P. R., & Després, C. (2006). The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. *The Plant Cell*, *18*(12), 3670-3685.

Scott, I. M., Clarke, S. M., Wood, J. E., & Mur, L. A. (2004). Salicylate accumulation inhibits growth at chilling temperature in *Arabidopsis*. *Plant Physiology*, *135*(2), 1040-1049.

Saslowsky, D. E., Warek, U., & Winkel, B. S. (2005). Nuclear localization of flavonoid enzymes in *Arabidopsis*. *Journal of Biological Chemistry*, *280*(25), 23735-23740.

Seo, P. J., Kim, M. J., Park, J. Y., Kim, S. Y., Jeon, J., Lee, Y. H., Kim, J., & Park, C. M. (2010). Cold activation of a plasma membrane - tethered NAC transcription factor induces a pathogen resistance response in *Arabidopsis*. *The Plant Journal*, *61*(4), 661-671.

Seo, P. J., Kim, M. J., Ryu, J. Y., Jeong, E. Y., & Park, C. M. (2011). Two splice variants of the IDD14 transcription factor competitively form nonfunctional heterodimers which may regulate starch metabolism. *Nature Communications*, *2*, 303.

Shah, J. (2003). The salicylic acid loop in plant defense. *Current opinion in plant biology*, *6*(4), 365-371.

Shin, R., Alvarez, S., Burch, A. Y., Jez, J. M., & Schachtman, D. P. (2007). Phosphoproteomic identification of targets of the *Arabidopsis* sucrose nonfermenting-like kinase SnRK2.8 reveals a connection to metabolic processes. *Proceedings of the National Academy of Sciences*, *104*(15), 6460-6465.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I., & Dean, C. (2003). FY is an RNA 3′ end-processing factor that interacts with FCA to

control the *Arabidopsis* floral transition. *Cell*, *113*(6), 777-787.

Song, Y. H., Smith, R. W., To, B. J., Millar, A. J., & Imaizumi, T. (2012). FKF1 conveys timing information for CONSTANS stabilization in photoperiodic flowering. *Science*, *336*(6084), 1045-1049.

Sonmez, C., Bäurle, I., Magusin, A., Dreos, R., Laubinger, S., Weigel, D., & Dean, C. (2011). RNA 3′ processing functions of *Arabidopsis* FCA and FPA limit intergenic transcription. *Proceedings of the National Academy of Sciences*,*108*(20), 8508-8513.

Spoel, S. H., & Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology*, *12*(2), 89-100.

Spoel, S. H., Mou, Z., Tada, Y., Spivey, N. W., Genschik, P., & Dong, X. (2009). Proteasome-mediated turnover of the transcription co-activator NPR1 plays dual roles in regulating plant immunity. *Cell*, *137*(5), 860.

Stavang, J. A., Gallego-Bartolomé, J., Gómez, M. D., Yoshida, S., Asami, T., Olsen, J. E., García-Martínez, J. L., Alabadí, D., & Blázquez, M. A. (2009). Hormonal regulation of temperature-induced growth in Arabidopsis. *The Plant Journal*, *60*(4), 589-601.

Stein, E., Molitor, A., Kogel, K. H., & Waller, F. (2008). Systemic resistance in *Arabidopsis* conferred by the mycorrhizal fungus *Piriformospora indica* requires jasmonic acid signaling and the cytoplasmic function of NPR1. *Plant and Cell Physiology*, *49*(11), 1747-1751.

Sun, J., Qi, L., Li, Y., Chu, J., & Li, C. (2012). PIF4–mediated activation of

YUCCA8 expression integrates temperature into the auxin pathway in regulating *Arabidopsis* hypocotyl growth. *PLoS Genetics*, *8*(3), e1002594.

Tada, Y., Spoel, S. H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., & Dong, X. (2008). Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. *Science*, *321*(5891), 952-956.

Torres, M. A., Jones, J. D., & Dangl, J. L. (2005). Pathogen-induced, NADPH oxidase–derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis* thaliana. *Nature genetics*, *37*(10), 1130-1134.

Udvardi, M. K., Czechowski, T., & Scheible, W. R. (2008). Eleven golden rules of quantitative RT-PCR. *The Plant Cell*, *20*(7), 1736-1737.

Vogel, J. P., Woeste, K. E., Theologis, A., & Kieber, J. J. (1998). Recessive and dominant mutations in the ethylene biosynthetic gene *ACS5* of *Arabidopsis* confer cytokinin insensitivity and ethylene overproduction, respectively. *Proceedings of the National Academy of Sciences*, *95*(8), 4766-4771.

Wang, W., Ye, R., Xin, Y., Fang, X., Li, C., Shi, H., Zhou, X. & Qi, Y. (2011). An importin β protein negatively regulates microRNA activity in *Arabidopsis*. *The Plant Cell*, *23*(10), 3565-3576.

Wigge, P. A. (2013). Ambient temperature signalling in plants. *Current Opinion in Plant Biology*, *16*(5), 661-666.

Woldemariam, M. G., Onkokesung, N., Baldwin, I. T., & Galis, I. (2012). Jasmonoyl - l - isoleucine hydrolase 1 (JIH1) regulates jasmonoyl - l - isoleucine levels and attenuates plant defenses against herbivores. *The Plant Journal*, *72*(5), 758-767.

Xie, C., Zhou, X., Deng, X., & Guo, Y. (2010). PKS5, a SNF1-related kinase, interacts with and phosphorylates NPR1, and modulates expression of *WRKY38* and *WRKY62*. *Journal of Genetics and Genomics*, *37*(6), 359-369.

Yan, S., Wang, W., Marqués, J., Mohan, R., Saleh, A., Durrant, W. E., Song, J., & Dong, X. (2013). Salicylic acid activates DNA damage responses to potentiate plant immunity. *Molecular Cell*, *52*(4), 602-610.

Yoo, S. D., Cho, Y. H., & Sheen, J. (2007). *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nature Protocols*, *2*(7), 1565-1572.

Yu, C. W., Liu, X., Luo, M., Chen, C., Lin, X., Tian, G., Lu, Q., Cui, Y., & Wu, K. (2011). HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in *Arabidopsis*. *Plant Physiology*, *156*(1), 173-184.

Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W., & Chua, N. H. (2006). Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nature Protocols*, *1*(2), 641-646.

Zipfel, C. (2009). Early molecular events in PAMP-triggered immunity. *Current Opinion in Plant Biology*, *12*(4), 414-420.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G., & Boller, T. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature*, *428*(6984), 764-767.

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1. Lee S, Seo PJ, **Lee HJ**, Park CM. A NAC transcription factor NTL4 promotes reactive oxygen species production during drought-induced leaf senescence in *Arabidopsis*. (2012) *Plant J*. **70**, 831-844.

2. Ryu JY, **Lee HJ**, Seo PJ, Jung JH, Ahn JH, Park CM. The *Arabidopsis* floral repressor BFT delays flowering by competing with FT for FD binding under high salinity. (2014) *Mol. Plant* **7**, 377-387.

3. Jung JH*, **Lee HJ***, Park MJ*, Park CM. Beyond ubiquitination: proteolytic and nonproteolytic roles of HOS1. (2014) *Trends Plant Sci.* **19**, 538-545.

4. Lee S*, **Lee HJ***, Huh SU, Paek KH, Ha JH, Park CM. The *Arabidopsis* NAC transcription factor NTL4 participates in a positive feedback loop that induces programmed cell death under heat stress conditions. (2014) *Plant Sci*. **227**, 76-83.

5. Lee S*, **Lee HJ***, Jung JH, Park CM. The *Arabidopsis thaliana* RNA-binding protein FCA regulates thermotolerance by modulating the detoxification of reactive oxygen species. (2014) *New Phytol*. doi: 10.1111/nph.13079.

6. **Lee HJ**, Jung JH, Llorca LC, Kim SG, Lee S, Baldwin IT, Park CM. FCA mediates thermal adaptation of stem growth by attenuating auxin action in *Arabidopsis*. (2014) *Nat. Commun.* **5**, 5473.

ABSTRACT IN KOREAN (국문 초록)

식물은 자신이 뿌리 내린 곳에서 움직일 수 없기 때문에 다양한 환경 변화에 대해 적응하기 위해 다양한 시스템을 구축하였다. 전사 과정을 조절하는 것은 식물의 환경 적응 메커니즘 중에서도 중요한 요소 중 하나로 꼽힌다. 전사 과정을 조절하는 전사 조절 인자 혹은 전사 보조 인자는 직접 혹은 간접적으로 유전자의 발현을 조절하기 때문에 이들의 활성을 조절하는 것은 전사 연구에 있어 핵심 주제 중 하나이다. 그 동안의 분자 생물학적, 유전적 연구를 통해 식물 적응 반응에 관여하는 다양한 전사 인자들이 밝혀졌다. 하지만 식물이 변화하는 환경에 따라 이들의 활성을 어떻게 조절하는지는 아직 많은 부분이 의문으로 남아 있다. 이 연구에서는 두 가지 대표적인 환경 요소인 온도와 병원체에 대한 반응을 조절하는 전사 인자들을 연구하여 이들의 활성 조절 메커니즘을 증명하였다.

1장에서는 28°C 정도의 약한 고온에 대한 반응에 중요한 역할을 하는 *PHYTOCHROME-INTERACTING FACTOR 4* (*PIF4*) 에 대한 연구를 하였다. 애기장대에서 PIF4는 고온에 의한 옥신 (auxin) 합성을 촉진시켜 식물의 성장을 증대시키는 것으로 알려져 있다. PIF4는 옥신 합성 유전자인 *YUCCA8* (*YUC8*) 의 발현을 직접

조절하여 옥신을 합성한다. 이 연구에서는 기존에 RNA 결합 단백질이자 개화 시기를 조절한다고 알려진 FCA가 PIF4의 DNA 결합을 저해하여 활성을 억제하는 역할을 한다는 사실을 밝혀내었다. FCA의 기능이 사라진 돌연변이 식물에서는 28ºC에 의한 옥신 합성이 촉진되고 반대로 FCA를 과발현시킨 유전자 조작 식물에서는 옥신 합성이 저해된다. 이것은 줄기의 성장에 영향을 끼쳐, FCA 돌연변이체는 고온에서 줄기가 길어지는 반면 FCA 과발현체는 줄기가 길어지지 않는다. 따라서, FCA는 PIF4가 *YUC8*의 DNA에 붙는 것을 억제하여 고온에서 지나치게 줄기가 길어지는 것을 억제하는 역할을 한다고 볼 수 있다.

2장에서는 식물의 병 저항에 중요한 역할을 하는 전사 보조 인자인 NONEXPRESSER OF PR GENES 1 (NPR1) 의 세포 내 이동에 대해 기술하였다. 식물은 병에 감염되었을 때 감염된 부위를 괴사시켜 병원균이 더 퍼지지 못하게 하는 동시에 다른 부위에 신호를 전달해 면역 반응을 일으키는데 이것을 systemic acquired resistance (SAR) 라고 한다. 살리실산 (salicylic acid) 은 NPR1을 통해 SAR을 일으키는데, 많은 양의 살리실산을 합성할 경우 식물의 성장과 발달에 해가 될 수 있다. 따라서 식물은 병원체가 감염된 잎에서는 살리실산을 많이 합성하는 반면 다른 잎에서는 소량의 살리실산만을 만들어낸다. 하지만 소량의

살리실산이 어떻게 강한 면역 신호를 만들어내는지에 대해서는 많은 부분이 의문으로 남아 있다. 이 연구에서는 SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8) 이 낮은 살리실산 농도가 유지되는 잎에서 NPR1을 인산화시켜 핵으로의 이동에 중요한 역할을 한다는 사실을 밝혀내었다. 즉, 애기장대는 성장에 해가 되는 살리실산을 낮은 농도로 유지하는 대신 SnRK2.8을 발현시켜 NPR1의 활성을 유지함으로써 성장과 면역 반응이라는 두 가지 효과를 모두 이루어내는 것으로 생각된다.

주요어: 전사 조절 인자, 옥신, 주위 온도 (ambient temperature), 면역 반응, 전신성 획득 저항성 (systemic acquired resistance), 세포 내 이동

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