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Research

The *Arabidopsis thaliana* RNA-binding protein FCA regulates thermotolerance by modulating the detoxification of reactive oxygen species

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Summary

- Heat stress affects various aspects of plant growth and development by generating reactive oxygen species (ROS) which cause oxidative damage to cellular components. However, the mechanisms by which plants cope with ROS accumulation during their thermotolerance response remain largely unknown. Here, we demonstrate that the RNA-binding protein FCA, a key component of flowering pathways in *Arabidopsis thaliana*, is required for the acquisition of thermotolerance.
- Transgenic plants overexpressing the *FCA* gene (*35S:FCA*) were resistant to heat stress; the *FCA*-defective *fca-9* mutant was sensitive to heat stress, consistent with induction of the *FCA* gene by heat. Furthermore, total antioxidant capacity was higher in the *35S:FCA* transgenic plants but lower in the *fca-9* mutant compared with wild-type controls.
- FCA interacts with the ABA-INSENSITIVE 5 (ABI5) transcription factor, which regulates the expression of genes encoding antioxidants, including *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*). We found that FCA is needed for proper expression of the *PER1* gene by ABI5.
- Our observations indicate that FCA plays a role in the induction of thermotolerance by triggering antioxidant accumulation under heat stress conditions, thus providing a novel role for FCA in heat stress responses in plants.

Introduction

Heat stress leads to the disruption of cellular homeostasis and growth retardation in plants, which cause severe crop loss in agriculture (Wang *et al.*, 2003). Therefore, plants are forced to spend valuable resources to adjust their growth and development and prevent heat stress-induced cellular and metabolic damage, a process known as heat acclimation (Larkindale & Huang, 2005). The heat acclimation response is regulated through complex signaling networks in which heat stress transcription factors, stress hormones, and reactive oxygen species (ROS) play critical roles (Larkindale *et al.*, 2005; Volkov *et al.*, 2006). However, the mechanisms by which plants cope with the cellular and physiological damage caused by heat stress are largely unknown.

The plant stress hormone abscisic acid (ABA) plays a critical role in the heat stress response in several plant species (Larkindale & Huang, 2004). Under heat stress conditions, the endogenous ABA concentration is elevated in pea (*Pisum sativum*) and creeping bentgrass (*Agrostis stolonifera*) (Larkindale & Huang, 2005; Liu *et al.*, 2006), and genes encoding ABA biosynthetic enzymes, including 9-*cis*-EPOXYCAROTENOID DIOXYGENASE 9 (NCED9), are rapidly induced in *Arabidopsis thaliana* (Toh *et al.*, 2008). Accordingly, ABA biosynthetic mutants, such as *ABA deficient 1* (*aba1*), *aba2*, and *aba3*, and ABA signaling mutants, including *abi1* and *abi2*, exhibit reduced thermotolerance (Larkindale *et al.*, 2005).

ROS cause oxidative damage to cellular components, such as nucleic acids and cellular membranes (Møller *et al.*, 2007). Intracellular concentrations of ROS, such as superoxide, hydrogen peroxide and the hydroxyl radical, are significantly elevated in plants that are exposed to heat stress (Møller *et al.*, 2007; Zhou *et al.*, 2012). An oxidative burst occurs shortly after exposure to high temperatures, which is obviously attributable to NADPH oxidase activity (Miller *et al.*, 2009). In addition, pretreatments with H_2O_2 have been shown to lead to the induction of thermotolerance in *A. thaliana* (Larkindale & Huang, 2004).

Plants have developed an array of ROS scavenging/detoxifying enzymes and various antioxidants to manage ROS that accumulate under stress conditions. Superoxide dismutase (SOD) converts superoxide into hydrogen peroxide. Ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) then decompose hydrogen peroxide to water (Mittler, 2002). Ascorbate and glutathione (GSH) act as antioxidants. *Arabidopsis thaliana* mutants that are defective in antioxidant production,

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such as *ascorbate peroxidase 1* (*apx1*), *apx2*, *vitamin C defective 1* (*vtc1*) and *vtc2*, exhibit symptoms of increased oxidative damage when exposed to high temperatures (Larkindale *et al.*, 2005; Suzuki *et al.*, 2013). However, peroxiredoxins, which are thiol-based peroxidases, enhance plant tolerance to oxidative and heat stresses (Kim *et al.*, 2010).

FCA is a plant-specific RNA-binding protein that constitutes the autonomous flowering pathway in *A. thaliana*. 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).

The components of the autonomous flowering pathway are involved in environmental adaptation responses through RNA processing and chromatin modification. *Arabidopsis thaliana* FVE, a component of the histone deacetylase complexes that mediate transcriptional silencing, negatively regulates cold-responsive genes (Kim *et al.*, 2004). In addition, *A. thaliana fy* mutants exhibit ABA-insensitive phenotypes (Jiang *et al.*, 2012). The barley (*Hordeum vulgare*) *FCA* gene is induced by ABA in embryos, and FCA has been shown to enhance the promoter activity of ABA-responsive genes (Kumar *et al.*, 2011). Notably, the promoter driving the expression of antisense *FLC* RNA, the processing of which is regulated by FCA, is temperature-responsive (Swiezewski *et al.*, 2009).

In this work, we demonstrate that FCA regulates thermotolerance by modulating antioxidant activity under heat stress conditions. FCA interacts with ABA-INSENSITIVE 5 (ABI5) to induce the *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*) gene, which encodes the antioxidant 1-Cys peroxiredoxin (Haslekås *et al.*, 2003). The total antioxidant capacity was reduced in *fca* mutants but increased in *FCA*-overexpressing plants. These observations indicate that FCA plays a role in the stimulation of thermotolerance by enhancing antioxidant activity under heat stress conditions, thus demonstrating a novel role for FCA in the plant stress response.

Materials and Methods

Plant material and growth conditions

All *Arabidopsis thaliana* (L.) Heynh lines used were in the Columbia (Col-0) background. The plants were grown in a controlled culture room at 23°C with a relative humidity of 55% under long days (LDs; 16 h light : 8 h dark) with white light illumination (120 μmol photons m^{-2} s^{-1}) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). The *fca-9*, *fca-11*, and *abi5-3* mutants and *35S:FCA* transgenic plants have been described previously (Bäurle *et al.*, 2007; Piskurewicz *et al.*, 2008; Jung *et al.*, 2012). To generate the *35S:FCA* transgenic plant, a full-size *FCAγ* cDNA was fused in-frame to the 3′ end of the MYC-coding sequence in the myc-pBA vector (Seo *et al.*, 2010). The expression construct was transformed into Col-0 plants.

For heat treatments, 1-wk-old plants grown on half-strength Murashige and Skoog (MS)-agar plates were covered with aluminum foil to expose the plants to homogeneous heat conditions in the dark. The plates were transferred to a heat chamber set at 45°C and incubated for 90 min. After heat treatments, the plants were allowed to recover at 23°C for 3 d with white light illumination as described in the 'Plant material and growth conditions' section.

Analysis of gene transcript levels

Gene transcript levels were determined using reverse transcription–quantitative real-time PCR (RT-qPCR). Reverse transcription and quantitative PCR reactions were carried out according to recommendations that have been proposed to guarantee reproducible and accurate measurements of transcript levels (Udvardi *et al.*, 2008). The RNA samples were pretreated with RNase-free DNase to remove any contaminating genomic DNA before use.

RT-qPCR reactions were carried out in 96-well blocks using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the SYBR Green I master mix in a volume of 20 μl. The PCR primers were designed using the PRIMER EXPRESS software (Bioneer, Dae-Jeon, Korea) installed with the system and are listed in Supporting Information Table S1. The two-step thermal cycling profile used was conducted at 94°C for 15 s and at 68°C for 1 min. An *EUKARYOTIC TRANSLATION INITIATION FACTOR 4A (eIF4A)* gene (*At3g13920*) was included in the reactions as an internal control to normalize the variations in the cDNA amounts used (Gutierrez *et al.*, 2008). All RT-qPCR reactions were performed in biological triplicates using RNA samples extracted from three independent plant materials grown under identical conditions. The comparative $\Delta\Delta C_T$ method was employed to evaluate the relative quantity of each amplified product in the samples. The threshold cycle (C_T) was automatically determined for each reaction by the Applied Biosystems 7500 Real-Time PCR system according to the default parameters. The specificity of the PCR reactions was determined based on a melting curve analysis of the amplified products using the standard method installed with the system.

Yeast two-hybrid assay

The BD Matchmaker system (Clontech, Mountain View, CA, USA) was used for the assays. The pGADT7 vector was used to express the GAL4 activation domain (AD), and the pGBKT7 vector was used to express the GAL4 DNA-binding domain (BD). The yeast strain AH109 (Leu–, Trp–, Ade–, His–), which contains the chromosomally integrated reporter genes *lacZ* (β-D-

galactosidase) and *HIS* under the control of the GAL1 promoter, was used for transformation. The full-length *FCA* cDNA was amplified by PCR and subcloned into the pGBKT7 vector. Similarly, full-length *ABI3*, *ABI4*, and *ABI5* cDNAs were subcloned into the pGADT7 vector.

In vitro pull-down assay

Recombinant FCA protein was prepared as a maltose-binding protein (MBP)-FCA fusion in *Escherichia coli* Rosetta2 (DE3) pLysS strain (Novagen, Madison, WI, USA) and partially purified as described previously (Seo *et al.*, 2011). [^{35}S]Methionine-labeled ABI3, ABI4, and ABI5 polypeptides were prepared using *in vitro* translation based on the TNT-coupled reticulocyte lysate system (Promega, Madison, WI, USA).

In vitro pull-down assays were performed as described previously (Seo *et al.*, 2011). Bound proteins were eluted with ×1 SDS-loading buffer by boiling for 5 min and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Bimolecular fluorescence complementation (BiFC) assay

A full-size *FCA* cDNA fragment was fused in-frame to the 3′ end of the gene sequence encoding the N-terminal half of Enhanced yellow fluorescent protein (EYFP) in the pSATN-nEYFP-C1 vector (E3081). A full-size *ABI5* cDNA fragment was fused in-frame to the 5′ end of the gene sequence encoding the C-terminal half of EYFP in the pSATN-cEYFP-N1 vector (E3084). nYFP-FCA and ABI5-cYFP vectors were cotransfected into *A.thaliana* mesophyll protoplasts using the polyethylene glycol (PEG)-calcium transfection method (Yoo *et al.*, 2007).

Measurement of chlorophyll content

Chlorophyll content was measured as described previously (Lee *et al.*, 2012).

Measurement of antioxidant contents

To measure ascorbate and glutathione contents, heat-treated whole plants were ground in liquid nitrogen, and 1 ml of 6% (w/v) Trichloroacetic acid (TCA) was added per 40 mg of whole plants. The crude extract was centrifuged at 4°C for 10 min at 16 000 ***g***. The supernatants were measured as described previously (Gillespie & Ainsworth 2007; Queval & Noctor, 2007).

To measure anthocyanin content, heat-treated whole plants were ground in liquid nitrogen and incubated at 4°C for 16 h in 1 ml of methanol containing 1% (v/v) HCl per 40 mg of plant material. The mixture was centrifuged at 4°C for 10 min at 16 000 ***g***. The supernatants were measured as described previously (Solfanelli *et al.*, 2006).

Determination of cell death

Trypan blue staining was employed to visualize dead cells in the rosette leaves of 1-wk-old plants, as described previously (Koch & Slusarenko, 1990). The staining was quantified using the LabWorks software (UVP, Upland, CA, USA) based on 15 leaves. The signal intensity for each leaf was calculated as the average of the index of blue pixels measured at three points inside the leaf area minus the average values at three points on the opposite side of each leaf.

For electrolyte leakage assays, 1-wk-old plants grown on MS-agar plates were used. The aerial parts of five seedlings were floated on deionized water for 12 h in complete darkness before the conductivity was measured using the Orion 5-star conductivity meter (Thermo, Beverly, MA, USA).

Determination of hydrogen peroxide and thiobarbituric acid reactive substance (TBARS) contents

Hydrogen peroxide was detected as described previously (Lee *et al.*, 2012). Plant samples were incubated in a 3,3′-diaminobenzidine (DAB) staining solution (0.5 mg ml^{-1}) for 24 h at room temperature in complete darkness. The plant samples were then destained by incubating the samples in 95% ethanol at 70°C for 15 min. The staining density was quantified using the LabWorks software (UVP). Thiobarbituric acid reactive substances (TBARSs) were assayed using the OXI-TEK TBARS assay kit (ZeptoMetrix, Buffalo, NY, USA).

Measurement of total antioxidant capacity

Total antioxidant capacity was evaluated using the oxygen radical absorbance capacity (ORAC) assay (Gillespie *et al.*, 2007). Briefly, *c.* 20 mg of plant material was ground in liquid nitrogen and resuspended in 2 ml of ice-cold 50% acetone. The resuspension was centrifuged at 4°C for 30 min at 4500 ***g***, and the supernatant was transferred to a fresh 2-ml tube. For the ORAC reactions, 150 µl of 0.08 µM fluorescein was added to each well of a black microplate. Next, 25 µl of 75 mM phosphate buffer, pH 7.0 (blank), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (standard solution, 6.25–50 µM), or samples (20–100 dilution series) was added to the mixture. The mixtures were incubated at 37°C for 10 min, and 25 µl of 2,2′-azobis(2-amidinopropane) dihydrochloride was then added to each well to initiate the ORAC reactions. The fluorescence was measured at 10-min intervals for 70 min at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a multimode microplate reader (Berthold, Bad Wildbad, Germany).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out as described previously (Lee *et al.*, 2012) using 1-wk-old plants grown on MS-agar plates. Whole plants were vacuum-infiltrated with 1% (v/v) formaldehyde for cross-linking and ground in liquid nitrogen after quenching the cross-linking process. Chromatin preparations were sonicated to produce 0.4- to 0.7-kb fragments. An anti-MYC antibody (Millipore, Billerica, MA, USA) was added to the chromatin solutions, which were precleared with salmon sperm DNA/

Protein A agarose beads (Roche, Indianapolis, IN, USA). 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 a Promega spin column (Promega). Quantitative PCR was used to determine the amounts of genomic DNA enriched in the chromatin preparations. The primers used are listed in Table S1.

Transient expression assays in *Arabidopsis thaliana* protoplasts

For the transcriptional activation activity assays, a series of reporter and effector vectors were constructed. In the reporter vector, four copies of the GAL4 upstream activation sequence (UAS) were fused to the β-*glucuronidase* (*GUS*) gene. The *ABI5* gene sequence was fused to the GAL4 DNA-binding domain-coding sequence driven by the cauliflower mosaic virus (CaMV) 35S promoter in the effector vector. The *FCA* gene was subcloned into the expression vector harboring the CaMV 35S promoter. The Auxin response factor 5 middle region (ARF5M) construct, in which the *ARF5M* gene was subcloned into the GAL4 expression vector (Tiwari *et al.*, 2003), was used as a positive control. The reporter, effector and expression vectors were cotransfected into *A. thaliana* mesophyll protoplasts using the PEG-calcium transfection method (Yoo *et al.*, 2007). The CaMV 35S promoter–luciferase construct was also cotransfected as an internal control. GUS activity was measured using the fluorometric method as described previously (Lee *et al.*, 2012), and the luciferase assay was performed using the Luciferase Assay System kit (Promega).

For the transient expression assays, the promoter sequence regions harboring the ABA-responsive element (ABRE) element of the *PER1* gene was subcloned into the reporter vector. The *ABI5* or *FCA* gene sequence was subcloned into the effector vector. The reporter and effector vectors were cotransfected into *A. thaliana* mesophyll protoplasts. The luciferase expression construct was included as an internal control in the transfection. GUS and luciferase activity assays were performed after incubation for 16 h as described in the paragraph above.

Coimmunoprecipitation assay

Leaves of 4-wk-old *Nicotiana benthamiana* plants grown in soil were used for *Agrobacterium tumefaciens*-mediated infiltration to transiently express FLAG-FCA and MYC-ABI5 constructs as has been described previously (Sparkes *et al.*, 2006). Coimmunoprecipitation assays were performed as described previously (Song *et al.*, 2012).

RNA-Seq analysis

One-week-old seedlings grown on MS-agar plates were exposed to 45°C for 90 min, and whole seedlings were ground in liquid nitrogen. Total RNA was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA). After extensive treatment with RNase-free DNase I, the total RNA samples were submitted to the Beijing Genomics Institute (BGI) at Hong Kong for sequencing. Only genes with P-value < 0.05 and an absolute value of fold-change ≥ 2 were considered as differentially expressed genes.

Results

FCA mediates the thermotolerance response

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

We first examined the effects of high temperatures on plant growth using *FCA*-overexpressing transgenic plants (*35S:FCA*) and *FCA*-deficient mutants (*fca-11* and *fca-9*) (Fig. S1). Under nonacclimation conditions, whereas the *35S:FCA* transgenic plants exhibited increased resistance to heat (45°C), the *fca-11* and *fca-9* mutants exhibited reduced resistance to heat (Fig. 1a, b). Consistent with the altered heat survival rates, the chlorophyll content of the *35S:FCA* transgenic leaves was higher but that of the *fca* mutant leaves was lower than that of Col-0 leaves under heat stress (Fig. 1c). The decrease in heat resistance of the *FCA*-defective mutants was also evident under mild heat conditions (37°C for 2 d; Fig. S2). To examine whether FCA is also involved in the heat acclimation response, we first treated plants at 37°C for 120 min before heat (45°C) treatment to induce heat acclimation. *35S:FCA* transgenic plants continued to exhibit increased resistance to heat, but the *fca-9* and *fca-11* mutants exhibited decreased resistance, as observed using a basal thermotolerance test (Fig. S3). Therefore, these results indicate that FCA is involved in both basal and acquired thermotolerance responses.

Gene expression analysis under various abiotic stress conditions showed that the *FCA* gene was induced > 3-fold by heat (45°C) but was suppressed by *c.* 60% by cold (4°C) (Fig. 1d). The *FCA* gene was also induced gradually upon exposure to 37°C (Fig. S4), supporting the notion that a broad range of high temperatures induces *FCA* gene expression. By contrast, this gene was not affected by drought or high salt. We also examined the effects of ABA and SA on *FCA* gene expression. The *FCA* gene was induced *c.* 3-fold by ABA but was not affected by salicylic acid (SA) (Fig. 1e). In addition, *FCA* gene expression was not affected by heat in the ABA-deficient *aba3-1* mutant (Fig. 1f), showing that the induction of the *FCA* gene by heat depends on ABA.

The exogenous application of ABA is known to induce chlorophyll degradation (Nagira *et al.*, 2006). We therefore examined the ABA responses of *fca* mutants and *35S:FCA* transgenic plants. Under normal growth conditions, the leaves of *fca* mutants and *35S:FCA* transgenic plants were green, similar to Col-0 leaves (Figs 1g (left panel), S5). When grown in the presence of ABA,

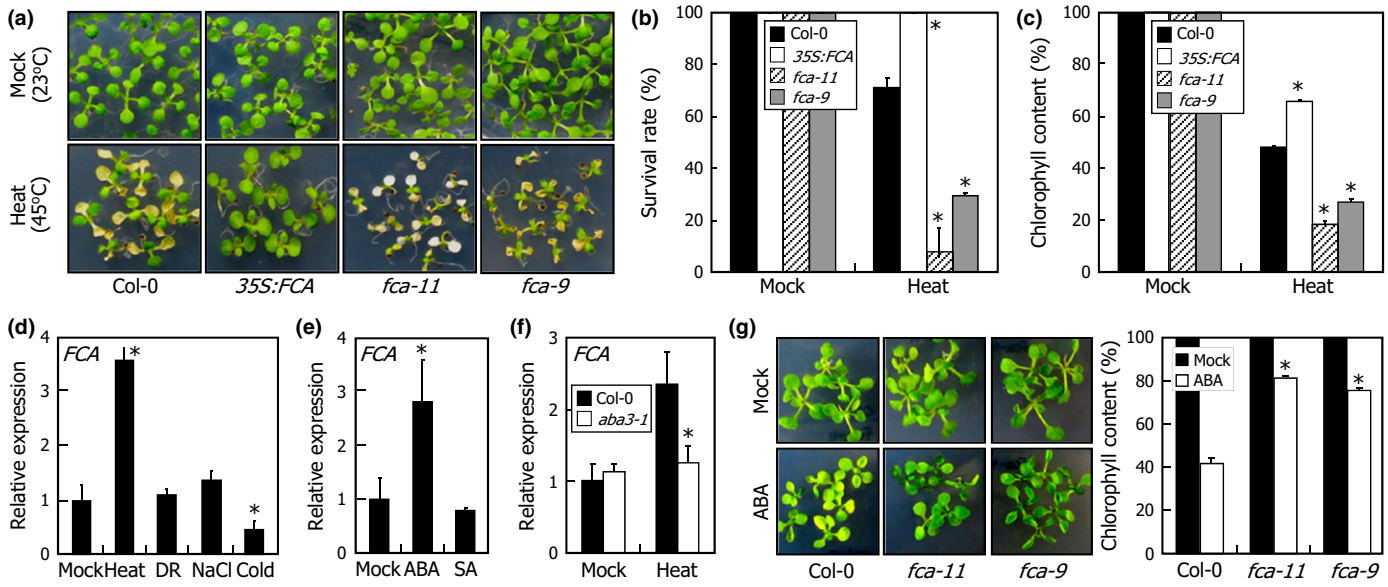


Fig. 1 FCA mediates the thermotolerance response. In (b–f), the statistical significance of the measurements was determined using Student's *t*-test. Bars indicate the SE of the mean. (a, b) Survival rates. One-week-old *Arabidopsis thaliana* plants grown on Murashige and Skoog (MS)-agar plates were exposed to heat (45°C for 90 min) and allowed to recover at 23°C for 3 d. Survival rates were calculated based on the average results from 50 plants (*t*-test; *, $P < 0.01$; difference from heat-treated Col-0). (c) Chlorophyll content. The rosette leaves of 1-wk-old plants grown on MS-agar plates were harvested immediately after heat treatment (45°C for 90 min), and the chlorophyll content was measured. Five measurements were averaged (*t*-test; *, $P < 0.01$; difference from heat-treated Col-0). (d) Effects of abiotic stresses on *FCA* expression. Two-week-old Col-0 plants grown on MS-agar plates were exposed to heat, drought (DR), NaCl, or cold. For heat treatment, plants were exposed to 45°C for 2 h. For drought treatment, plants were air-dried for 2 h. For NaCl treatment, plants were soaked in MS liquid medium containing 150 mM NaCl for 2 h. For cold treatment, plants were exposed to 4°C for 24 h. Total RNA was extracted from entire plants. Transcript levels were determined using reverse transcription–quantitative real-time PCR (RT-qPCR). Biological triplicates were averaged (*t*-test; *, $P < 0.01$; difference from mock). (e) Effects of abscisic acid (ABA) on *FCA* expression. Two-week-old plants grown on MS-agar plates were transferred to MS liquid cultures containing ABA (20 μM) or salicylic acid (SA; 100 μM) and incubated for 24 h. Transcript levels were determined using RT-qPCR as described in (d) (*t*-test; *, $P < 0.01$; difference from mock). (f) Expression of the *FCA* gene in the *aba3-1* mutant under heat stress. One-week-old plants grown on MS-agar plates were exposed to 45°C for 2 h before harvesting whole-plant material. Transcript levels were determined using RT-qPCR as described in (d) (*t*-test; *, $P < 0.01$; difference from mock). (g) Chlorophyll content after ABA treatment. Three-day-old plants grown on MS-agar plates were transferred to MS-agar plates containing 10 μM ABA and then grown for a further 2 wk (left panel). The aerial plant parts were harvested, and their chlorophyll contents were measured (right panel). Three measurements were averaged (*t*-test; *, $P < 0.01$; difference from ABA-treated Col-0).

the *fca* leaves remained green; by contrast, the leaves of Col-0 and *35S:FCA* transgenic plants were pale green. The chlorophyll content was significantly reduced in the Col-0 and *35S:FCA* transgenic leaves but was only slightly reduced in the *fca* leaves (Figs 1g (right panel), S5). These observations suggest that FCA is involved in plant responses to ABA.

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

Heat stress disrupts the integrity of cellular membranes, leading to the inevitable leakage of inorganic and organic solutes from the cell (Liu & Huang, 2000). Our data showed that FCA mediates plant responses to heat stress.

To further examine the involvement of FCA in the thermotolerance response, we performed electrolyte leakage assays on *35S:FCA*, *fca-9*, and *fca-11* leaves after heat treatment (45°C for 90 min). The degree of electrolyte leakage was not detectably different in the Col-0, *35S:FCA* and *fca* mutant leaves under normal conditions (Fig. 2a). However, after exposure to heat, the degree of electrolyte leakage was significantly lower in the *35S:FCA* leaves but markedly higher in the *fca* mutant leaves compared with that in the Col-0 leaves. We visualized cell death using lactophenol trypan blue staining, which selectively stains dead cells. The *fca* leaves were stained dark blue, but the *35S:FCA* leaves were stained light blue (Figs 2b,c, S6), indicating that cell death was reduced in the *35S:FCA* leaves but increased in the *fca* leaves compared with that in Col-0 leaves after heat treatment.

Heat shock proteins (HSPs) play a critical role in plant responses to heat stress (Zhou *et al.*, 2012). We therefore examined whether FCA is associated with HSPs in the thermotolerance response. RT-qPCR analysis revealed that the expression of a selected set of *HSP* genes, such as *HSP60*, *HSP70*, *HSP90.1*, *HSP90.2*, and *HSP101*, was unchanged in *35S:FCA* transgenic plants and in the *fca-9* mutant (Fig. S7), suggesting that FCA is not associated with *HSP* expression.

ROS accumulates in *fca* mutants under heat stress

During the hypersensitive responses caused by pathogen infection, high light and exposure to drought and heat, ROS rapidly accumulate in plant cells (Mittler, 2002; Mittler *et al.*, 2004).

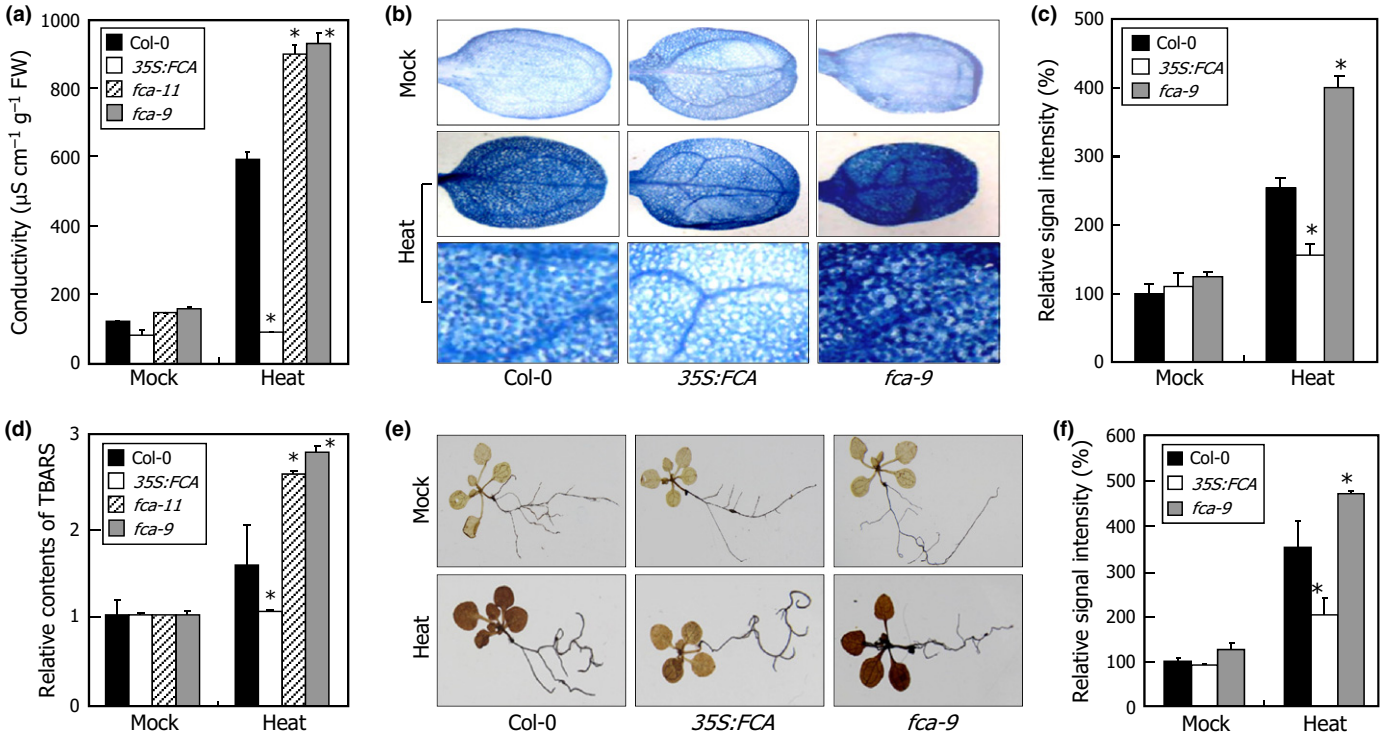


Fig. 2 Cell death is accelerated in the *fca* mutants under heat stress. In (a, c, d, f), the statistical significance of the measurements was determined using Student's *t*-test (*; $P < 0.01$; difference from heat-treated Col-0). Bars indicate the SE of the mean. (a) Electrolyte leakage assays. One-week-old *Arabidopsis thaliana* plants were exposed to heat (45°C for 90 min). The aerial parts of five seedlings were used for each measurement. Relative electrolyte leakage was calculated by dividing the conductivity by total fresh weight. Five measurements were averaged. (b, c) Trypan blue staining. Two-week-old plants were exposed to heat (45°C for 90 min) and allowed to recover by incubating at 23°C for 2 d. The second rosette leaves of the heat-treated plants were used for trypan blue staining. The signal intensities of 15 representative rosette leaves were averaged. (d) 2-Thiobarbituric acid reactive substance (TBARS) assays. One-week-old plants were exposed to heat. After recovering at 23°C for 2 d, entire plants were assayed and five measurements were averaged. (e, f) 3,3′-diaminobenzidine (DAB) staining. Two-week-old plants were treated with heat (45°C for 90 min) and subjected to DAB staining. Fifteen representative rosette leaves were quantified, and the results were averaged.

Under heat stress conditions, the accumulated ROS cause oxidative damage (Mittler, 2002). Considering the hypersensitivity of *fca* mutants to heat, we hypothesized that ROS would accumulate to a higher level in the *fca* mutants under heat stress.

To examine the oxidative damage caused by ROS after exposure to heat, we measured the degree of lipid peroxidation using the reagent thiobarbituric acid. Malondialdehyde (MDA) is formed by the oxidation of polyunsaturated lipids and is frequently used as an indicator of ROS-triggered oxidative damage (Zhang *et al.*, 2009). MDA, which is a TBARS, generates a red fluorescent derivative after condensation with thiobarbituric acid. The amount of TBARS was lower in the *35S:FCA* transgenic plants but higher in the *fca* mutants compared with that in the Col-0 plants under heat stress conditions (Fig. 2d), indicating that a high degree of oxidative damage occurs in the *fca* mutants, possibly as a result of high ROS accumulation.

We next measured the concentration of endogenous H_2O_2 using the DAB staining method (Torres *et al.*, 2005). As expected, heat treatment rapidly induced H_2O_2 accumulation in the Col-0 plants (Fig. 2e,f). However, although H_2O_2 accumulation was more prominent in the *fca* mutants, the concentration of H_2O_2 was lower in the *35S:FCA* plants (Figs 2e,f, S8). These observations indicate that FCA mediates ROS metabolism under heat stress.

FCA modulates ROS detoxification

Plants produce various antioxidants, such as ascorbate and glutathione, to cope with ROS-triggered cellular damage under abiotic stress conditions (Mittler *et al.*, 2004). Based on the role of FCA in ROS accumulation, we postulated that antioxidant activity is altered in the *35S:FCA* transgenic plants and in the *fca-9* mutant.

To examine whether FCA modulates antioxidant activity, we examined the oxidative stress tolerance of the *35S:FCA* transgenic plants and the *fca-9* mutant by growing them in the presence of paraquat (*N,N′*-dimethyl-4,4′-bipyridinium dichloride), a chemical that induces ROS production in plant cells. The growth of *35S:FCA* transgenic seedlings, which was slower than that in Col-0 and *fca-9* seedlings (Fig. 3a), was less sensitive to paraquat, whereas *fca-9* and *fca-11* seedling growth was more sensitive to paraquat than that of the Col-0 seedlings (Figs 3a, S9). Consistent with these observations, the heat-sensitive property of the *fca-9* mutant was rescued in the presence of ascorbate, indicating that FCA mediates the ROS detoxification process (Fig. S10).

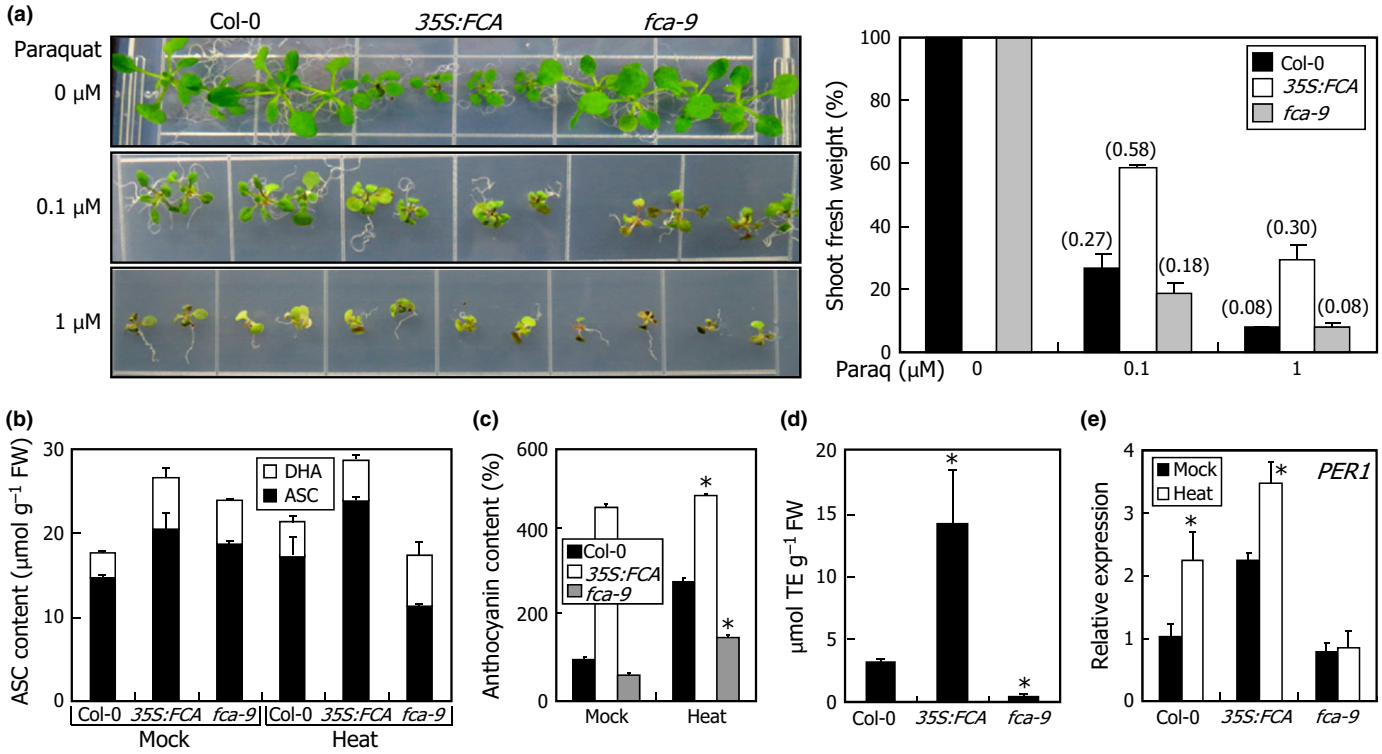


Fig. 3 FCA modulates reactive oxygen species (ROS) detoxification. (b–e) One-week-old *Arabidopsis thaliana* whole plants grown on Murashige and Skoog (MS)-agar plates were used for the measurements or the heat treatments. The statistical significance of the measurements was determined using Student's *t*-test. Bars indicate the SE of the mean. (a) Oxidative stress response of *35S:FCA* transgenic plants and the *fca-9* mutant. Three-day-old plants grown on MS-agar plates were transferred to MS-agar plates containing various concentrations of paraquat and then grown for a further 2 wk (left panel). The shoot fresh weights of 30 plants were averaged (right panel). Numbers in parentheses represent ratios of the shoot fresh weights. (b) Ascorbate (ASC) content. Plants were exposed 45°C for 90 min and harvested immediately after the heat treatment. Three measurements of the ASC and dehydroascorbate (DHA) contents were averaged. (c) Anthocyanin content. Plants were exposed to heat and allowed to recover at 23°C for 2 d before harvesting entire plants. Three measurements were averaged (*t*-test; *, $P < 0.01$; difference from heat-treated Col-0). (d) Total antioxidant capacity. Five measurements were averaged (*t*-test; *, $P < 0.01$; difference from Col-0). TE, Trolox equivalent. (e) Expression of the *PER1* gene. Plants were exposed to 45°C for 90 min before harvesting whole-plant material. Transcript levels were determined using RT-qPCR. Biological triplicates were averaged (*t*-test; *, $P < 0.01$; difference from mock).

We next measured the antioxidant contents of the plants to investigate whether FCA is associated with antioxidant metabolism. The amounts of total and reduced ascorbate were higher in the *35S:FCA* transgenic plants but slightly lower in the *fca-9* mutant than that in Col-0 plants after heat treatment (Fig. 3b). In addition, the glutathione content was slightly higher in the *35S:FCA* transgenic plants (Fig. S11). Anthocyanins are also important antioxidants in plants (Gould *et al.*, 2002). Endogenous anthocyanin concentrations were significantly higher in the *35S:FCA* transgenic plants but lower in the *fca-9* mutant under both normal and heat stress conditions (Figs 3c, S12). Furthermore, total antioxidant capacity was significantly higher in the *35S:FCA* transgenic plants but lower in the *fca-9* mutant, suggesting that the overall antioxidant system is affected by FCA (Fig. 3d). These observations support the notion that FCA regulates ROS accumulation by modulating the endogenous concentrations of various antioxidants.

To investigate how the antioxidant capacity is altered in the *35S:FCA* transgenic plants and in the *fca-9* mutant, we examined the expression patterns of genes encoding antioxidant metabolizing enzymes, such as *VITAMIN C DEFECTIVE1* (*VTC1*), *VTC2*, *GLUTAMATE-CYSTEINE LIGASE* (*GSH1*), *GLUTATHIONE SYNTHETASE2* (*GSH2*), *ASCORBATE PEROXIDASE1* (*APX1*), *GLUTATHIONE PEROXIDASE3* (*GPX3*), *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*), *CHALCONE SYNTHASE* (*CHS*), *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), and *PER1* (Haslekås *et al.*, 2003; Foyer & Noctor, 2005). Although the expression patterns of the *VTC1*, *GSH1*, *GSH2*, *APX1*, and *GPX3* genes were not significantly different in the *35S:FCA* transgenic plants and in the *fca-9* mutant, the transcript levels of the *VTC2*, *DFR*, *CHS*, and *PAP1* genes were significantly induced in the *35S:FCA* transgenic plants; these findings are consistent with the high concentrations of ascorbate and anthocyanins in the transgenic plants (Fig. S13). Notably, the *PER1* expression was increased by *c.* 2-fold in the *35S:FCA* transgenic plants compared with the transcript level in Col-0 plants under normal conditions (Fig. 3e). It was also induced in the heat-treated Col-0 and *35S:FCA* transgenic plants, but the inductive effects of heat were not observed in the *fca-9* and *fca-11* mutants (Figs 3e, S14). These observations indicate that the heat induction of the *PER1* gene is dependent on FCA.

FCA modulates ABI5-mediated *PER1* expression under heat stress conditions

Our data show that the *PER1* gene is induced under heat stress conditions in a FCA-dependent manner. We therefore examined the mechanism by which FCA regulates *PER1* induction. The *PER1* gene has been suggested to be a putative target of the ABI5 transcription factor (Haslekås *et al.*, 2003). Therefore, we anticipated that FCA would be involved in ABI5-mediated ABA signaling under heat stress conditions.

We first examined whether FCA interacts with ABI3, ABI4, and ABI5. Yeast two-hybrid assays showed that FCA interacts with ABI5 but not with ABI3 and ABI4 (Fig. 4a). The specific interaction of FCA with ABI5 was also examined in *in vitro* pull-down assays using recombinant MBP-FCA fusion proteins prepared in *E. coli* cells and *in vitro* translated, ^{35}S-labeled ABI3, ABI4, and ABI5 polypeptides (Fig. 4b). In addition, we found that the FCA–ABI5 interaction occurs in the nucleus (this was verified in BiFC assays using *A. thaliana* protoplasts; Figs 4c, S15). To verify that FCA and ABI5 interact *in planta*, we performed coimmunoprecipitation assays by transiently overexpressing FLAG-FCA and MYC-ABI5 fusions using *A. tumefaciens* (Sparkes *et al.*, 2006). The results showed that FCA and ABI5 associate with each other *in vivo* (Fig. 4d).

To study the functional link between FCA and ABI5, we generated *abi5-3 35S:FCA* plants and performed heat survival assays on these plants. Under heat stress conditions, the *abi5-3 35S:FCA* plants exhibited a lower survival rate than the *35S:FCA* transgenic plants but a higher survival rate than that in the *abi5-3* mutant (Fig. 4e). We next examined the expression patterns of the *PER1* gene. *PER1* gene expression was induced by heat, but the heat induction of *PER1* was absent in both the *fca-9* and *abi5-3* mutants (Fig. 4f). Additionally, the *PER1* gene was highly expressed in the *35S:FCA* transgenic plants under both normal and heat stress conditions, but this high-level expression was largely compromised in the *abi5-3 35S:FCA* plants. These observations indicate that ABI5 is required for the FCA regulation of *PER1* expression under heat stress conditions.

We next performed transcriptional activation activity assays in *A. thaliana* protoplasts to investigate whether FCA affects the activity of ABI5. The *ABI5* gene sequence was fused in-frame to the 3′ end of the GAL4 DNA-binding domain-coding sequence, and the resulting fusion was then cotransformed into *A. thaliana*

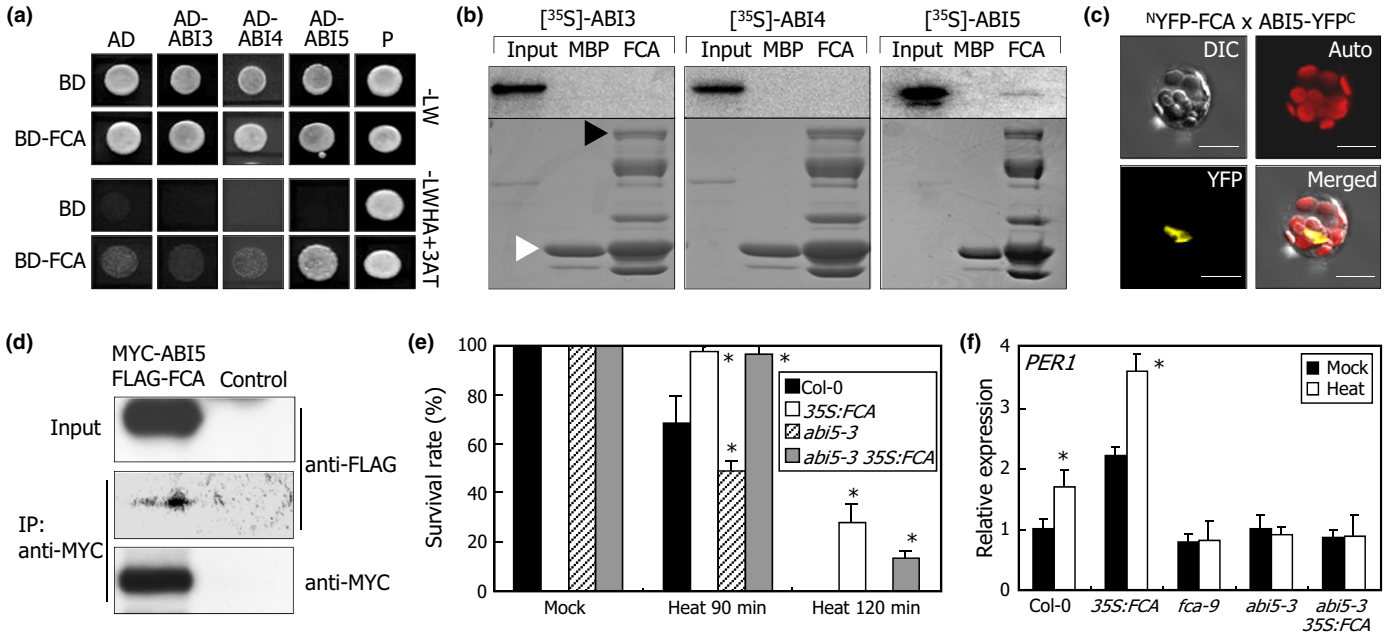


Fig. 4 FCA modulates ABA-INSENSITIVE 5 (ABI5)-mediated abscisic acid (ABA) signaling under heat stress. (e, f) The statistical significance of the measurements was determined using Student's *t*-test. Bars indicate the SE of the mean. (a) Interaction of FCA with ABI5 in yeast cells. Yeast cell growth on selective media without Leu, Trp, His, and Ade (–LWHA) but with 14 mM 3-amino-1,2,4-triazole (3-AT) represents a positive interaction. P, positive control; AD, activation domain; BD, binding domain. (b) *In vitro* pull-down assay. FCA protein was prepared as a maltose-binding protein (MBP) fusion in *Escherichia coli* cells. ABI3, ABI4, and ABI5 polypeptides were prepared as [^{35}S]methionine-labeled *in vitro* translation products. In the assay, the MBP protein was included as a control. Black arrowheads, MBP-FCA fusion protein; white arrowheads, MBP protein. The input represents 5% of the labeled ABI5 polypeptide used in the binding assay. (c) Bimolecular fluorescence complementation (BiFC) assay. Partial YFP constructs were fused with FCA or ABI5 and transiently coexpressed in *Arabidopsis thaliana* protoplasts; these constructs were then visualized using differential interference contrast (DIC) microscopy and fluorescence microscopy. Bar, 10 μm. (d) Coimmunoprecipitation assay. MYC-ABI5 and FLAG-FCA fusion constructs were transiently coexpressed in tobacco leaves. Control, protein extract without transient expression; Input, 10% of the protein extracts. (e) Heat survival test of *abi5-3 35S:FCA* plants. Heat survival rates were calculated as described in Figure 1 (*t*-test; *, $P < 0.01$; difference from heat-treated Col-0). (f) Expression of the *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*) gene. One-week-old *A. thaliana* plants grown on Murashige and Skoog (MS)-agar plates were exposed to 45°C for 90 min before harvesting; total RNA was then extracted from whole-plant material. Transcript levels were determined using reverse transcription–quantitative real-time PCR (RT-qPCR). Biological triplicates were averaged (*t*-test; *, $P < 0.01$; difference from mock).

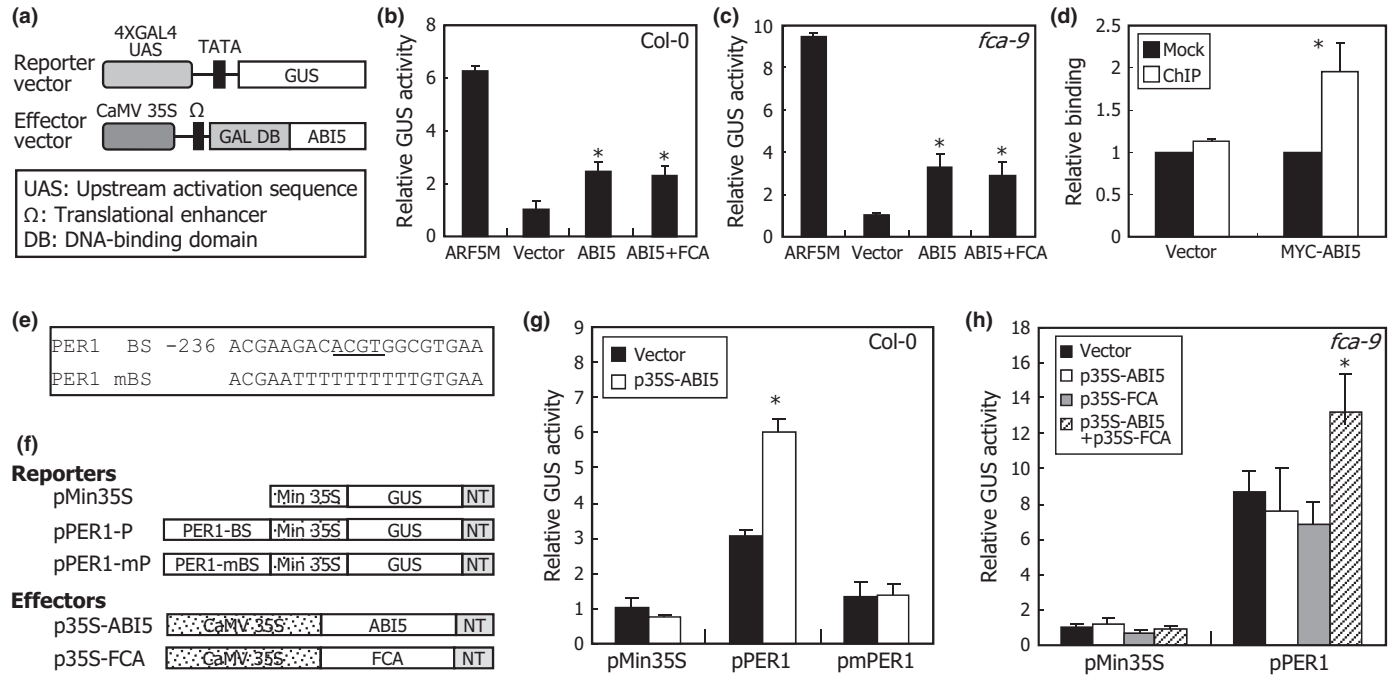


Fig. 5 FCA facilitates the binding of ABA-INSENSITIVE 5 (ABI5) to DNA. (b–d, g, h) The statistical significance of the measurements was determined using Student's *t*-test (*, $P < 0.01$; difference from vector control). Bars indicate the SE of the mean. (a) Illustration of the reporter and effector vectors. (b, c) GAL4 transient expression assays using (b) Col-0 and (c) *fca-9* protoplasts. ARF5M, transformation with the effector vector containing the *ARF5M* gene (activator control) (Tiwari *et al.*, 2003); Vector, transformation with the effector vector without gene inserts. A luciferase vector was cotransformed in each assay to normalize the transformation efficiency. Three measurements were averaged. (d) Chromatin immunoprecipitation (ChIP) assay of ABI5 binding to the *1-CYSTEINE PEROXIREDOXIN 1* (*PER1*) gene promoter. *35S:MYC-ABI5* transgenic plants grown on Murashige and Skoog (MS)-agar plates at 23°C for 10 d were harvested for ChIP assays. Transgenic plants harboring the 6 X MYC-pBA vector were used as control plants. The no antibody control is presented as a mock. MYC-specific antibody was used for immunoprecipitation. Three measurements were averaged. (e) ABI5-binding sequences in the proximal promoter regions of the *PER1* gene. Putative core ABRE elements are underlined. (f) Expression constructs. The promoter sequence elements shown in (e) were fused to a minimal 35S promoter and were used as reporters. In the effector vector, the *ABI5* and *FCA* genes were transcriptionally fused to the cauliflower mosaic virus (CaMV) 35S promoter. NT, Nos terminator. (g, h) Transient expression assays in *Arabidopsis thaliana* protoplasts using (g) Col-0 and (h) *fca-9* protoplasts. β-glucuronidase (GUS) activity was determined fluorimetrically. A luciferase vector was cotransformed in each assay to normalize the transformation efficiency. Three measurements were averaged.

protoplasts with a reporter plasmid containing the *GUS* gene (Fig. 5a). A plasmid containing the *Renilla reniformis* luciferase gene was also included for assay normalization. The transcriptional activation activity of ABI5 in the Col-0 protoplast was similar to that in the *fca-9* protoplast (Fig. 5b,c). In addition, coexpression of the *FCA* gene did not alter the activity of ABI5 in either of the genetic backgrounds, indicating that FCA does not affect the transcriptional activation activity of ABI5.

The *PER1* gene promoter contains a potential ABI5-binding motif (Haslekås *et al.*, 2003). We therefore examined whether ABI5 binds to the *PER1* gene promoter and, if so, whether FCA affects the DNA-binding property of ABI5. ChIP assays using *35S:MYC-ABI5* transgenic plants showed that ABI5 binds to the *PER1* gene promoter (Fig. 5d).

We next performed transient expression assays in *A. thaliana* protoplasts to determine whether FCA affects the DNA-binding activity of ABI5. The ABI5-binding sequence (BS) within the *PER1* gene promoter was used in the assays. The core binding sequence (ACGT) was mutated, resulting in PER1-mBS, to confirm the binding specificity (Fig. 5e). BS and mBS were fused to the CaMV 35S minimal promoter (pMin35S), resulting in the pPER1-P or pPER1-mP constructs (Fig. 5f). Reporter vectors and effector vectors, such as p35S-ABI5 and p35S-FCA, were transiently coexpressed in *A. thaliana* protoplasts. The coexpression of p35S-ABI5 with the pPER1-P reporter increased GUS activity *c.* 2-fold. By contrast, the coexpression of p35S-ABI5 with the pPER1-mP reporter did not alter reporter gene expression (Fig. 5g), consistent with the hypothesis that ABI5 is a transcriptional activator of the *PER1* gene (Haslekås *et al.*, 2003) (Fig. 5d).

Notably, the inductive effects of the coexpression of p35S-ABI5 with the pPER1-P reporter on *GUS* expression were absent in the *fca-9* protoplasts but were recovered when p35S-FCA was coexpressed (Fig. 5h). However, FCA did not directly bind to the *PER1* gene promoter (Fig. S16a,b). These observations suggest that FCA regulates the binding of ABI5 to the *PER1* gene.

It is critical to determine whether PER1 is associated with the thermotolerance response. To examine this issue, we measured the survival rates of the *PER1*-deficient *per1-1* and *per1-2* mutants under heat stress conditions (Fig. S17). The survival rates of the mutants were evidently reduced under the assay conditions (Fig. S18), supporting the notion that PER1 at least partly contributes to the FCA-mediated thermotolerance response.

fca mutation affects a variety of ROS-responsive genes

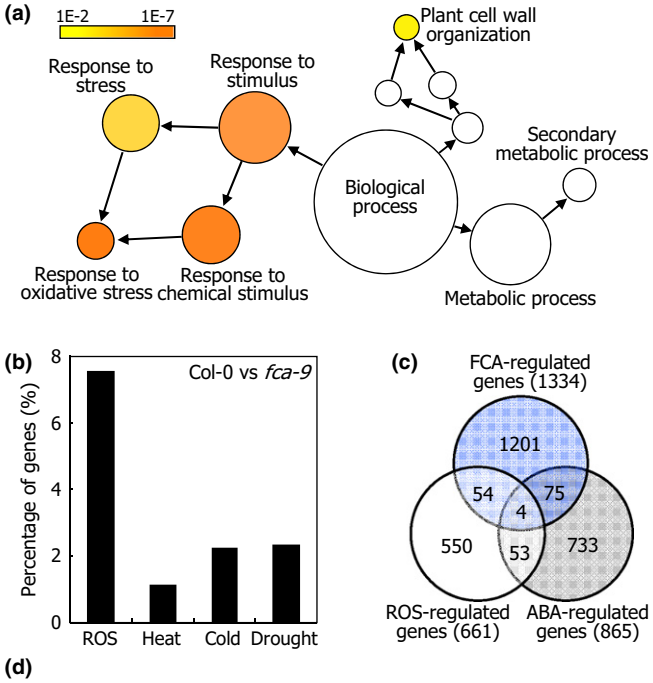
Based on the observation that *abi5-3 35S:FCA* plants are more resistant to heat stress than the *abi5-3* mutant (Fig. 4e), we anticipated the existence of an FCA-mediated thermotolerance pathway that is independent of ABI5. To obtain clues regarding the ABI5-independent role of FCA in thermotolerance, we performed RNA-Seq analysis. By comparing the RNA-Seq reads of Col-0 plants versus the *fca-9* mutant, we found that the expression of 1334 genes was altered by the *fca* mutation by > 2-fold (Table S2). Analysis of the gene ontology (GO) annotation, which is based on the Biological Networks Gene Ontology tool (BiNGO; Maere *et al.*, 2005), revealed that genes that are involved in responses to oxidative stress were most significantly overrepresented (Fig. 6a), supporting that idea that FCA is associated with the ROS response.

Among the 1334 genes that were affected by FCA, 970 were up-regulated and 364 were down-regulated (Table S2). BiNGO analysis showed that oxidative stress-responsive genes and cell wall organization-related genes were significantly up-regulated in the *fca-9* mutant, implying that feedback regulation exists between antioxidant capacity and oxidative stress-responsive genes (Fig. S19). Genes that are responsive to cold stress and biotic stress were significantly down-regulated in the *fca-9* mutant, suggesting that FCA might also be associated with stress responses other than the oxidative stress response (Fig. S20).

To confirm the BiNGO data, we analyzed GO terms using the GO annotation search tool in The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org), and stress genes were selected from the 1334 genes. It was found that ROS-related genes were most prominent among the selected stress genes (Fig. 6b). At least 22 genes, such as *RECEPTOR-LIKE PROTEIN KINASE 1* and *TYPE ONE SERINE/THREONINE PROTEIN PHOSPHATASE 2*, were annotated as belonging to ROS metabolic and signaling pathways (Table 1).

Notably, 12 genes encoding peroxidase members and their potential homologs were up-regulated in the *fca-9* mutant (i.e. *At2g38380*, *At5g66390*, *At5g17820*, *RARE COLD INDUCIBLE GENE3*, *At4g26010*, *At5g64100*, *At4g08770*, *At5g42180*, *At3g01190*, *At5g19890*, *At2g39040* and *At4g08780*; Table 1). Furthermore, *At2g21640*, which is annotated as a marker gene for the oxidative stress response in TAIR, was induced by > 4-fold in the *fca-9* mutant even under normal growth conditions, suggesting that the ROS response is constantly turned on in the mutant. However, expression of the *PER1*, *HSP60*, *HSP70*, *HSP90.1*, *HSP90.2*, *HSP101*, *GSH1*, *GSH2*, *VTC1*, *VTC2*, *APX1*, *DFR*, *CHS* and *PAP1* genes was not discernibly altered in the *fca-9* mutant under normal conditions, as observed using RT-qPCR analysis (Figs 3e, S7, S13).

We next compared the 1334 genes that were affected by the *fca* mutation in our RNA-Seq analysis with those in the previously reported microarray data, which were used to identify ROS-regulated and ABA-regulated genes (Davletova *et al.*, 2005; Li *et al.*, 2006). The comparative analysis showed that *RELATED TO AP 2.6* (*RAP2.6*), *URIDINE DIPHOSPHATE GLYCOSYL TRANSFERASE 74E2* (*UGT74E2*), *CALCIUM EXCHANGER 7* (*CAX7*) and *NITRATE TRANSPORTER* (*NRT1.5*) were predicted to be responsive to FCA, ROS and ABA (Fig. 6c,d). The RAP2.6 transcription factor is a component of ABA-dependent abiotic stress signaling pathways (Zhu *et al.*, 2010). CAX7 is a calcium/cation antiporter that participates in regulating calcium ion flux (Shigaki *et al.*, 2006), which is a key player in protecting plant cells from oxidative damage under heat stress conditions (Larkindale & Knight, 2002). UGT74E2 and NRT1.5 have been reported to mediate plant responses to various abiotic stresses, such as water deficit and salt and drought stresses (Tognetti *et al.*,



(d)

Locus	Gene annotation
AT5G13330	RAP2.6 (related to AP2 6)
AT5G17860	CAX7 (calcium exchanger 7)
AT1G05680	UGT74E2 (uridine diphosphate glycosyltransferase 74E2)
AT1G32450	NRT1.5 (nitrate transporter 1.5)

Fig. 6 FCA is related to the reactive oxygen species (ROS) response. (a) Enriched gene ontology (GO) terms of FCA-regulated genes. The network diagram presented visualizes the results of a Biological Networks Gene Ontology tool (BiNGO) analysis and shows significantly overrepresented GO terms for genes whose expression was altered in the *fca-9* mutant (1334 genes). The GO biological process term was used for analysis. Colored nodes represent significantly overrepresented GO terms (Benjamini & Hochberg corrected $P < 0.01$). Colored bars show the significance level (*P*-value). (b) GO analyses of stress-related genes regulated by FCA under normal growth conditions. The GO annotation search tool in TAIR (http://www.arabidopsis.org) was used for the analysis. Percentages represent the fractions of GO-annotated genes among the genes whose expression was altered in the *fca-9* mutant. (c) Venn diagram representing the overlap between FCA-, ROS-, and abscisic acid (ABA)-regulated genes. FCA-regulated genes were identified based on our RNA sequencing. ROS- and ABA-regulated genes were identified based on previously reported microarray data (Davletova *et al.*, 2005; Li *et al.*, 2006). (d) List of genes that are co-regulated by FCA, ROS and ABA.

Table 1 Selected reactive oxygen species (ROS)-related genes, the expression levels of which were significantly altered in the *fca-9* mutant grown under normal conditions

Locus	Fold(log_2)	*P*-value	Gene annotation	GO annotation
Genes involved in ROS metabolism				
At5g59160	−5.38	0.032340	TOPP2 (type one serine/threonine protein phosphatase2)	H_2O_2 biosynthetic process
At5g60900	−4.96	0.032340	RLK1 (receptor-like protein kinase1)	Regulation of H_2O_2 metabolic process
At2g40750	−1.14	0.046085	WRKY54 (WRKY DNA-binding protein54)	Regulation of H_2O_2 metabolic process
At1g49860	1.03	1.88E-07	GSTF14 (glutathione S-transferase)	Regulation of H_2O_2 metabolic process
At1g77120	1.05	0.005139	ADH1, ADH (alcohol dehydrogenase1)	Regulation of H_2O_2 metabolic process
At5g24090	2.02	0.006660	CHIA (chitinase A)	H_2O_2 biosynthetic process
At4g02430	5.54	0.030190	SR34b (RNA-binding family protein)	H_2O_2 biosynthetic process
ROS-responsive genes				
At1g60470	−1.38	0.017552	GolS4 (galactinol synthase4)	Response to oxidative stress
At4g31870	1.02	6.24E-05	GPX7 (glutathione peroxidase7)	Response to oxidative stress
At2g38380	1.20	6.35E-17	Peroxidase superfamily protein	Response to oxidative stress
At5g66390	1.33	4.16E-08	Peroxidase superfamily protein	Response to oxidative stress
At5g17820	1.39	3.29E-11	Peroxidase superfamily protein	Response to oxidative stress
At1g05260	1.53	3.15E-13	RCI3, RCI3A (peroxidase superfamily protein)	Response to oxidative stress
At4g26010	1.72	7.32E-06	Peroxidase superfamily protein	Response to oxidative stress
At5g64100	1.82	3.09E-25	Peroxidase superfamily protein	Response to oxidative stress
At4g08770	1.94	0.003728	Prx37 (peroxidase superfamily protein)	Response to oxidative stress
At2g21640	2.28	2.16E-07	A marker for oxidative stress response	Response to oxidative stress
At5g42180	2.42	2.11E-11	Peroxidase superfamily protein	Response to oxidative stress
At3g01190	2.82	6.50E-24	Peroxidase superfamily protein	Response to oxidative stress
At5g19890	5.77	0.030190	Peroxidase superfamily protein	Response to oxidative stress
At2g39040	6.53	0.003709	Peroxidase superfamily protein	Response to oxidative stress
At4g08780	7.57	5.60E-05	Peroxidase superfamily protein	Response to oxidative stress

GO, gene ontology.

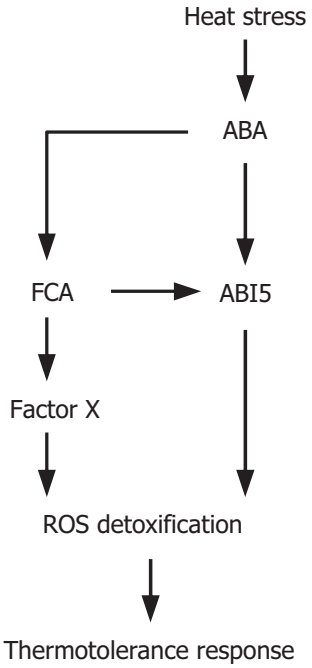


Fig. 7 Proposed scheme of the role of FCA in the thermotolerance response. Under heat stress, FCA interacts with ABA-INSENSITIVE 5 (ABI5), which regulates genes encoding reactive oxygen species (ROS) detoxifying enzymes, resulting in thermotolerance. In this working scenario, FCA facilitates the binding of ABI5 to DNA. It is likely that FCA also modulates an additional factor(s) (i.e. factor X) that mediates the ROS detoxification pathway.

2010; Chen *et al.*, 2012). It is therefore possible that RAP2.6, UGT74E2, CAX7, and NRT1.5 might be additional targets of FCA, other than ABI5, in the thermotolerance response.

Taken together, these findings suggest that FCA induces thermotolerance by promoting antioxidant capacity via ABA-mediated heat stress signaling pathways. In this signaling scheme, FCA promotes the binding of ABI5 to the promoter of the *PER1* gene, which is involved in ROS scavenging (Fig. 7). Our data provide evidence for a previously unknown role for FCA, in addition to its major role in flowering time control, in the ABA-mediated thermotolerance response in plants.

Discussion

The role of FCA in the induction of thermotolerance

Controlled RNA metabolism and editing, which are mediated by various RNA-binding proteins and riboregulators, constitute a critical part of the gene regulatory networks that govern plant responses to developmental cues and environmental constraints. Several RNA-binding proteins function as key regulators of diverse stress responses in plants. A small set of glycine-rich RNA-binding proteins (GRPs) have been shown to play a role in plant responses to abiotic stresses. For example, AtRZ-1a and GRP2 enhance freezing tolerance and promote seed germination and seedling growth at low temperatures (Kim *et al.*, 2005, 2007). It is notable that GRP2 regulates the activity of ROS-metabolizing enzymes under stress conditions (Kim *et al.*, 2007),

although the underlying molecular mechanisms have not yet been defined.

In this work, we report a distinct role for FCA in the induction of thermotolerance in *A. thaliana*. We found that the *FCA* gene is induced by high temperatures through the ABA signaling pathway that mediates antioxidant metabolism. The *FCA* gene is also affected by cold temperatures but is not affected by drought or high salinity (Fig. 1d). FCA is therefore postulated to be a constituent of the signaling pathways that mediate plant adaptation responses to a physiological range of high temperatures and temperature extremes. Extensive examination of the effects of various temperatures on *fca* mutant phenotypes and on *FCA* gene expression and protein stability will help to elucidate the physiological relevance of FCA in the temperature stress response.

Detoxification of ROS by FCA signaling

Heat stress triggers the accumulation of ROS in plants; thus, plants have evolved several defense mechanisms to cope with ROS-induced oxidative damage (Mittler, 2002; Kim *et al.*, 2012). We found that FCA mediates ROS detoxification. ROS accumulated to high levels in the *fca* mutants (Figs 2e,f, S8), and total antioxidant capacity was significantly reduced by the *fca* mutation (Fig. 3d). These observations are explained at least in part by transcriptional regulation of the *PER1* gene by FCA. Whereas the *PER1* gene is induced by heat and *FCA* overexpression, the heat induction of the *PER1* gene is compromised in the *fca* mutants (Figs 3e, S14).

It is notable that genes encoding peroxidase superfamily proteins other than PER1 are induced in the *fca-9* mutant, as found using RNA-Seq (Table 1). This might be attributable to feedback regulation between antioxidant capacity and oxidative stress genes. Alternatively, FCA might regulate antioxidant genes at the translational level. In fact, the FCA RNA-binding protein has been shown to act as a transcriptional regulator by modulating RNA processing or chromatin modification (Liu *et al.*, 2007, 2010). It is also possible that the binding of FCA to its target RNAs might enhance the translation of target mRNAs. It has been shown that a mammalian RNA-binding protein, HuR, enhances the translation of p53 by binding to its mRNA in UV-treated cells (Mazan-Mamczarz *et al.*, 2003). The p53 transcription factor induces many tumor-suppressor genes; thus, the HuR-mediated enhancement of p53 translation protects mammalian cells from UV-induced tumorization. It will be interesting to examine whether FCA binds to the mRNAs of oxidative stress genes and enhances their translation under heat stress conditions.

Functional mechanism of FCA in antioxidant metabolism

FCA physically interacts with the ABI5 transcription factor, which activates the *PER1* gene. It is important to understand the mechanism by which FCA modulates ABI5 activity in antioxidant biosynthesis.

FCA mediates the action of a diverse class of enzymes and regulators that are involved in the transcriptional and posttranscriptional control of plant responses to developmental and environmental signals. FCA largely functions in these processes through RNA metabolism and chromatin modification. On the basis of previous observations, we propose that FCA acts as a transcriptional coactivator in the ABI5 induction of downstream genes. Transcriptional coregulators are frequently used in eukaryotic gene expression. NONEXPRESSER OF PR GENES 1 (NPR1) is an ankyrin repeat-containing protein that serves as an SA receptor (Wu *et al.*, 2012) and that regulates the transcriptional activity of the TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 2 (TGA2) transcription factor through protein–protein interactions (Després *et al.*, 2000). Similar to the role of FCA in the regulation of ABI5 activity (this work), NPR1 enhances the binding of TGA2 to the promoter of target genes (Després *et al.*, 2000). The human RRM-containing coactivator activator (CoAA) protein, which belongs to the heterogeneous nuclear ribonucleoprotein (hnRNP)-like protein family (Iwasaki *et al.*, 2001), is an RNA-binding protein that interacts with the transcriptional coactivator thyroid hormone receptor-binding protein (TRBP) to regulate gene transcription and RNA splicing (Auboeuf *et al.*, 2004; Verreman *et al.*, 2011).

We observed that FCA enhances the DNA-binding of ABI5. However, it remains unclear how FCA regulates ABI5 activity. FCA has been shown to bind to the 3′ sequence region of the *FLC* locus (Liu *et al.*, 2007). The FCA–ABI5 heterodimer would have a higher affinity for DNA than ABI5 monomers and homodimers. We cannot exclude the possibility that FCA affects the protein stability or conformation of ABI5.

Our data show that the *PER1* gene, which is regulated by the ABI5 transcription factor (Haslekås *et al.*, 2003), plays a key role in FCA-mediated ABA signaling. However, it appears that FCA-mediated ABA signals are not mediated solely by ABI5. We observed that, although increased *PER1* expression in *35S:FCA* transgenic plants was completely suppressed by the *abi5-3* mutation (Fig. 4f), the thermotolerance of the *FCA*-overexpressing plant was only partially affected by the *abi5-3* mutation (Fig. 4e). It is therefore clear that FCA is also involved in ABI5-independent signaling pathways, possibly through interactions with a transcription factor that has not yet been identified (factor X; see Fig. 7). Our RNA-Seq data, together with the previously reported microarray data (Davletova *et al.*, 2005; Li *et al.*, 2006), predicted that expression of the *RAP2.6*, *CAX7*, *UGT74E2*, and *NRT1.5* genes is affected by FCA-mediated ABA signaling. It is currently unknown whether these genes are regulated by ABI5. The coordinated action of FCA and factor X might target the *RAP2.6*, *CAX7*, *UGT74E2*, and *NRT1.5* genes, which are involved in various aspects of abiotic stress responses (Shigaki *et al.*, 2006; Tognetti *et al.*, 2010; Zhu *et al.*, 2010; Chen *et al.*, 2012), in an ABI5-independent manner.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Expression of *FCA* and *FLC* genes in *35S:FCA* transgenic plants and *fca* mutants.

Fig. S2 Measurements of survival rate after heat treatment (37°C).

Fig. S3 Measurements of survival rate after heat acclimation.

Fig. S4 Expression of the *FCA* gene at 37°C.

Fig. S5 Measurement of chlorophyll contents in *35S:FCA* transgenic plants after ABA treatments.

Fig. S6 Trypan blue staining of *35S:FCA* transgenic plants and the *fca-11* mutant after heat treatment.

Fig. S7 Expression of heat shock genes in *35S:FCA* transgenic plants and the *fca-9* mutant after heat treatments.

Fig. S8 3,3′-Diaminobenzidine (DAB) staining of *35S:FCA* transgenic plants and the *fca-11* mutant after heat treatment.

Fig. S9 Oxidative stress response of *35S:FCA* transgenic plants and the *fca-11* mutant.

Fig. S10 Effects of ascorbate (ASC) under heat stress.

Fig. S11 Measurements of glutathione content.

Fig. S12 Accumulation of anthocyanins in *35S:FCA* transgenic plants.

Fig. S13 Expression of the genes encoding antioxidant-metabolizing enzymes.

Fig. S14 Expression of the *PER1* gene in the *fca-11* mutant.

Fig. S15 Bimolecular fluorescence complementation (BiFC) assays on control expression constructs.

Fig. S16 FCA does not bind to the *PER1* gene promoter.

Fig. S17 RT-PCR analysis of *PER1* transcripts in *per1-1* and *per1-2* mutants.

Fig. S18 PER1 confers a thermotolerance response.

Fig. S19 Enriched gene ontology (GO) terms of up-regulated genes in the *fca-9* mutant.

Fig. S20 Enriched GO terms of down-regulated genes in the *fca-9* mutant.

Table S1 Primers used in RT-qPCR

Table S2 FCA-regulated genes identified by RNA-Seq

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