MYB30 transcription factor regulates oxidative and heat stress responses through ANNEXIN-mediated cytosolic calcium signaling in *Arabidopsis*

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**Summary**

- Cytosolic calcium signaling is critical for regulating downstream responses in plants encountering unfavorable environmental conditions.
- In a genetic screen for *Arabidopsis thaliana* mutants defective in stress-induced cytosolic free Ca²⁺ ([Ca²⁺]ₙ) elevations, we identified the R2R3-MYB transcription factor MYB30 as a regulator of [Ca²⁺]ₙ in response to H₂O₂ and heat stresses.
- Plants lacking MYB30 protein exhibited greater elevation of [Ca²⁺]ₙ in response to oxidative and heat stimuli. Real-time reverse transcription–polymerase chain reaction (RT-PCR) results indicated that the expression of a number of ANNEXIN (ANN) genes, which encode Ca²⁺-regulated membrane-binding proteins modulating cytosolic calcium signatures, were upregulated in *myb30* mutants. Further analysis showed that MYB30 bound to the promoters of *ANN1* and *ANN4* and repressed their expression. *myb30* mutants were sensitive to methyl viologen (MV) and heat stresses. The H₂O₂- and heat-induced abnormal [Ca²⁺]ₙ in *myb30* was dependent on the function of ANN proteins. Moreover, the MV and heat sensitivity of *myb30* was suppressed in mutants lacking ANN function or by application of LaCl₃, a calcium channel blocker.
- These results indicate that MYB30 regulates oxidative and heat stress responses through calcium signaling, which is at least partially mediated by ANN1 and ANN4.

**Introduction**

The calcium ion (Ca²⁺) is an important intracellular secondary messenger in plants (Poovaiah & Reddy, 1993) and plays important roles in the response to both environmental and internal signals. Various abiotic and biotic stimuli, including cold (Knight et al., 1991), heat (Gong et al., 1998), osmotic stress (Kiegle et al., 2000), salt and drought (Knight et al., 1998), light (Shacklock et al., 1992), plant hormones (Schroeder et al., 2001), fungal elicitors and nodulation factors (Knight et al., 1991), trigger elevations of the cytosolic free Ca²⁺ concentration ([Ca²⁺]ₙ). Such increasing cytosolic free Ca²⁺ is released from internal Ca²⁺ stores and/or the extracellular matrix (Kudla et al., 2010), and vary in duration, magnitude, frequency, and spatial distribution depending on the stimuli (Reddy et al., 2011). [Ca²⁺]ₙ signals are decoded by intracellular Ca²⁺ sensors such as calmodulins (CaMds) (Zielinski, 1998; Luan et al., 2002; McCormack et al., 2005), calcium-dependent protein kinases (CDPKs) (Harmon et al., 2000; Romeis et al., 2001) and salt overly sensitive 3 (SOS3)-like calcium-binding proteins (SCaBPs)/calcineurin B-like proteins (CBLs) (Weinl & Kudla, 2009), and lead to the modulation of downstream phosphorylation and transcriptional events (Knight et al., 1991; Trewavas, 1999; White & Broadley, 2003; DeFalco et al., 2010).

Reactive oxygen species (ROS) are highly reactive molecules including H₂O₂, HClO and free radicals (Valko et al., 2007). Enhanced concentrations of ROS are generated endogenously in response to a number of biotic and abiotic stresses, such as pathogenic elicitors, salt, ABA, heat, cold, drought or high light intensity (Lamb & Dixon, 1997; Pei et al., 2000; Zhu, 2001; Volkov et al., 2006; Leshem et al., 2007). ROS are potent regulators of plasma membrane Ca²⁺ channels. In *Arabidopsis*, ROS treatment activates the Ca²⁺-permeable channels (Pei et al., 2000) and triggers Ca²⁺ influx (Rentel & Knight, 2004). Multiple proteins with transmembrane transport activity for calcium ions have been identified in plants, such as cyclic nucleotide-gated channels (CNGCs), Mid1-complementing activities (MCAs), and ANNEXINs (ANNs) (Hua, 2003; Ali et al., 2007; Nakagawa et al., 2007; Laohavisit et al., 2012). ANNs genes, encoding Ca²⁺-dependent phospholipid-binding proteins, comprise a multigene family in both plants and animals and participate in diverse cellular processes (Gerke & Moss, 2002; Rescher & Gerke, 2004; Mor-timer et al., 2008; Divya et al., 2010; Jami et al., 2012). Plant ANNs are reported to be involved in abiotic stress responses such as...
as responses to oxidative, heat, drought and salt stresses (Lee et al., 2004; Konopka-Postupska et al., 2009; Divya et al., 2010; Huh et al., 2010; Richards et al., 2014; Qiao et al., 2015; Wang et al., 2015a) and participate in H$_2$O$_2$-activated Ca$^{2+}$ fluxes that mediate the cross-talk between calcium and ROS in stress signaling pathways (Konopka-Postupska et al., 2009; Laohavisit et al., 2010). AtANN1 has been proposed to act as a H$_2$O$_2$ sensor (Konopka-Postupska et al., 2009) and has Ca$^{2+}$-permeable transporter activity (Laohavisit et al., 2012). The loss-of-function atann1 mutant fails to produce a OH$^-$ and NaCl-induced [Ca$^{2+}$]$_{cyt}$ elevation in root epidermal cells (Laohavisit et al., 2013). In the presence of Ca$^{2+}$, AtANN1 and AtANN4 bind to each other and function cooperatively to regulate the osmotic stress response, ABA signaling (Lee et al., 2004), and drought and salt stress responses (Huh et al., 2010). AtANN1 and Brassica juncea Annexin 3 (AnnB3) are reported to exhibit antioxidative activity under oxidative stress (Dalal et al., 2014; Richards et al., 2014). ANN1 positively regulates the heat shock (HS) response and mediates the heat-induced [Ca$^{2+}$]$_{cyt}$ elevation (Qiao et al., 2015; Wang et al., 2015a) in Arabidopsis and rice (Oryza sativa). However, it is not clear how ANNs are regulated at transcriptional and post-translational levels.

The MYB30 (MYB) family is a key family of transcription factors functioning in the regulation of development, metabolism and stress response in Arabidopsis (Dubos et al., 2010), among which the R2R3-MYB type is the largest subfamily (Stracke et al., 2004). Arabidopsis MYB30 is one of the best characterized R2R3-MYB transcription factors and is involved in multiple stress responses. MYB30 plays an important role in the initiation of cell death (Daniel et al., 1999) and regulation of the hypersensitive response (HR) (Vailleau et al., 2002). MYB30 also participates in salicylic acid (SA), brassinosteroid (BR) and abscisic acid (ABA) signaling (Raffaele et al., 2006; Li et al., 2009; Zheng et al., 2012), mediates the cross-talk between flowering time and biotic stress perception (Liu et al., 2014), and functions in the very-long-chain fatty acid (VLCPA)/acyl-CoA-binding protein 3 (ACBP3)-mediated hypoxia response (Xie et al., 2015). However, it is not clear how MYB30 mechanistically functions in different signaling pathways.

In this study, we found that MYB30 participates in the regulation of stress-induced [Ca$^{2+}$]$_{cyt}$ elevation through forward-genetic screening. The disordered H$_2$O$_2$- and heat-induced [Ca$^{2+}$]$_{cyt}$ elevation in the myb30 mutant was probably caused by the enhanced expression of ANV genes resulting from the transcriptional repression release of MYB30 which directly bound to their promoters. These disordered calcium signals were probably responsible for the phenotypic alterations of the myb30 mutant under H$_2$O$_2$- and heat-stress treatments. Taken together, our results reveal a novel function of MYB30 in the generation of stress-induced [Ca$^{2+}$]$_{cyt}$ signals and highlight the specific function of precisely fine-tuned calcium signals generated by various calcium channels.

Materials and Methods

Plant material and growth

Arabidopsis thaliana L. Heynh. Columbia (Col-0) constitutively expressing Aequorin (AQ) (Knight et al., 1991) driven by the cauliflower mosaic virus 35S promoter was used as the wild-type. Seeds were sterilized in a solution containing 20% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 for 10 min, washed five times with sterilized distilled water and sown on Murashige and Skoog (MS) medium containing 2.5% (w/v) sucrose and 0.3% (w/v) phytagar (Sigma-Aldrich), kept in the dark for 3 d at 4°C, and then grown in a growth chamber at 23°C, 4000 lux under continuous white light. High-throughput screening of [Ca$^{2+}$]$_{cyt}$ response mutants from a T-DNA insertion pool (pSKI015) in the wild-type background was described previously (Pan et al., 2012). Mutants myb30-1 (SALK_122884), ann1 (SALK_015426) and ann4 (SALK_019725) were obtained from the Arabidopsis Biological Resource Center (ABRC).

Cytosolic Ca$^{2+}$ measurements

For Aequorin assays, 9-d-old seedlings growing on MS medium were used for [Ca$^{2+}$]$_{cyt}$ measurement. The [Ca$^{2+}$]$_{cyt}$ luminescence measurements were performed as described previously (Zhao et al., 2013), and the quantification of [Ca$^{2+}$]$_{cyt}$ (in μmol l$^{-1}$) was performed as described by Rentel & Knight (2004). The plates were sprayed with 10 mM H$_2$O$_2$ and the photon emissions were immediately collected for 3 min. For heat treatment, 30 ml of H$_2$O at 45°C was poured into the plates and the photon emissions were immediately collected for 8 min.

For the Fluo-4 assay, the 7-d-old vertically cultured seedlings of wild-type and myb30 were incubated with 10 μM Fluo-4-AM in the presence of 0.02% (v/v) pluronic F-127 for 2 h at 37°C. Fluorescence in the roots of stained seedlings was captured using the Andor IQ confocal microscope (Andor, Belfast, Northern Ireland) (excitation at 488 nm and emission at 520 nm) after stress treatments.

Complementation assays

The MYB30 genomic DNA fragment (corresponding to the sequence from 2480 bp upstream of the MYB30 translation start codon to 500 bp downstream of the stop codon) was cloned into the pCAMBIA1300 vector between the SacI and BamHI sites. The resulting construct was introduced into the myb30-2 mutant using Agrobacterium tumefaciens-mediated floral transformation (Clough & Bent, 1998). The T$_4$ transgenic plants (COM) were used for complementation analyses. Primers used for cloning are listed in Supporting Information Table S1.

Plasmid construction and generation of transgenic plants

The MYB30 coding region was cloned into the pCAMBIA1307-MYC binary vector driven by the 35S promoter. MYB30 over-expressing transgenic plants were generated using A. tumefaciens-mediated floral transformation. Reverse transcription–polymerase chain reaction (RT-PCR) and immunoblot analysis were used to detect the abundance of the transgenes and proteins. Stable MYB30 overexpression (OE) T$_4$ lines were used for analyses. Primers used for cloning are listed in Table S1.
Real-time and RT-PCR analysis

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) from 9-d-old seedlings grown on MS medium. Total RNA was treated with RNase-free DNase I (Takara, Kuusatsu, Japan) to remove genomic DNA and then reverse transcribed with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s instructions. cDNA was used for quantitative real-time PCR or RT-PCR analysis.

Real-time PCR was performed using SYBR Green PCR Master Mix (Takara) on a 7500 fast real-time PCR System (Applied Biosystems, Foster City, CA, USA) following the manufacturer’s protocol. Relative quantitative results were calculated by normalization to Actin. Actin was used as an internal control. Primers used for real-time PCR and RT-PCR are listed in Table S1.

Immunoblot analysis

Total proteins were extracted from 9-d-old seedlings. Equal amounts of each sample were separated on a 12% (w/v) sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride membranes. Proteins were probed by immunoblot analysis with anti-AQ, anti-MYC, anti-FLAG (DYKDDDDK; Sigma-Aldrich), anti-ACTIN or anti-ANN1 antibodies. The chemiluminescence signals were detected by autoradiography.

Transactivation activity assays in Arabidopsis protoplasts

A reporter plasmid containing the ANN promoter upstream of the reporter gene LUCIFERASE (LUC) was used. Protoplast preparation and transformation were performed as described previously (Sheen, 2001). After overnight incubation at 23°C, the protoplasts were transferred to a 12-well plate. Fifty microliters of D-luciferin (1 mM) (Promega) was added into each sample and the plate was kept in the dark for 5 min. A CCD camera (Roper Scientific, Martinsried, Germany) was used to obtain the LUC signal. Primer sequences for cloning ANN promoters are listed in Table S1.

Chromatin immunoprecipitation—quantitative PCR (ChIP-qPCR) assay

The full-length MYB30 genomic DNA fragment derived from the MYB30 native promoter (corresponding to the sequence 2480 bp upstream of the MYB30 translation start codon) was cloned into the pCAMBIA1305-FLAG vector. The resulting construct was introduced into the myb30-2 mutant and the stable MYB30-FLAG T4 transgenic plants were used for ChIP assay. Primers used for cloning are listed in Table S1.

The ChIP assay was performed as described previously (Blecher-Gonen et al., 2013). Chromatin isolation was performed using 9-d-old seedlings of the myb30-2 mutant and the MYB30-FLAG transgenic line. Monoclonal anti-FLAG antibody (Sigma-Aldrich) was used. Both immunoprecipitated DNA and input DNA were analyzed by real-time quantitative PCR; Actin was amplified as a control. At least three independent experiments were performed with similar results. The primers used for this assay are listed in Table S1.

Protein expression and electrophoretic mobility shift assay (EMSA) experiment

The 1–477 bp coding sequence of MYB30 was amplified and cloned into the pET28a vector. The plasmid was introduced to Escherichia coli strain BL21. Escherichia coli cells were treated with 0.2 mM isopropyl-β-d-thiogalactopyranoside (IPTG) overnight at 16°C and collected by centrifugation. The His-MYB30 fusion protein was purified. Primers used for cloning are listed in Table S1.

For EMSA, the fragments of the promoters were obtained by annealing using biotin-labeled or -unlabeled primers. Biotin-unlabeled fragments of the same sequences were used as competitors. The reaction mixture (10 μl) for EMSA contained 0.5 μg purified protein, 1 μl 50 μg ml−1 biotin-labeled annealed oligonucleotide, 2 μl 5× binding buffer (Beyotime, Shanghai, China), and ultrapure water. The reactions were incubated at 22°C for 30 min. The reactions were fractionated on a 6% native polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). The EMSA was conducted using a Chemiluminescent EMSA Kit (Beyotime) following the manufacturer’s protocol. The primers used for this assay are listed in Table S1.

Yeast one-hybrid assay

Yeast one-hybrid assays were performed according to the instructions provided with the Matchmaker LexA two-hybrid system (Clontech, Mountain View, CA, USA). In brief, the full-length coding sequence (CDS) of MYB30 was cloned into the pB42AD vector, and different fragments of ANN promoters were cloned into the pLacZi2 vector (Clontech). The MYB30 and ANN promoter plasmids were co-transformed into the EGY48 yeast strain, which contains a reporter plasmid (p8op-lacZ). Yeast transformants were then plated on minimal SD/-His-Trp-Ura agar plates for 3 d at 28°C. Finally, well-grown colonies were plated onto minimal SD/Gal/Raf/-His-Trp-Ura agar plates with X-Gal for interaction tests.

Germination assay

Arabidopsis seeds were sown on half-strength MS medium without or with the indicated concentration of MV (Sigma-Aldrich) or ABA (Sigma-Aldrich), kept in the dark at 4°C for 3 d, and then transferred to growth chambers. The percentage of green cotyledons was determined at the indicated time.

Heat shock assay

Sterilized seeds were sown in glass plates containing half-strength MS, 1.5% (w/v) sucrose and 0.36% (w/v) phytagar (Sigma-
Aldrich). After being kept at 4°C in darkness for 3 d, seeds were allowed to grow at 23°C under long-day conditions (16 h:8 h, light:night) for 6 d. For the heat treatment, the plates were wrapped in general kitchen plastic wrap and submerged in a water bath at 45°C for 16 min. Then the seedlings were returned to 23°C to recover for 1 wk before photographs were taken.

For chlorophyll content analysis, seedlings were weighed and put into 1.5-ml Eppendorf tubes containing 1 ml of 80% (v/v) acetone. Samples were incubated in the dark overnight at room temperature. The samples were centrifuged at 13 000 g for 10 min and each supernatant was transferred into a single cuvette. The absorption was measured at 663 and 645 nm with a spectrophotometer. The concentration of chlorophyll (total chlorophyll, Chl$\text{a}$ and Chl$\text{b}$) was calculated according to the following formula: Chl$\text{a}$ = $12.7A_{665} - 2.69A_{645}$; Chl$\text{b}$ = $22.9A_{645} - 4.68A_{633}$; and total chlorophyll = Chl$\text{a}$ + Chl$\text{b}$.

Lanthanum chloride/EGTA treatment

For Ca$^{2+}$ luminescence measurements, before the H$_2$O$_2$ or HS treatment was applied, 30 ml of 50 lM LaCl$_3$/500 lM EGTA solutions (Sigma-Aldrich) or H$_2$O was poured into the plates in the dark for pretreatment. After 20 min of incubation, the liquid in the plates was discarded, 10 mM H$_2$O$_2$ was sprayed onto the seedlings or 30 ml of H$_2$O at 4°C was poured into the plates, and then the photon emissions were collected immediately.

For the oxidative stress assay, a piece of filter paper was put into the culture dish, and 3 ml of half-strength MS liquid medium containing the indicated concentration (as shown in each figure) of MV and LaCl$_3$/EGTA was added to each culture dish, respectively. Arabidopsis seeds were sown on these media. After being kept at 4°C in darkness for 3 d, seeds were allowed to grow in a growth chamber. The percentage of green cotyledons was determined on the seventh day.

For the heat shock assay, 6-d-old seedlings grown at 23°C were submerged into 50 lM LaCl$_3$ or H$_2$O. After 20 min of incubation, the treated seedlings were transferred to a new plate containing half-strength MS medium. Then the plates were wrapped in general kitchen plastic wrap and submerged in a water bath at 45°C for 16 min. Finally, the plates were transferred to the growth chamber at 23°C to recover for 5 d before photographs were taken.

Results

The 1128 mutant exhibits enhanced [Ca$^{2+}$]$_{cyt}$ elevation in response to H$_2$O$_2$ treatment

Arabidopsis ecotype Col-0 with stable expression of AQ was used as the wild-type to monitor the change in the cytosolic free calcium concentration ([Ca$^{2+}$]$_{cyt}$) in response to stresses (Knight et al., 1991). A pooled T-DNA (A. tumefaciens-transformed DNA) insertion library based on this line was screened for mutants with abnormal [Ca$^{2+}$]$_{cyt}$ changes (Zhao et al., 2013). For screening, 9-d-old seedlings were treated with 10 mM H$_2$O$_2$ and luminescence was measured immediately with a cold charge-coupled device (CCD) imaging system. One of the detected mutants, 1128, showed a significantly greater [Ca$^{2+}$]$_{cyt}$ elevation
upon H$_2$O$_2$ treatment (Fig. 1a). The mean peak value of the calcium signal in 1128 was three times as high as that in the wild-type ($P \leq 0.05$) (Fig. 1b). Next, the [Ca$^{2+}$]$_{cyt}$ changes in wild-type and 1128 under H$_2$O$_2$ treatment were measured using a luminometer. The peak value of [Ca$^{2+}$]$_{cyt}$ in 1128 was as high as 0.93 ± 0.13 μmol l$^{-1}$ (mean ± SD), much higher than that in wild-type (0.54 ± 0.04 μmol l$^{-1}$) (Fig. 1c). To rule out the possibility that the difference in calcium signals between wild-type and 1128 was attributable to unequal amounts of total AQ, the protein levels of AQ were determined using an anti-AQ antibody. The obtained data indicated that the AQ level in wild-type was similar to that in the 1128 mutant (Fig. 1d). We also studied H$_2$O$_2$-induced [Ca$^{2+}$]$_{cyt}$ elevation in 1128 using the fluorescent dye Fluo-4 acetoxymethyl (AM) ester, which specifically detects cytosolic free calcium (Wang et al., 2015b). Before H$_2$O$_2$ treatment, no significant difference was observed in [Ca$^{2+}$]$_{cyt}$ in roots between wild-type and 1128 (Fig. 1e). However, [Ca$^{2+}$]$_{cyt}$ increased much more strongly in 1128 compared with the wild-type after H$_2$O$_2$ treatment. These results suggest that 1128 participates in cytosolic calcium signaling regulation under oxidative stress.

MYB30 functions as a negative regulator of calcium signaling upon H$_2$O$_2$ exposure

To identify the mutation causing this phenotype, we performed thermal asymmetric interlaced PCR (TAIL-PCR) to detect the insertion of the T-DNA fragment in the 1128 mutant. The T-DNA in 1128 was detected in exon 1 of At3g28910, which is the same as in the myb30-2 mutant that we identified previously from screening ABA-sensitive mutants (Zheng et al., 2012). To confirm that the phenotype observed in 1128 is attributable to the disruption of MYB30, MYB30 genomic DNA expressed under the control of the native MYB30 promoter was transformed into the myb30-2 mutant for the complementation assay. Two independent T$_4$ transgenic complement lines, COM-1 and COM-2, were used for tests, in which both the MYB30 mRNA and AQ protein levels were equal to those of the wild-type (Fig. S1a,b). The results showed that the H$_2$O$_2$-induced aberrant [Ca$^{2+}$]$_{cyt}$ elevation in the myb30-2 mutant was recovered to the wild-type level by expressing MYB30 genomic DNA (Fig. 2a,b).

To further determine the role of MYB30 in H$_2$O$_2$-induced [Ca$^{2+}$]$_{cyt}$ elevation, a Pro35S:MYC-MYB30 plasmid was transferred into the wild-type. Two independent T$_4$ transgenic lines (MYB30 OE-1 and MYB30 OE-2) with equivalent expression of AQ protein and increased expression of MYB30 were chosen for further analyses of [Ca$^{2+}$]$_{cyt}$ dynamics (Fig. S1c,d). The results showed that the overexpression of MYB30 could repress the [Ca$^{2+}$]$_{cyt}$ elevation to a very low level (Fig. 2c,d). These results suggest that MYB30 is a negative regulator of calcium signaling in response to H$_2$O$_2$ treatment.

MYB30 negatively regulates the expression of ANNEXIN genes

MYB30 is an R2R3-MYB transcription factor and functions in HR and ABA signaling pathways by regulating the expression of target genes (Daniel et al., 1999; Vailleau et al., 2002; Zheng et al., 2012). To determine the potential target genes of MYB30 in regulating [Ca$^{2+}$]$_{cyt}$ elevation, RNA sequencing (RNA-seq) analysis of wild-type and MYB30 OE-2 was performed. Focusing on the genes functioning in [Ca$^{2+}$]$_{cyt}$ regulation, three ANN genes,
ANN1, ANN3 and ANN4, the expression levels of which were upregulated in myb30-2, were identified and selected for further studies. As ANNs have been identified to participate in ROS-activated [Ca^{2+}]_{cyt} signaling in plants (Laohavisit et al., 2012, 2013; Richards et al., 2014), we speculated that they might work downstream of MYB30 to mediate H_2O_2-induced [Ca^{2+}]_{cyt} elevation.

To confirm the repression of ANN genes by MYB30, the expression levels of ANN1, ANN3 and ANN4 were examined by real-time RT-PCR (Fig. 3a). The expression of ANN1, ANN3 and ANN4 increased significantly in the myb30-1 and myb30-2 mutants, and decreased in two OE lines. There are eight ANN genes in the Arabidopsis genome (Clark et al., 2001). We also detected the expression of other ANN genes in myb30 mutants and OE lines. The results showed that ANN2 was also upregulated in myb30 mutants and downregulated in OE lines significantly, but the expression of ANN5, ANN6, ANN7 and ANN8 exhibited no significant difference among these lines (Fig. 3a). Moreover, based on our RNA-seq results, the expression of a cation exchanger (CAX1; AT2G38170) or Ca^{2+}-ATPases (ACAs; AT2G41560 and AT5G57110), which function in Ca^{2+} efflux, appeared likely to be regulated by MYB30. However, real-time RT-PCR results indicated that the expression of these genes showed no significant difference in wild-type compared to myb30 backgrounds (Fig. S2). Together, these results suggest that MYB30 represses the expression of ANN1, ANN2, ANN3 and ANN4.

It has been reported that ANN1 is the most abundant ANN protein in Arabidopsis (Clark et al., 2005). Therefore, we generated anti-ANN1 antibodies using an ANN1-specific peptide and analyzed the ANN1 protein level in wild-type, myb30-1, myb30-2, MYB30 OE-1, MYB30 OE-2 and ann1 (SALK_015426), a knockout mutant of ANN1 (Fig. S3). There was no ANN1 protein detected in the ann1 mutant (Fig. S3b). The protein level of ANN1 was increased in myb30-1 and myb30-2, and was decreased in the two OE lines (Fig. 3b). These results are consistent with the detected mRNA expression level of ANN1 in different MYB30 lines.
MYB30 binds to the ANNEXIN promoters and represses their gene expression

In Arabidopsis, ANN1 may function as an H₂O₂ sensor (Konopka-Postupolska et al., 2009) and has Ca²⁺-permeable transporter activity (Laohavisit et al., 2012). ANN1 interacts with ANN4 to regulate drought and salt stress responses (Huh et al., 2010). However, the function of ANN2 and ANN3 in stress responses is not well studied. Therefore, we chose ANN1 and ANN4 for further analysis in this study. To determine if MYB30 directly represses the expression of ANN genes, MYB30-mediated transcriptional repression was tested. To this end, a LUC reporter gene was fused with the ANN1 promoter (ProANN1). The resulting plasmid was expressed in the wild-type and the myb30-2 mutant, and coexpressed with the Pro35S:FLAG-MYB30 plasmid in Arabidopsis protoplasts. When the ProANN1:LUC plasmid was transformed into the myb30-2 mutant, the LUC intensity was about two times higher than that in the wild-type. When Pro35S:FLAG-MYB30 was coexpressed with ProANN1:LUC, the LUC intensity reduced significantly (Figs 4a,b, S4a). When the promoter of ANN4 was used in the same system, similar results were obtained (Figs 4c,d, S4a). These results suggest that MYB30 represses the transcription of ANN1 and ANN4.

To determine whether MYB30 directly binds to ANN promoters in vivo, a ChIP assay was performed. The ProMYB30: MYB30-FLAG plasmid was transformed into the myb30-2 mutant. The MYB30 and DNA fragments were cross-linked and anti-FLAG antibody was used to immuno-precipitate the MYB30-FLAG protein. The enrichment of ANN1 and ANN4 promoters was detected by quantitative real-time PCR in the pull-down products (Fig. 4e). The results indicated that MYB30 specifically associated with the promoter fragments of ANN1-F2, ANN4-F2 and ANN4-F4 (Fig. 4f), in which several putative MYB binding sites were predicted by AGRIS (http://arabidopsis.med.ohio-state.edu/ArsidsDB/) (Fig. S3a,b). These results suggest that MYB30 binds to the promoter regions of ANN1 and ANN4 in Arabidopsis.

We then used an EMSA to determine whether the MYB30 protein binds directly to the ANN promoters in vitro. The MYB30 protein containing the DNA-binding domain was fused to a His tag and expressed in E. coli. The ANN1-F1 fragment used in the ChIP assay contains a putative MYB-binding site (AACAAAC, named EMSA-1), which was reported to be targeted by MYB30 (Li et al., 2009). There were also several putative MYB-binding sites predicted by AGRIS in the ANN4-F2 (TATCC and TTGGT, named EMSA-2) and ANN4-F4 (CAGTTG, named EMSA-3) fragments (Figs 4g, S5a,b). EMSA-1, -2 and -3 were labeled with biotin and used for binding assays. The His-MYB30 fusion protein bound to these probes, and the biotin binding signal could be reduced by competition with unlabeled probes (Fig. 4b). These results indicate that MYB30 can bind directly to ANN1 and ANN4 promoter fragments in vitro.

Yeast one-hybrid assays were further performed to determine if MYB30 specifically binds to the segments of ANN genes containing MYB-binding sites. We truncated the 0.9-kb ANN1 promoter and 1.5-kb ANN4 promoter into 300-bp and 500-bp segments, respectively, and detected the binding of MYB30 protein to each segment in the yeast system. As a result, the second fragment of the ANN1 promoter was found to be bound by MYB30, which was consistent with the ChIP assay and EMSA results (Fig. S4b,c). The last two segments of the ANN4 promoter, which contained the ANN4-F2 and ANN4-F4 fragments in the ChIP assay, were bound by MYB30. Taken together, these results further support the conclusion that MYB30 binds directly to specific elements within the ANN1 and ANN4 promoters.

ANNEXINs mediate MYB30-dependent calcium signaling regulation under oxidative stress

Previous studies showed that ANN1 participates in calcium signaling regulation through its transporter activity (Laohavisit et al., 2009, 2012; Richards et al., 2014). To test whether ANNs mediate the MYB30-dependent calcium signaling regulation, ann1 (SALK_015426) and ann4 (SALK_019725), a knockout mutant of ANN4, were crossed to the myb30-2 mutant to generate myb30-2 ann1 and myb30-2 ann4 double mutants, and the myb30-2 ann1 ann4 triple mutant (Fig. S3). We obtained the myb30-2 ann4 and myb30-2 ann1 ann4 mutant lines expressing equal levels of AQ compared with the wild-type (Fig. S6a,b), but failed to obtain appropriate ann1 single mutant, ann4 single mutant, ann1 ann4 double mutant and myb30 ann1 double mutant lines with equal AQ expression. When measured with the CCD camera, both the myb30-2 ann4 double mutant and myb30-2 ann1 ann4 triple mutant displayed decreased H₂O₂-induced [Ca²⁺]ₜₜ elevation compared with myb30-2; however, it was still higher than that of the wild-type (Fig. 5a). When the H₂O₂-induced [Ca²⁺]ₜₜ change was measured using the lumimeter, the peak values of [Ca²⁺]ₜₜ in myb30-2 ann4 (0.71 ± 0.05 μmol l⁻¹) and myb30-2 ann1 ann4 (0.60 ± 0.05 μmol l⁻¹) were significantly lower than that in myb30-2 (0.90 ± 0.08 μmol l⁻¹), and the peak value of [Ca²⁺]ₜₜ in the wild-type was the lowest (0.50 ± 0.05 μmol l⁻¹) (Fig. 5b).

The decrease of [Ca²⁺]ₜₜ caused by ann1 ann4 double mutations in myb30-2 was significantly more pronounced than that caused by ann4 single mutation (Fig. 5a,b), suggesting that both ANN1 and ANN4 are involved in the H₂O₂-induced MYB30-dependent calcium signaling pathway and work downstream of MYB30. As H₂O₂ induces artificial [Ca²⁺]ₜₜ elevations in the myb30-2 mutant, we speculated that MYB30 may function in the oxidative stress response. Seeds of myb30 mutants, ann mutants and myb30-2 ann double mutants were germinated on MS medium with different concentrations of MV, which is a major ingredient of parquat and induces ROS accumulation in cells. The results showed that both myb30-1 and myb30-2 mutants were hypersensitive to MV, with reduced cotyledon greening relative to wild-type (Fig. S7a,b). In contrast, the ann4 single mutant and ann1 ann4 double mutant were hyporesponsive to MV (Fig. S7c,d). The hypersensitivity of myb30-2 to MV could be partially suppressed by either ann4 single mutation or ann1 ann4 double mutations (Fig. 5c–f). These results indicate that MYB30 positively
Fig. 4 MYB30 binds to the ANNXIN (ANN) promoters and represses their gene expression. (a, c) Repression of ANN1 and ANN4 transcription by MYB30 in Arabidopsis protoplasts. Wild-type (WT) (left) and myb30-2 (middle) were transformed with both ProANN1:LUC (LUCIFERASE) / ProANN4:LUC and Pro35S:MYC-NRPB3 plasmids. WT (right) was co-transformed with the Pro35S:FLAG-MYB30 plasmid. The expression of MYC-NRPB3 was used as an internal control. Three independent biological experiments were performed and luminescence signals were collected with the cold charge-coupled device (CCD) imaging system. (b, d) Analyses for luminescence signal intensity in (a, c). Data represent mean ± SD of at least three replicate experiments. Statistical significance was determined by Student’s t-test; significant differences (P ≤ 0.05) are indicated by different lowercase letters. (e) ANN1 and ANN4 DNA fragments used for chromatin immunoprecipitation (ChIP) assay. The translation start sites are designated +1. (f) Quantitative real-time ChIP PCR assay showing that MYB30 interacts with ANN1 and ANN4 promoters in vivo. Anti-FLAG antibody was used to precipitate chromatin prepared from 9-d-old myb30-2 and myb30-2:ProMYB30:MYB30-FLAG seedlings. The experiments were repeated three times and data are presented as mean ± SD (n = 3). (g) The sequences of three probes, EMSA-1, EMSA-2 and EMSA-3. The predicted MYB binding sites are underlined. (h) Electrophoretic mobility shift assay (EMSA) for MYB30 binding to the ANN promoter sequences in vitro. Each biotin-labeled DNA probe was incubated with His-MYB30 protein. The unlabeled probe (cold probe) was added to compete with the labeled probe.
regulates the oxidative stress response at least partially through ANN1 and ANN4, and that other ANN genes may also be potential targets.

ANNEXINs mediate MYB30-dependent calcium signaling regulation under heat stress

Heat shock is a stimulus that induces [Ca$^{2+}$]$_{cyt}$ elevation (Gong et al., 1998; Liu et al., 2003, 2008). It has been reported that AtANN1 is involved in heat-induced [Ca$^{2+}$]$_{cyt}$ elevation and the heat stress response (Wang et al., 2015a). In order to investigate if the heat-induced [Ca$^{2+}$]$_{cyt}$ elevation in Arabidopsis is also altered in myb30-2 and myb30-2 ann mutants, the AQ luminescence was recorded using a CCD camera following a 45°C treatment. The myb30-2 mutant displayed an enhanced HS-induced [Ca$^{2+}$]$_{cyt}$ elevation, which was also confirmed by using the Fluo-4 fluorescent dye (Figs 6a, S8). In addition, both the myb30-2 ann4 double mutant and myb30-2 ann1 ann4 triple mutant displayed decreased heat-induced [Ca$^{2+}$]$_{cyt}$ elevations compared with myb30-2. However, it was still higher than that of the wild-type (Fig. 6a). When the heat-induced [Ca$^{2+}$]$_{cyt}$ change was measured using the luminometer, the pattern of [Ca$^{2+}$]$_{cyt}$ elevation induced by heat was different from that induced by the H$_2$O$_2$ treatment. The [Ca$^{2+}$]$_{cyt}$ increased very quickly and decreased slightly, then formed another peak (Fig. 6b). The peak values of [Ca$^{2+}$]$_{cyt}$ in myb30-2 ann4 (0.26 ± 0.04 μmol l$^{-1}$) and myb30-2 ann1 ann4 (0.21 ± 0.02 μmol l$^{-1}$) were significantly lower than that in myb30-2 (0.33 ± 0.02 μmol l$^{-1}$), and the peak value of [Ca$^{2+}$]$_{cyt}$ in the wild-type was the lowest (0.14 ± 0.01 μmol l$^{-1}$) (Fig. 6b). The decrease of [Ca$^{2+}$]$_{cyt}$ caused by ann1 ann4 double mutations in myb30-2 was significantly less pronounced than that caused by ann4 single mutation (Fig. 6a,b), suggesting that both ANN1 and ANN4 are involved in the heat-induced MYB30-dependent calcium signaling pathway and work downstream of MYB30.

Subsequently, we analyzed the sensitivity of myb30 mutants, ann mutants and myb30-2 ann double mutants to HS (Figs S9a, b, 6c–f). The results of these experiments indicated that both myb30-1 and myb30-2 mutants were more resistant to HS treatment than wild-type, with increased fresh weight and chlorophyll content compared with wild-type (Fig. S9a,b). In contrast, the ann4 mutant was hypersensitive to heat stress, and ann4...
mutation in myb30-2 could suppress the HS hyposensitivity of myb30-2 (Fig. 6c,d). Similar results were observed for the myb30-2 ann1 ann4 triple mutant (Fig. 6e,f). The hypersensitivity of the ann1 ann4 double mutant was more pronounced than that of either the ann1 or ann4 single mutant (Fig. S9c,d), indicating that both ANN1 and ANN4 function in the HS response. These results suggest that MYB30 negatively regulates the HS response at least partially through ANN1 and ANN4, and other ANN genes may also be potential targets.

The enhanced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation in myb30-2 increases the sensitivity of the mutant plants to oxidative and heat stresses

We performed further experiments to investigate the association between the HS/H2O2-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation and plant HS/MV sensitivity. When wild-type and myb30-2 seedlings were pretreated with LaCl3 (lanthanum chloride), a nonselective Ca2+ channel blocker, and then subjected to H2O2 or HS treatment, the \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation in myb30-2 decreased significantly to the wild-type level (Fig. 7a–d).

To study the oxidative sensitivity of myb30-2 in the presence of LaCl3, wild-type and myb30-2 seeds were germinated on liquid MS medium with MV and LaCl3. The results showed that the cotyledon greening of myb30-2 in medium containing MV and LaCl3 was significantly higher than that of myb30-2 growing with MV only, suggesting that the oxidative hypersensitivity of myb30-2 was partially rescued by LaCl3 (Fig. 7e,f).

When pretreated 6-d-old wild-type and myb30-2 seedlings with LaCl3 before subjecting them to HS, the heat hyposensitivity of myb30-2 was also partially rescued, with reduced chlorophyll content compared to the myb30-2 seedlings pretreated with H2O (Fig. 7g–k), suggesting that an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) contributes to the heat-tolerant phenotype of the myb30-2 mutant. These results suggest that a faithfully adjusted Ca2+ signal is required for appropriate stress responses and that the MYB30 transcription factor plays a crucial role in fine-tuning calcium signals in response to oxidative and heat stresses in plants.
In this study, we provide evidence that ANN-mediated calcium signals are important for Arabidopsis to properly respond to oxidative and heat stresses. The transcription factor MYB30 crucially functions in these processes by directly regulating ANN gene expression. Based on our results, we propose a working model for the MYB30/ANN regulatory pathway (Fig. 8).

MYB30 binds to the promoters of ANN genes and maintains their expression at a functional level. ANNs modulate the oxidative- and heat-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation, which further triggers downstream oxidative and heat stress responses.

Abiotic stresses such as oxidative, temperature, salt and drought stresses induce the elevation of \([\text{Ca}^{2+}]_{\text{cyt}}\) with specific patterns or signatures (Knight et al., 1991; Gong et al., 1998; Kiegle et al., 2000; Ranf et al., 2008). The generation of a \([\text{Ca}^{2+}]_{\text{cyt}}\) signal is the initial step to trigger calcium signaling and plant response. Several proteins have been identified to participate in the stress-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) generation, such as Flagellin-Sensitive 2 (FLS2) and Brassinosteroid receptor 1-Associated Kinase (BAK1) under pathogen stress (Ranf et al., 2012), the Actin-Related Protein2/3 (Arp2/3) complex under salt stress (Zhao et al., 2013), reduced hyperosmolality-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) increase 1 (OSCA1) under hypoosmotic stress (Yuan et al., 2014), K+ exchange antiporter 1/2 (KEA1/2) and KEA3 under hyperosmotic stress (Stephan et al., 2016) and Mda1-complementing activity 1 (Mca1) under hypoosmotic stress (Nakagawa et al., 2007). However, how the intensity of a specific

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**Discussion**

In this study, we provide evidence that ANN-mediated calcium signals are important for *Arabidopsis* to properly respond to oxidative and heat stresses. The transcription factor MYB30 crucially functions in these processes by directly regulating ANN gene expression. Based on our results, we propose a working model for the MYB30/ANN regulatory pathway (Fig. 8). MYB30 binds to the promoters of ANN genes and maintains their expression at a functional level. ANNs modulate the oxidative- and heat-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation, which further triggers downstream oxidative and heat stress responses.

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calcium signal regulates stress responses in plant is poorly understood. Through the identification of the Arabidopsis transcription factor MYB30, we found that the adjustment of ANN-mediated calcium signals is critical for appropriate plant responses to oxidative and heat stresses. The oxidative- and heat-induced elevations of \([\text{Ca}^{2+}]_{\text{c}}\) are both enhanced in the myb30 mutant. However, the myb30 mutant is hypersensitive to MV and hyposensitive to heat. The reduction of \([\text{Ca}^{2+}]_{\text{c}}\) by either EGTA/LaCl3 treatment or deletion of ANN genes partially rescued the oxidative and heat phenotypes of the myb30 mutant. MYB30/ANN-mediated calcium signals directly contribute to the oxidative and heat stress responses. These results suggest that the Ca2+ signals generated by ANN proteins, which may be different in duration, magnitude, frequency, and spatial distribution, are translated into different responses depending on the stress stimuli. However, calcium sensors that decode the specific calcium signatures remain to be uncovered.

Both Ca2+ influx and efflux systems contribute to the generation of \([\text{Ca}^{2+}]_{\text{c}}\) signatures (Edel & Kudla, 2015; Costa et al., 2017). The expression of CAX1, ACA4 and ACA8 in myb30 mutants and the OE lines was similar to that in wild-type. Because it seems that only the amplitude of the Ca2+ signal is affected in the myb30-2 mutant, but the rate (shape and speed) of the signal-declining phase is not affected, we propose that alterations of the Ca2+ signature observed in the myb30-2 mutant are mainly caused by effects on the Ca2+ influx. However, we cannot exclude the possibility of posttranscriptional downregulation of Ca2+ efflux systems in myb30 mutants.

The disruption of both ANN1 and ANN4 in the myb30-2 mutant could not fully rescue the levels of \([\text{Ca}^{2+}]_{\text{c}}\) elevation and oxidative and heat sensitivity of the myb30-2 mutant to the wild-type levels, which indicates that there are other components working together with ANN1 and ANN4 downstream of MYB30 to regulate the MYB30-mediated calcium signal that further triggers plant oxidative and heat stress responses. Among eight ANN genes in Arabidopsis, ANN1, ANN2, ANN3 and ANN4 were found to be downregulated by MYB30, and their expression was not dramatically increased in the myb30 mutants. It is possible that all of ANN1, 2, 3 and 4 may be involved in this regulation, and they function together to generate the calcium signal and also play a unique role in this regulation, as the deletion of either ANN1 or ANN4 partially rescued the myb30-2 mutant phenotypes. It remains to be elucidated how the oxidative- or heat-induced calcium signal is generated in the ANN1–4 combination. It is possible that MYB30 regulates the level of ANNs under different stimuli in Arabidopsis to generate ANNs combinations, which in turn generates a specific calcium signal for plant response to the corresponding stresses.

AtANN1 localizes to the plasma membrane, vacuole, glyoxysome, mitochondria, stroma and thylakoid (Laohavisit & Davies, 2011), and AtANN4 localizes to the endoplasmic reticulum and mitochondria (Huh et al., 2010). Therefore, ANN1 and ANN4 may mediate the Ca2+ influx from both the apoplast and the internal calcium stores. EGTA is reported to hinder the influx of Ca2+ from the apoplast specifically, while LaCl3 is reported to be a Ca2+ channel blocker (Rentel & Knight, 2004). When we pretreated the seedlings with 500 μM EGTA before detecting an H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) elevation, the H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) elevation in wild-type and myb30-2 reduced significantly compared with that in seedlings pretreated with H2O2 but the H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) elevation in myb30-2 was still much higher than that of the wild-type (Fig. S10a,c), suggesting that the calcium in the apoplast contributes to the H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) elevation. The 50 μM LaCl3 treatment also repressed the H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) elevation in both the myb30-2 mutant and the wild-type to significantly reduced levels compared with the wild-type pretreated with H2O2 (Fig. S10b,c). As LaCl3 can penetrate the cell (Friedman et al., 1998), and the repression of H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) elevation by LaCl3 was stronger than that by EGTA, it appears reasonable to conclude that internal calcium stores also contribute to the elevated H2O2-induced \([\text{Ca}^{2+}]_{\text{c}}\) in myb30-2.

MYB30 is strongly expressed in roots, cotyledons and hypocotyls (Li et al., 2009), which suggests that MYB30 could play important roles in multiple stress responses during the early stages of plant development. Our study reveals that MYB30 positively regulates the oxidative stress response and negatively regulates the heat stress response through the ANN-mediated calcium signal, which indicates that MYB30 also functions in the plant oxidative stress response and heat stress response.

Previous reports showed that MYB30 plays an important role in the pathogen defense response by mediating VLCFA-related target gene expression and Arabidopsis secretory phospholipase A 2-alpha nuclear relocalization (Froidure et al., 2010; Canonne et al., 2011). We analyzed the physiological functions of ANN1 and ANN4 under pathogen stress. No HR phenotype was detected in the ann mutants, and the HR phenotype of the myb30-2 mutant could not be suppressed by the disruption of ANN genes, indicating that neither ANN1 nor ANN4 functions in this process. MYB30, ANN1 and ANN4 function in the ABA response in Arabidopsis, and MYB30 mediates the ABA response by repressing the expression of...
ABA-responsive genes (Lee et al., 2004; Zheng et al., 2012). However, deletion of ANN1 and ANN4 did not alter the myb30-2 mutant ABA sensitivity (Fig. S1a,b). These results suggest that MYB30 functions in different biological processes by regulating various targets.

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Author contributions

Y.G., Y.Z. and C.L. planned and designed the research. C.L. and Y.G. and Y.Z. performed experiments and analyzed data. C.L., Y.G. and Y.Z. wrote the manuscript.

References


**Fig. S4** MYB30 binds to *ANNEXIN* promoters.

**Fig. S5** Predicted MYB binding sites exist in promoters of *ANNEXIN* genes.

**Fig. S6** Expression levels of AEQUORIN in *myb30-2 ann4* and *myb30-2 ann1 ann4*.

**Fig. S7** The MV sensitivity of *myb30* and *annexin* mutants.

**Fig. S8** Heat-induced [Ca$^{2+}$]$_{cyt}$ elevation in roots using Fluo-4.

**Fig. S9** The heat sensitivity of *myb30* and *annexin* mutants.

**Fig. S10** EGTA and LaCl$_3$ repress the H$_2$O$_2$- and heat-induced [Ca$^{2+}$]$_{cyt}$ elevation of WT and *myb30-2* to varying degrees.

**Fig. S11** The ABA sensitivity of *myb30* and *annexin* mutants.

**Table S1** Primers used in this study

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