Control of proline accumulation under drought via a novel pathway comprising the histone methylase CAU1 and the transcription factor ANAC055

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Abstract

Proline plays a crucial role in the drought stress response in plants. However, there are still gaps in our knowledge about the molecular mechanisms that regulate proline metabolism under drought stress. Here, we report that the histone methylase encoded by CAU1, which is genetically upstream of P5CS1 (encoding the proline biosynthetic enzyme Δ1-pyrroline-5-carboxylate synthetase 1), plays a crucial role in proline-mediated drought tolerance. We determined that the transcript level of CAU1 decreased while that of ANAC055 (encoding a transcription factor) increased in wild-type Arabidopsis under drought stress. Further analyses showed that CAU1 bound to the promoter of ANAC055 and suppressed its expression via H4R3sme2-type histone methylation in the promoter region. Thus, under drought stress, a decreased level of CAU1 led to an increased transcript level of ANAC055, which induced the expression of P5CS1 and increased proline level independently of CAS. Drought tolerance and the level of proline were found to be decreased in the caul1 anac055 double-mutant, while proline supplementation restored drought sensitivity in the anac055 mutant. Our results reveal the details of a novel pathway leading to drought tolerance mediated by CAU1.

Keywords: ANAC055, CAU1, drought tolerance, histone methylase, P5CS1, proline.

Introduction

In plants under osmotic stress, proline is mainly synthesized by P5CS (Δ1-pyrroline-5-carboxylate synthetase) (Savouré et al., 1995; Yoshiba et al., 1995; Székely et al., 2008) and P5CR (P5C reductase) from glutamate in chloroplasts (Szoke et al., 1992; Verbruggen et al., 1993). Proline catabolism is controlled by PDH (proline dehydrogenase) (Kiyosue et al., 1996; Verbruggen et al., 1996) and P5CDH (P5C dehydrogenase) in Arabidopsis (Deuschle et al., 2001). As well as functioning as a compatible osmolyte, proline may act as a metabolic signal that regulates the stabilization of proteins and antioxidant enzymes, the direct scavenging of ROS (reactive oxygen species), and the balance of intracellular redox homeostasis, such as the ratios of NADP+/NADPH and GSH/GSSG (Hamilton and Heckathorn, 2001; Kavi Kishor et al., 2005; Miller et al., 2009; Szabados and Savouré, 2010; Alves et al., 2011).
Several studies have shown that the transcription of \textit{P5CS1} is activated by H$_2$O$_2$-derived signals, the calcium signal, PLC (phospholipase C), PLD (phospholipase D), and by the ABA-dependent pathway (Yoshida \textit{et al.}, 1995; Savouré \textit{et al.}, 1997; Strizhov \textit{et al.}, 1997; Parre \textit{et al.}, 2007; Verslues \textit{et al.}, 2007; Ghars \textit{et al.}, 2008). \textit{ABI1} (ABA-INSENSITIVE 1) and the CaM4 calmodulin-MYB2 regulatory pathway are involved in the control of \textit{P5CS1} transcription (Knight \textit{et al.}, 1997; Strizhov \textit{et al.}, 1997; Yoo \textit{et al.}, 2005; Parre \textit{et al.}, 2007). Although much is known about the biological functions of proline in stress tolerance, its regulation needs further investigation.

In plants, histone modifications have been implicated in the response to drought stress (Sokol \textit{et al.}, 2007; van Dijk \textit{et al.}, 2010; Kim \textit{et al.}, 2012; Sani \textit{et al.}, 2013; Zong \textit{et al.}, 2013). The dehydration-stress response gene \textit{ATX1} encodes a protein that trimethylates histone H3 at lysine 4 (H3K4me3) in NCE3 (\textit{NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3}) (Ding \textit{et al.}, 2009, 2011). The H3K4 demethylase homolog HvPKDM7-1 may be involved in drought tolerance in barley (Papaefthimiou and Tsafaritis, 2012). The histone acetylation levels increase in \textit{RD20} (RESPONSIVE TO DEHYDRATION 20), \textit{RD29A} (RESPONSIVE TO DESICCATION 29A), and \textit{RD29B} (RESPONSIVE TO DESICCATION 29B) in Arabidopsis, and in four HATs (Histone acetyltransferases) genes \textit{OsHAC703}, \textit{OsHAG703}, \textit{OsHAF701}, and \textit{OsHAM701} in rice under drought stress (Kim \textit{et al.}, 2008; Fang \textit{et al.}, 2014). Histone deacetylase 2, encoded by \textit{AtHD2C}, is involved in tolerance to drought stress in Arabidopsis (Sridha and Wu, 2006). Histone H4 deacetylation is also involved in ABA-induced stomatal closing (Sridha and Wu, 2006; Zhu \textit{et al.}, 2008). However, there is still much to learn about the mechanisms of histone modification under drought stress.

Previously, we showed that the H4R3sme2-type histone methylase \textit{CAU1/PRMT5/SKB1} mediates stomatal closure by repressing the expression of \textit{CAS} (CALCIUM SENSOR, mediating the sensing of extracellular Ca$^{2+}$ in guard cells) in response to extracellular calcium (Han \textit{et al.}, 2003; Fu \textit{et al.}, 2013). However, the \textit{cas-1} mutant showed a partly restored water-loss rate and the same rate of stomatal closure as that of the wild-type (Fu \textit{et al.}, 2013), suggesting that other components may function in the drought tolerance pathway mediated by \textit{CAU1}.

In this study, we show that the transcription factor encoded by \textit{ANAC055} acts as a downstream component of \textit{CAU1} independently of \textit{CAS}. Our results show that drought stress represses the levels of \textit{CAU1} RNA and CAU1 protein, which lead to decreased H4R3sme2 methylation levels of chromatin in the \textit{ANAC055} promoter. The subsequent increase in \textit{ANAC055} expression leads to increased expression of its genetically downstream gene, \textit{P5CS1}, resulting in proline accumulation and drought tolerance.

Materials and methods

\textit{Plant material, growth conditions, and physiological analyses}

Plants of \textit{Arabidopsis thaliana} were grown in soil at 22 °C with 16-h light/8-h dark cycles. At 14 d after emergence, drought stress was induced by withholding watering for 14 d, and the survival rate was scored at 7 d after watering recommenced. Rosette leaves of 22-d-old sample plants were collected and used to determine rates of water loss by time-course measurements of their fresh weights (Vartanian \textit{et al.}, 1994; Pei \textit{et al.}, 1998). Stomatal assays were performed as previously described (Pei \textit{et al.}, 2000; Fu \textit{et al.}, 2013). Stomatal apertures were determined by measuring the pore widths and lengths with a digital ruler in Image-Pro Plus 6.0 (MediaCybernetics).

Alternatively, plants were grown in quarter-strength hydroponics as previously described (Arteca and Arteca, 2000; Gong \textit{et al.}, 2003). At 4 weeks of age, plants were treated with 10%, 20%, 30%, or 40% PEG-6000 and/or 50 mM proline for 12 h, or with 10% PEG-6000 for 0, 1, 3, 6, 12 h. Shoots were sampled and subjected to further analyses as indicated. Plants were weighed at the start of treatment (initial weight) and then reweighed at the end of treatment (final weight). Plants were then dried to a constant weight at 80 °C and reweighed to obtain dry weight. RWC was calculated as: (final weight – dry weight)/(initial weight – dry weight) ×100%.

\textit{DNA constructs and plant transformation}

The \textit{ANAC055} cDNA was amplified by RT-PCR. The two restriction sites for \textit{BamHI} and \textit{SacI} were introduced using \textit{ANAC055-1} primers and for \textit{Xhol} and \textit{EcoRI} using \textit{CAU1-1} (see Table S1 available at the Dryad Digital Repository, https://doi.org/10.5061/dryad. he4bj). The resulting fragments were confirmed by sequencing and then sub-cloned into the binary vector pBI121 (pre-digested with \textit{BamHI} and \textit{SacI}) or \textit{35S:EYFP/pMON530} (pre-digested with \textit{Xhol} and \textit{EcoRI}). The \textit{ANAC055} promoter was amplified by RT-PCR. The two restriction sites for \textit{PstI} and \textit{BamHI} were introduced using \textit{ANAC055-2} primers (see Table S1 at Dryad). The resulting fragments were confirmed by sequencing and then sub-cloned into the binary vector \textit{GUS/SpCAMBIA1300} (pre-digested with \textit{PstI} and \textit{BamHI}). The generated constructs \textit{35S:ANAC055/pBI121, 35S:EYFP-CAU1/pMON530} and \textit{pANAC055-GUS/SpCAMBIA1300} were transformed into \textit{Col-0} or \textit{cau1} using the floral dip method (Clough and Bent, 1998). Transgenic lines with a segregation rate of 3:1 grown on kanamycin or hygromycin plates were used for further homozygote and strong allele screenings.

\textit{RT-PCR/quantitative RT-PCR}

Total RNA from plants was isolated using the TRIzol reagent (Invitrogen). First-strand cDNA synthesis, RT-PCR, and quantitative RT-PCR (qPCR) were performed as previously described (Aggarwal \textit{et al.}, 2010). \textit{ANAC055-QP, CAU1-QP, P5CS1-QP, SAND-QP}, and drought-related gene primers were used in quantitative RT-PCR (see Table S1 at Dryad).

\textit{Histochemical analysis}

Transgenic plants of the \textit{pANAC055:GUS/cau1} mutant were subjected to histochemical analysis as previously described (Aggarwal \textit{et al.}, 2010).

\textit{Isolation of anac055 and cau1 anac055 mutant plants}

The T-DNA insertion line \textit{SALK_014331} obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu) was screened for the homozygous knockout mutant \textit{anac055} as previously described (Weinl \textit{et al.}, 2008). To generate the \textit{cau1 anac055} double-mutant, \textit{cau1} was crossed to \textit{anac055} to make an F$_2$ population; \textit{cau1}-like plants were further analysed to isolate the genotype \textit{anac055/anac055} using the PCR primers \textit{ANAC055-SALK} and \textit{LBA1} as previously described (Krysan \textit{et al.}, 1999).

\textit{Determination of proline levels}

Proline concentrations were determined as described by Bates \textit{et al.} (1973). Leaves were freeze-dried and then homogenized in 3%
sulfosalicylic acid, and were then centrifuged at 3000 g for 20 min. The sample supernatant, acetic acid, and 2.5% acid ninhydrin solution were boiled for 30 min, and the absorbance was measured at 520 nm.

Chromatin immunoprecipitation (ChIP) assay

For the ChIP assay, 21-d-old Col-0, caul1, and 35S:YEFP-CAU1/caul plants grown under long-day conditions were harvested. Approximately 4 g of plant material was cross-linked for 20 min in 1% formaldehyde. ChIP assays were performed as previously described (Vartanian et al., 1994; Ascenzi and Gantt, 1999). The sonicated chromatin extractions were immunoprecipitated overnight with antisymmetric dimethyl-H4R3 antibody (Abcam) for plants of Col-0 and caul1, with an anti-GFP (green fluorescent protein) antibody (Invitrogen) for plants of 35S:YEFP-CAU1/caul, or without antibody. Incubation of chromatin with mouse IgG (Abcam) served as a mock immunoprecipitation control. Protein A beads (Millipore) were used to capture the immunocomplexes. After reverse cross-linking and proteinase-K digestion, the DNA was extracted with phenol-chloroform and then precipitated with ethanol. The immunoprecipitated DNA was subsequently used for qPCR. The sequences were amplified from –1388 to 646 bp of the ANAC055 gene and each DNA fragment was approximately 120 bp in length. Primers used for ChIP-qPCR were as follows: Region A (ANAC055-1), region B (ANAC055-2), region C (ANAC055-3), region D (ANAC055-8), region E (ANAC055-9), region F (ANAC055-10), region G (ANAC055-11), and the primer sequences are given in Table S1 at Dryad. TUB8 was used as a control (Mathieu et al., 2005).

Protein gel blotting analysis

Transgenic 35S:YEFP-CAU1/caul plants were grown in hydroponics to 24 days of age, and then exposed to 10% PEG treatments. Total proteins were extracted from leaf samples using buffer E [125 mMTris-HCl pH 8.0; 1% (w/v) SDS, 10% (v/v) glycerol, 50mM Na3S2O5]. From each sample 30 μg total proteins were separated on 12% SDS-PAGE (Beyotime) gel and analysed by protein gel blotting according to the manufacturer’s instructions. Mouse anti-Actin2 (Abcam) antibodies were used as a control (Mathieu et al., 2005). TUB8 was used as a control (Mathieu et al., 2005).

Statistical analysis

Data were statistically analysed using one-way ANOVA with LSD tests (for multiple comparisons) or two-tailed Student’s t-tests (for comparisons of two sets of data).

Results

ANAC055 expression is enhanced in the caul1 mutant

Previously, we showed that the caul1 mutant is resistant to drought stress (Fu et al., 2013). To investigate the role of CAU1 in drought tolerance, the levels of CAU1 transcripts and CAU1 protein were determined in the wild-type (Col-0) and the caul1 mutant under drought stress conditions. The CAU1 transcript levels (Fig. 1A) and CAU1 protein levels (Fig. 1B) in Col-0 decreased under drought stress. Next, we analysed the transcript levels of drought tolerance-related genes including ANAC055, AREB2, CIPK1, CIPK3, CIPK21, DREB2, ERD1, MYC2, NCE3, RD26, RD29A, ANAC019, and ANAC072 by quantitative RT-PCR. The transcript level of ANAC055, which encodes a transcription factor, was enhanced in the caul1mutant (Fig. 1C), compared with that in Col-0. Histochemical analyses showed that the activity of GUS driven by the ANAC055 promoter was higher in caul leaves than in Col-0 leaves (Fig. 1D). The transcript level of ANAC055 in Col-0 was enhanced under drought stress (Fig. 1E). These results indicated that the ANAC055 level was enhanced in caul1, and that drought stress suppressed CAU1 expression but increased ANAC055 expression.

CAU1 regulates ANAC055 expression by H4R3sme2 methylation in its promoter region

To explore the mechanism by which CAU1 regulates ANAC055 expression, we performed a ChIP-qPCR assay to analyse the H4R3sme2 level in the ANAC055 promoter region, using an H4R3sme2 antibody (Fig. 2A, B). The H4R3sme2 level in region C of the ANAC055 promoter was significantly reduced in caul1 (Fig. 2A, B). This result suggested that CAU1 regulated ANAC055 transcription through histone methylation.

We also conducted a ChIP-qPCR assay using a GFP antibody to determine whether CAU1 binds to the ANAC055 chromatin (Fig. 2A, C). CAU1 strongly associated with region B of the ANAC055 promoter, whereas a similar CAU1–ANAC055 interaction was not detected in regions A or C–G. These results confirmed that CAU1 bound directly to the ANAC055 chromatin in region B, and mediated the level of histone methylation in region C (Fig. 2A–C).

Given that ANAC055 expression was up-regulated in response to drought stress (Fig. 1E), we analysed the correlation between drought stress and the level of H4R3sme2 in the ANAC055 promoter. As shown in Fig. 2D, drought stress significantly decreased the H4R3sme2 level in region C of the ANAC055 promoter in Col-0, while no change was observed in the caul1 mutant. Further analyses showed that there were significant decreases in CAU1 binding to the ANAC055 promoter (Fig. 2E) as well as significant decreases in CAU1 mRNA and CAU1 protein levels in Col-0 under drought stress (Fig. 1A, B). These data indicated that drought stress decreased the CAU1 mRNA and CAU1 protein levels and decreased CAU1 binding to the ANAC055 promoter, thus decreasing H4R3sme2 methylation of the ANAC055 chromatin and enhancing ANAC055 expression.

CAU1 acts with ANAC055 in response to drought stress

A previous study showed that the NAC gene family member ANAC055 was up-regulated by drought stress, and its over-expression increased drought tolerance (Tran et al., 2004). Given that caul1 was shown to be insensitive to drought stress (Fu et al., 2013) and showed enhanced ANAC055 expression (Fig. 1C), we sought to determine whether these phenotypes were genetically correlated with ANAC055. A T-DNA insertion line for ANAC055 was isolated (see Fig. S1A, B at Dryad). RT-PCR analysis confirmed that ANAC055 mRNA was not detectable in the anac055 mutant (see Fig. S1C at Dryad).
Further analyses showed that Col-0 (Fig. 3A, F) and anac055, the loss-of-function mutant of ANAC055 (Fig. 3C, F), were sensitive to drought stress, while caul plants were drought tolerant (Fig. 3B, F). However, the drought tolerance conferred by the caul mutation was abolished in the double mutant caul anac055 (Fig. 3D, F), even though the visible developmental phenotypes of caul anac055 were similar to those of caul. A drought-tolerant phenotype was also observed in 35S:ANAC055 in 4-week-old plants treated with 10% PEG-6000 for 0, 1, 3, 6, and 12 h. These results indicated that ANAC055 is downstream of CAU1, and plays an important role in stomatal closure and consequently in drought tolerance.

Further analyses showed that Col-0 (Fig. 3A, F) and anac055, the loss-of-function mutant of ANAC055 (Fig. 3C, F), were sensitive to drought stress, while caul plants were drought tolerant (Fig. 3B, F). However, the drought tolerance conferred by the caul mutation was abolished in the double mutant caul anac055 (Fig. 3D, F), even though the visible developmental phenotypes of caul anac055 were similar to those of caul. A drought-tolerant phenotype was also observed in 35S:ANAC055 (Fig. 3E, F). These results indicated that ANAC055 is downstream of CAU1, which functions in drought tolerance.

Next, we evaluated differences in stomatal apertures among the mutants and Col-0. As shown in Fig. 3G, the stomatal aperture was smaller in caul than in Col-0, while that of anac055 was larger than that of Col-0. In the double-mutant caul anac055, stomatal aperture was restored to a level between those of Col-0 and anac055 (Fig. 3G). The rate of water loss from the leaves was decreased in caul and 35S:ANAC055, and increased in anac055 compared with that of Col-0 (Fig. 3H). These results suggested that ANAC055 functions downstream of CAU1, and plays an important role in stomatal closure and consequently in drought tolerance.

CAU1 affects proline accumulation via its effects on P5CS1

To elucidate the molecular mechanism of CAU1 in the drought response, we measured the proline levels in Col-0 and caul under drought stress imposed by PEG-6000. Proline accumulated in both Col-0 and caul under drought stress with a clear dose-dependent effect, and to higher levels in caul than in Col-0 (Fig. 4A). In the absence of drought stress, there were higher proline contents in caul and 35S:ANAC055 than in Col-0 and caul anac055, but lower proline content in anac055 than in Col-0 (Fig. 4B). The transcript levels of P5CS1 in Col-0 and caul also increased under drought stress with a dose-dependent effect, and to higher levels in caul than in Col-0 (Fig. 4C). In the absence of drought stress, the P5CS1 transcript levels were higher in caul and 35S:ANAC055 than...
Fig. 2. Suppression of CAU1-mediated histone methylation in the ANAC055 promoter region under drought stress. (A) Diagram of the ANAC055 gene. The genomic regions A to G used in the ChIP assays are indicated. (B, C) ChIP assays with antibodies against H4R3sme2 (B) using Col-0 and cau1 plants, and GFP (C) using 35S:EYFP-CAU1/CAU1 plants. TUB8 was used as an internal control in (C). Three independent experiments were performed, and values are means ±SE. *P<0.05 and **P<0.01. (D, E) ChIP assays with antibodies against H4R3sme2 within the C region (D) or GFP within the B region (E) of ANAC055. Plants were treated with 10% PEG-6000 for the period indicated. Three independent experiments were performed. TUB8 was used as an internal control in (D). Values are means ±SE. Different letters above each bar indicate significant differences (ANOVA tests).

Fig. 3. CAU1–ANAC055 acts in the drought tolerance pathway. (A–E) Drought tolerance of Col-0 (A), cau1 (B), anac055 (C), cau1 anac055 (D), and 35S:ANAC055 (E). When plants were 14 d old watering was withheld for 14 d, and then watering recommenced for 7 d. (F) Survival rates of plants in (A–E) under drought stress. Values are means ±SD from three independent experiments. (G) Stomatal apertures on rosette leaves. Values are means ±SD (n=30). (H) Water loss rates from leaves of Col-0, cau1, anac055, cau1 anac055, and 35S:ANAC055. Values are means ±SD from three independent experiments (n=6 leaves per treatment). Different letters above each bar indicate significant differences (ANOVA tests).
in Col-0, but lower in anac055 and caul anac055 than in Col-0 (Fig. 4D). The proline level and P5CS1 transcript levels increased in plants in response to PEG-6000. Higher proline contents and P5CS1 transcript levels existed in caul and 35S:ANAC055 than in Col-0 and caul anac055, and lower proline content and P5CS1 transcript levels existed in anac055 than in Col-0 (Fig. 4B, D).

To identify the role of CAU1–ANAC055 in drought tolerance via the regulation of P5CS1, we tested whether proline could restore the sensitivity to osmotic stress imposed by PEG-6000 in caul plants. When treated with 10% (Fig. 5C, G) or 20% PEG (Fig. 5E, G) for 12 h, Col-0 and caul anac055 showed wilting symptoms and decreased RWC in a dose-dependent manner compared with their respective untreated controls (Fig. 5A, G) or with proline-treated plants (Fig. 5B, G). caul and 35S:ANAC055 plants showed higher RWC under 10% or 20% PEG treatment compared with those of Col-0 and caul anac055 (Fig. 5C, E, G). anac055 plants showed lower RWC under 10% or 20% PEG treatment compared with those of Col-0 and caul anac055 (Fig. 5C, E, G).

Proline restored the RWC of Col-0, caul, anac055, caul anac055, and 35S:ANAC055 plants treated with 10% or 20% PEG (Fig. 5G). Plants showed mild wilting in a dose-dependent manner when treated with 10% PEG and proline and 20% PEG and proline (Fig. 5D, F, G) compared to those treated with 10% and 20% PEG without proline (Fig. 5C, E, G). Under the same treatments, caul anac055 showed similar phenotypes to those of Col-0, and 35S:ANAC055 showed similar phenotypes to those of caul (Fig. 5). These results indicated that CAU1–ANAC055 acts in drought tolerance by regulating the expression of P5CS1 and consequently the proline level.

The CAU1–ANAC055 pathway acts independently of the CAU1–CAS pathway in drought tolerance

Previously, we showed that the CAU1–CAS pathway regulates stomatal closure (Fu et al., 2013). To investigate whether ANAC055 is involved in the CAU1–CAS pathway, we analysed the transcript levels of CAS in the anac055 mutant and the transcript levels of ANAC055 and P5CS1 in the cas-1 mutant. The transcript levels of CAS in the anac055 mutant (Fig. 6A) and of ANAC055 and P5CS1 in the cas-1 mutant (Fig. 6B) were similar to those in Col-0. These results suggested that CAU regulates ANAC055 independently of CAS in drought tolerance (Fig. 7).

Discussion

Proline acts as an osmolyte and a signaling molecule to modulate responses to abiotic and biotic stresses (Szabados and Savouré, 2010), but the regulation of proline synthesis is not completely understood. In this study, CAU1, an H4R3sme2-type histone methylase, was shown to bind to the promoter region of ANAC055 and repress its expression. The decrease in CAU1 under drought stress led to higher expressions of ANAC055 and its genetically downstream gene P5CS1.

CAU1 regulates expressions of ANAC055 and P5CS1 in response to drought stress

In plants, proline accumulates under abiotic stress (Saradhi et al., 1995; Yoshiba et al., 1995; Schat et al., 1997; Choudhary et al., 2005; Yang et al., 2009) and biotic stress (Fabro et al., 2004; Haudefcoeur et al., 2009). Proline synthetase (P5CS1) is regulated by calcium signals, H2O2-derived signals, and...
an ABA-dependent pathway (Yoshida et al., 1995; Savouré et al., 1997; Strizhov et al., 1997; Parre et al., 2007; Verslues et al., 2007). The known regulators in plants include CaM4-MYB2 (Yoo et al., 2005), PLC (Knight et al., 1997; Parre et al., 2007), PLD (Thiery et al., 2004; Ghars et al., 2008), ABI (Strizhov et al., 1997), LOS5/ABA3 (Xiong et al., 2001), LcMYB1 (Cheng et al., 2013), SpERD15 (Ziaf et al., 2011), GmbZIP132 (Liao et al., 2008), GsZFP1 (Luo et al., 2012), and TaABC1 (Wang et al., 2011).

Our results show that CAU1 represses the expression of ANAC055; thus, the decrease in CAU1 under drought stress leads to higher transcript levels of ANAC055 and the genetically downstream P5CS1, resulting in increased proline synthesis. However, the complete NACRS (NAC recognition sequence, TCNNNNNNACCCGATGT) was not determined in the P5CS1 region (Tran et al., 2004). P5CS1 might act genetically downstream of ANAC055 while not being directly bound in the upstream region with the ANAC055 protein.

CAU1–ANAC055 affects the gene expression involved in proline metabolism. The expression of P5CR, involved in the biosynthesis of proline, was higher in caul and 35S:ANAC055 than in Col-0 and caul anac055, and lower in anac055 than in Col-0 (see Fig. S2 at Dryad). In terms of the higher level of P5CS1, GSA/P5C might be the major factor that enhanced the level of P5CR in caul and 35S:ANAC055. The expression of PDH1 and P5CDH, involved in the catabolism of proline, tended to be lower in caul and 35S:ANAC055 than in Col-0 and caul anac055, and higher in anac055 than in Col-0 (see Fig. S2 at Dryad). These observations might be the result of changed levels of proline in the mutants. The results support a model wherein CAU1 functions upstream of a P5CS1–proline cascade (Fig. 7).

Proline functions as an osmolyte and also as a signaling molecule to regulate metabolite pools and the redox balance, to control gene expression, and ultimately to control drought tolerance (Szabados and Savouré, 2010). The transcript levels of P5CS1 were enhanced in the caul mutant and suppressed
in the anac055 and cau1 anac055 mutants (Fig. 4D). The proline level in cau1 anac055 was partly restored to a level similar to that in Col-0, but the level in the anac055 mutant was very low (Fig. 4B). These results indicate that there must be other components besides P5CS1 that function in CAU1-mediated proline synthesis.

A previous study showed that the transcript levels of three NAC transcription factors, ANAC055, ANAC019, and ANAC072, were increased under drought stress, and transgenic plants overexpressing these factors showed increased drought tolerance (Tran et al., 2004). In the present study, however, the transcript level of ANAC055 was up-regulated in cau1 while the transcript levels of ANAC019 and ANAC072 were similar to those in the wild-type (Fig. 1C). This result indicated that ANAC055, but not ANAC019 and ANAC072, is suppressed by CAU1 in drought tolerance. ANAC019 and ANAC072 may have redundant functions with ANAC055, and may be controlled by different regulators.

CAU1–ANAC055 plays a major role in drought response redundantly with CAU1–CAS

CAU1 may function as a signal junction. Previous studies have shown that CAU1 suppresses FLC to mediate flowering time (Pei et al., 2007; Wang et al., 2007; Schmitz et al., 2008; Deng et al., 2010), LSM4 to mediate salt tolerance (Zhang et al., 2011), bHLH to mediate iron homeostasis (Fan et al., 2014), CAS to mediate the [Ca2+]o signal (Fu et al., 2013), CRN to maintain the shoot apical meristem (Yue et al., 2013), PRR7/PRR9/GI to mediate circadian rhythms (Deng et al., 2016; Li et al., 2016), PRR8 to mediate diverse developmental processes (Deng et al., 2016), and SHR to maintain root stem cells after DNA damage (Li et al., 2016). These results indicate that CAU1 may serve as a signal junction to regulate different downstream genes in diverse biological and developmental processes.

Our results show that CAU1 acts in response to drought, and regulates two genetically downstream pathways to modulate tolerance to drought. One downstream pathway is CAS-[Ca2+]cyt, which might involve ROS or IP3 signals (Fu et al., 2013). The other downstream pathway is ANAC055–P5CS1, which leads to proline accumulation (Fig. 7). CAU1–ANAC055 may function redundantly with CAU1–CAS in drought tolerance. The expression of CAS was unaffected in the anac055 mutant (Fig. 6A), and the expressions of ANAC055 and P5CS1 were unaffected in the cas-1 mutant (Fig. 6B). The stomatal closure and water loss phenotypes in cau1 were partially restored in the cas-1 mutant (Fu et al., 2013) and restored in the anac055 mutant (Fig. 3). These results indicate that ANAC055 and CAS are independent genetically downstream genes of CAU1, and that CAU1–ANAC055 plays a major role in response to drought stress.

CAU1 suppresses ANAC055 expression via histone modification

CAU1/SKB1/AtPRMT5 is an arginine methyltransferase that regulates target genes by histone methylation or pre-mRNA splicing (Pei et al., 2007; Wang et al., 2007; Zhang et al., 2011; Fu et al., 2013; Yue et al., 2013; Fan et al., 2014; Deng et al., 2016; Li et al., 2016). The results of this study show that CAU1 suppresses the expression of ANAC055 by histone methylation. The transcript levels of ANAC055 and P5CS1 were significantly increased in cau1 (Figs 1C and 4D). Under PEG treatments, CAU1 mRNA and CAU1 protein levels significantly decreased (Fig. 1A, B), and the histone methylation level decreased in ANAC055 chromatin (Fig. 2B, D). Alternatively, spliced transcripts of ANAC055 and P5CS1 were not detected in cau1 (see Fig. S3 at Dryad).

In summary, these functional analyses reveal that CAU1 serves as an epigenetic suppressor of ANAC055, which regulates the expression of P5CS1, and hence proline accumulation in response to drought stress. The results also show that the CAU1–ANAC055 pathway is redundant with the CAU1–CAS pathway.

Data deposition

The following figures and table are available at the Dryad Data Repository: [https://doi.org/10.5061/dryad.h4b4j](https://doi.org/10.5061/dryad.h4b4j)

Fig. S1. Isolation of T-DNA insertion lines for anac055.

Fig. S2. Transcript levels of P5CR, PDH1 and P5CDH.

Fig. S3. Analysis of alternative splicing of ANAC055 and P5CS1.

Table S1. List of primer sequences.

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