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TCP transcription factors are critical for the coordinated regulation of *ISOCHORISMATE SYNTHASE 1* expression in *Arabidopsis thaliana*

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SUMMARY

Salicylic acid (SA) plays an important role in various aspects of plant development and responses to stresses. To elucidate the sophisticated regulatory mechanism of SA synthesis and signaling, we used a yeast one-hybrid system to screen for regulators of *ISOCHORISMATE SYNTHASE 1* (*ICS1*), a gene encoding the key enzyme in SA biosynthesis in *Arabidopsis thaliana*. A TCP family transcription factor AtTCP8 was initially identified as a candidate regulator of *ICS1*. The regulation of *ICS1* by TCP proteins is supported by the presence of a typical TCP binding site in the *ICS1* promoter. The binding of TCP8 to this site was confirmed by *in vitro* and *in vivo* assays. Expression patterns of *TCP8* and its corresponding gene *TCP9* largely overlapped with *ICS1* under pathogen attack. A significant reduction in the expression of *ICS1* during immune responses was observed in the *tcp8 tcp9* double mutant. We also detected strong interactions between TCP8 and SAR deficient 1 (SARD1), WRKY family transcription factor 28 (WRKY28), NAC (NAM/ATAF1, ATAF2/CUC2) family transcription factor 019 (NAC019), as well as among TCP8, TCP9 and TCP20, suggesting a complex coordinated regulatory mechanism underlying *ICS1* expression. Our results collectively demonstrate that TCP proteins are involved in the orchestrated regulation of *ICS1* expression, with TCP8 and TCP9 being verified as major representatives.

Keywords: Arabidopsis, TCP transcription factor, *ICS1* regulation, plant immune response, protein–protein interaction.

INTRODUCTION

Salicylic acid (SA) is involved in diverse biological processes in plants, including immune responses (Malamy *et al.*, 1990; Metraux *et al.*, 1990; Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Wildermuth *et al.*, 2001; Tsuda *et al.*, 2009), tolerance to abiotic stresses (Horvath *et al.*, 2007; Alonso-Ramirez *et al.*, 2009), seed germination (Rajjou *et al.*, 2006), trichome development (Traw and Bergelson, 2003), flowering (Martinez *et al.*, 2004) and leaf senescence (Morris *et al.*, 2000; Buchanan-Wollaston *et al.*, 2005; Zhang *et al.*, 2013). Considerable efforts have been devoted to the elucidation of SA biosynthesis and signaling in response to pathogen infection (Wildermuth *et al.*, 2001; Mou *et al.*, 2003; Fu *et al.*, 2012; Serrano *et al.*, 2013). Two SA biosynthesis pathways with chorismate as the initial substrate have been revealed in plants. SA is synthesized either via a series of enzymatic reactions with a rate-limiting step catalyzed by phenylalanine ammonia lyase (Leon *et al.*, 1995; Mauch-Mani and Slusarenko, 1996; Coquoz *et al.*, 1998; Ribnicky *et al.*, 1998) or through two sequential enzymatic reactions catalyzed by isochorismate synthase (ICS) and isochorimate pyruvate lyase, respectively (Verberne *et al.*, 2000; Mauch *et al.*, 2001). Although the gene(s) encoding isochorimate pyruvate lyase has yet to be identified, the ICS-dependent pathway is recognized as a major SA biosynthesis pathway in several species (Wildermuth *et al.*, 2001; Uppalapati *et al.*, 2007; Catinot *et al.*, 2008). There are two ICS paralogs (*ICS1* and *ICS2*) in the *Arabidopsis thaliana* genome, and ICS1 has been shown to play a predominant role in mediating systemic acquired resistance (Wildermuth *et al.*, 2001), abiotic stress tolerance (Garcion *et al.*, 2008; Lee *et al.*, 2010) and plant development (Martinez *et al.*, 2004; Garcion *et al.*, 2008; Li *et al.*, 2012).

When plants are subject to pathogen infection, *ICS1* expression is activated, and SA accumulation is subsequently enhanced, leading to the growth-to-defense transition via coordinated regulation of NPR1 (non-expressor of *PR* genes 1) and TBF1 (*TL1*-binding transcription factor 1) (Pajerowska-Mukhtar *et al.*, 2012). This process involves significant transcriptional reprogramming, particularly inhibition of the auxin signaling pathway, which results in growth retardation (Wang *et al.*, 2007). In order to ensure an efficient utilization of limited resources, it is plausible for plants to have evolved an elaborate regulatory network to effectively switch between normal growth/developmental status and emergent responses to pathogen attacks (Heil *et al.*, 2000; Vos *et al.*, 2013) and abiotic stresses (Su *et al.*, 2013). Regulation of *ICS1* expression may be one of the early regulatory nodes of the network. By using an *ICS1* promoter/luciferase reporter screening system, MUR3 was identified as a negative regulator of *ICS1* expression, but the regulation appears to be indirect (Tedman-Jones *et al.*, 2008). NPR1, a key regulator of SA signaling, has also been shown to be a negative feedback regulator of *ICS1* expression. Although it is clear that nuclear localization of NPR1 is required for *ICS1* regulation, the underlying mechanism remains unknown (Zhang *et al.*, 2010a). By more extensive genetic screening and/or molecular analyses, SAR deficient 1 (SARD1)/calmodulin binding protein 60g (CBP60g) and WRKY family transcription factor 28 (WRKY28) have been identified as direct positive regulators, whereas ethylene insensitive 3 (EIN3)/EIN3-like1 (EIL1) and NAC (NAM/ATAF1,ATAF2/CUC2) family transcription factor 019/055/072 (NAC019/NAC055/NAC072) act as direct negative regulators of *ICS1* expression (Chen *et al.*, 2009; Zhang *et al.*, 2010b; van Verk *et al.*, 2011; Zheng *et al.*, 2012). These findings suggest that a sophisticated network may be responsible for regulating *ICS1* expression. Nevertheless, how different regulators are dynamically and/or hierarchically coordinated remains to be explored.

Previously, we have shown that probenazole, an effective inducer of systemic acquired resistance, efficiently induces *ICS1* expression in Arabidopsis (Yu *et al.*, 2010). In the present study, we used the yeast one-hybrid (Y1H) system to screen for upstream direct regulators of *ICS1*, and identified TCP8, a transcription factor of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) family. We further confirmed that TCP8 was a direct positive regulator of *ICS1*. *TCP8*, along with *TCP9*, *TCP13* and *TCP20*, significantly respond to pathogen infection, with TCP8 and TCP9 redundantly regulating *ICS1* expression during the immune response. Importantly, we demonstrate that TCP8 interacts with most of the transcription factors involved in the regulation of *ICS1*, suggesting that TCP proteins may act as orchestrators to regulate the expression of *ICS1* during pathogen infection.

RESULTS

TCPs bind to a negative regulatory region of the *ICS1* promoter in yeast cells

To screen for the putative trans-regulators of *ICS1* using the Y1H system, we first analyzed the *ICS1* promoter and identified a short fragment with a significant regulatory effect. A 1.2 kb promoter–GUS fusion construct was generated, and stable transformants were obtained. Histochemical staining analysis revealed that there was no detectable GUS activity in these transformants; however, GUS activity was significantly induced in leaves infiltrated with *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*.ES4326) (Figure 1a and Figure S1a). The inducibility of the system was confirmed by simultaneous activation of endogenous *ICS1* expression (Figure S1b), and the co-localization of GUS staining with the subsequent chlorosis resulting from *Psm*.ES4326 infiltration (Figure S1c). The 1.2 kb promoter fragment was then dissected, and a series of 5′ truncated fragments was used to create GUS fusion constructs. Stable transformants were generated. Using histochemical staining, GUS activity assay and quantitative RT–PCR analysis, we observed a dramatic enhancement in GUS expression in the 128 bp–GUS transformants, but, unexpectedly, this occurred in the absence of pathogen challenge. This was in contrast to the results observed with the 316 bp–GUS transformants, as well as all other transformants harboring longer promoter–GUS fusions, for which GUS activity was barely detected throughout plant development (Figure 1b and Figure S2a,b). This indicates that there is a strong negative regulatory region between −128 and −316 bp, and the binding of repressor(s) to this region may be necessary for suppression of *ICS1* expression during plant growth and development.

Based on the results of promoter dissection and sequence alignment analysis, we amplified a 281 bp PCR fragment from the promoter region between −138 and −418 bp upstream of the translational start site of *ICS1* (Figure 1a and Figure S3). Using the fragment as a bait, we identified three independent positive clones of a TCP family transcription factor, TCP8 (At1g58100), in one Y1H screening, and verified its interaction with the promoter region by re-transformation (Figure 1c). To determine whether other TCPs are also capable of binding to this region, we performed an exhaustive Y1H verification. Except for the genes that we were unable to clone or caused a severe growth delay of yeast cells (*TCP12*, *TCP16*, *TCP18* and *TCP22*), a total of 17 TCP proteins showed varied binding abilities. In addition to TCP8, TCP3, TCP7, TCP9 and TCP15 also showed strong binding ability. In contrast, TCP5, TCP11 and TCP19 appeared not to bind to the promoter region (Figure 1d). In addition, transformants of a few TCP genes grew rather slowly

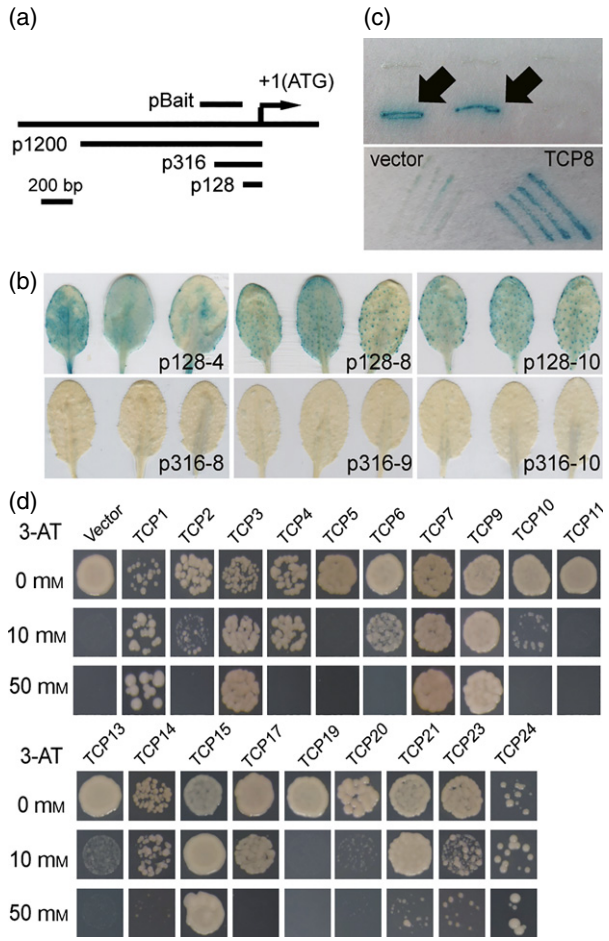

Figure 1. TCP transcription factors bind to a negative regulatory region of the *ICS1* promoter in yeast cells.
(a) Schematic diagram showing representative DNA fragments of the *ICS1* promoter used in this study. The position relative to the translation start site (+1) is indicated by the numbers after the letter p. pBait represents the DNA fragment between −138 and −418 bp used as the bait sequence for the Y1H assay.
(b) Histochemical indication of GUS activity in transgenic lines harboring the GUS reporter gene driven by 128 bp (*p128*) or 316 bp (*p316*) DNA fragments of the *ICS1* promoter. The numbers after *p128* and *p316* indicate independent transgenic lines.
(c) Staining of β–galactosidase activity (LacZ reporter) in yeast transformants. Upper panel: two representative positive colonies identified by Y1H screening (arrows). Lower panel: re-transformants of *TCP8* showing strong β–galactosidase activity. Empty vector was used as a negative control.
(d) Binding capabilities of various TCP proteins to the bait sequence tested by growth of yeast on SD medium lacking Trp, Leu and His supplemented with the indicated concentrations of 3–amino-1,2,4–triazole (3–AT). Yeast cell suspensions used in this assay were adjusted to OD_{600} = 0.1. Experiments were repeated twice with similar results.

compared with transformants of the empty vector, probably due to the toxic effect of their proteins on yeast cells, especially when over-expressed under the control of the strong T7 promoter. This result is consistent with a recent report that TCP4 blocks yeast cell division (Aggarwal *et al.*, 2011).

TCP8 specifically binds to the TCP binding site in the *ICS1* promoter *in vitro* and *in vivo*

TCP family transcription factors (TFs) characteristically bind to consensus sequences, with class I members binding to GGNCCCAC and class II members binding to GGGNCCAC (Kosugi and Ohashi, 2002). A dual TCP binding sequence (GGGCCCAC), which meets the binding requirement for both class I and class II members, is present in the *ICS1* promoter region at approximately −150 bp (Figure S3). To test whether the TCPs showing strong binding ability to the *ICS1* promoter region are capable of specifically binding to this site, we selectively constructed recombinant TCP8, TCP9 and TCP15 proteins (strong binding in the Y1H assay) and a TCP19 protein (no binding in the Y1H assay) with a maltose binding protein (MBP) fused to their N–termini and a 6xHis tag fused to their C–termini. Except for MBP–TCP9–6xHis, which could not be expressed in *Escherichia coli* BL21, MBP–TCP8–6xHis, MBP–TCP15–6xHis and MBP–TCP19–6xHis proteins were expressed and purified using both maltose and Ni–NTA columns. An electrophoretic mobility shift assay (EMSA) was then performed using the recombinant proteins and oligonucleotides harboring the TCP binding sequence. As shown in Figure 2(a) and Figure S4, a shift in mobility was clearly observed when labeled probe was pre-incubated with MBP–TCP8–6xHis, and addition of excess unlabeled wild-type probe competed with the binding. The binding specificity was further corroborated by the observation that addition of excess unlabeled probe containing a mutated TCP binding site failed to compete with the binding. A weak binding ability was also detected when the recombinant MBP–TCP15–6xHis was used (Figure S4a). In contrast, no binding ability was observed for MBP–TCP19–6xHis (Figure S4b), which is consistent with our Y1H assay results. These results indicate that TCP8 and TCP15, but not TCP19, are capable of specifically binding to the TCP binding sequence of *ICS1 in vitro*.

We further examined whether TCP8 targets the *ICS1* promoter *in vivo* using a chromatin immunoprecipitation (ChIP) assay. By creating TCP8–GFP transgenic plants and quantifying the immunoprecipitated DNA, *ICS1* promoter fragments around the TCP binding site (P3–P7) were found to be significantly enriched, compared with fragments further upstream or downstream of the site (Figure 2b), indicating that TCP8 directly binds to the TCP binding site of the *ICS1* promoter *in vivo*. In addition, we found that TCP8 clearly localized to the nucleus (Figure S5a). By fusing TCP8 to the GAL4 DNA-binding domain (GAL4–BD), we observed a trans-activation capability of TCP8 in the yeast strain AH109, which harbors a *HIS* reporter gene driven by an upstream activation sequence that is recognized by GAL4–BD (Figure S5b). These analyses collectively suggest that TCP8 and other related TCPs are putative regulators of *ICS1*.

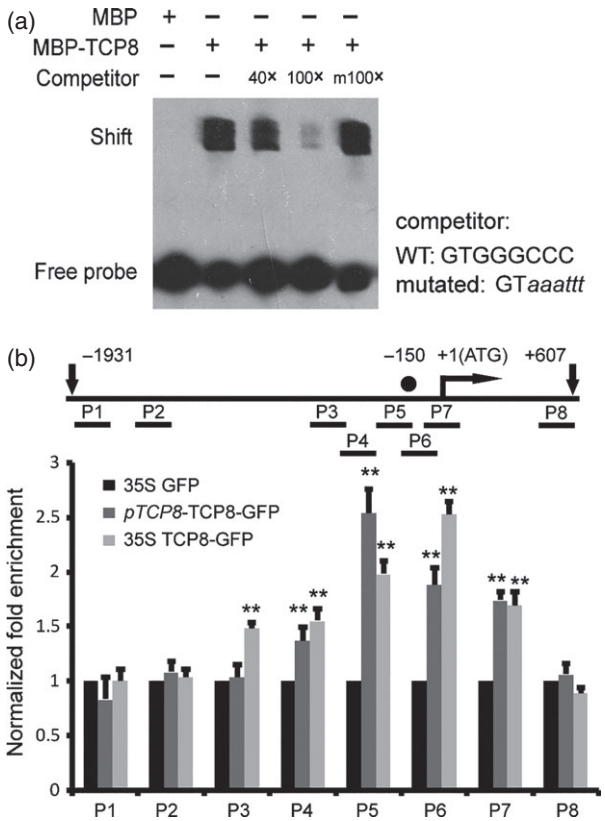

Figure 2. TCP8 binds to the *ICS1* promoter both *in vitro* and *in vivo*.
(a) TCP8 directly bound to a 25 bp *ICS1* promoter fragment containing the TCP binding site in an electrophoretic mobility shift assay (EMSA). The TCP binding sequence in the probe is shown in capitals, and the mutated nucleotides are shown in italic. Experiments were repeated twice with similar results.
(b) DNA fragments around the TCP binding site were co-precipitated with TCP8. The closed circle above the line (at approximately −150 bp) indicates the putative TCP binding site. P1–P8 indicate various DNA fragments amplified in the chromatin immunoprecipitation (ChIP) assay. The 18S rRNA gene was used to normalize the quantitative PCR results for each of the ChIP samples. Values are means and SE of three quantitative PCR measurements. Another biological repeat produced a similar result. Asterisks indicate statistically significant differences compared with the control (35S–GFP) (**$P < 0.01$, unpaired *t* test).

TCP8/TCP9 positively regulate *ICS1* expression with redundancy upon pathogen infection, and TCPs are involved in maintaining *ICS1* expression

Because *ICS1* expression is dramatically induced by pathogen infection, we first examined how *TCP8* and other *TCP* genes respond to *Psm*.ES4326 infection using quantitative RT–PCR. *TCP8* was significantly up-regulated 24 h after inoculation, together with *TCP9*, *TCP13* and *TCP20*, but most other TCP genes examined were down-regulated (Figure 3a). This result is in agreement with published microarray data (Figure S6). To confirm the role of TCP8 in the regulation of *ICS1* expression, a *TCP8* T–DNA insertion line named *tcp8–1* (*CS875709*) was identified and analyzed (Figure S7). However, no significant alterations in either *ICS1* expression or pathogen resistance were observed in *tcp8–1* under our experimental conditions (Figure 3e,f and Figure S8). Previous analyses have implied that there may be functional redundancy among TCP family members in the regulation of *ICS1* expression. According to phylogenetic analysis, pathogen response and a binding ability test in yeast cells, TCP9 is one of the proteins that may be redundant with TCP8. A *TCP9* T–DNA insertion line, designated *tcp9–3* (SALK_035853), was isolated (Figure S7) and a *tcp8–1 tcp9–3* double mutant was generated. No obvious alterations in *ICS1* expression and pathogen resistance were detected in *tcp9–3* (Figure 3e,f and Figure S8). However, a significant attenuation in resistance to *Psm*.ES4326 was observed in the *tcp8–1 tcp9–3* double mutant, compared with those in single mutants or wild-type plants (WT, Col–0). When pressure-infiltrated with pathogen, *tcp8–1 tcp9–3* plants partially mimicked the phenotype of the *ICS1* mutant *sid2–2* (Figure 3b) and a key SA signaling component mutant *npr1*, showing a significant increase in bacterial growth (Figure 3e). Introduction of a genomic fragment of *TCP8* fused to GFP at its C–terminus (p*TCP8*-TCP8-GFP) into *tcp8–1 tcp9–3* restored its resistance to *Psm*.ES4326 (Figure 3b,e,f). We then analyzed the dynamic changes of *ICS1* expression and SA content over 48 h after *Psm*.ES4326 inoculation. Both the *ICS1* transcript level and SA content reached their peaks at approximately 24 h, and intriguingly, although *ICS1* expression and SA content were only slightly compromised during their responsive phases, more dramatic decreases of *ICS1* expression and SA content were observed in *tcp8–1 tcp9–3* 36 and 48 h after inoculation during their declining phases (Figure 3c, d). Significant reductions in *PR* gene induction were also observed in *tcp8–1 tcp9–3* 36 h after inoculation (Figure 3f). These observations imply that TCP8 and TCP9 may play an important role in maintaining *ICS1* expression and consequently SA content during the dynamic cycle of pathogen infection. The decreases in *ICS1* expression and SA content are consistent with the observations of more severe disease symptoms and faster bacterial growth in *tcp8–1 tcp9–3*. Although *ICS1* expression was compromised to a significant extent in *tcp8–1 tcp9–3*, it was far from being abolished. This may suggests the involvement of other TCPs or other types of transcription (co-)factors in positively mediating *ICS1* regulation, which is in agreement with the finding that the defect in resistance to pathogen in *sid2–2* is more severe than that in *tcp8–1 tcp9–3*.

To further determine the importance of the TCP binding site in *ICS1* transcriptional regulation, we complemented *sid2–2* with the coding sequence (CDS) of *ICS1* driven by a 1529 bp promoter in which the TCP binding site was or was not mutated. Compared with plants complemented with *ICS1* under the control of the native promoter (*Comp.ICS1*), mutation of the TCP binding site (Com*p.ICS1m*) resulted in a significant reduction in pathogen resistance

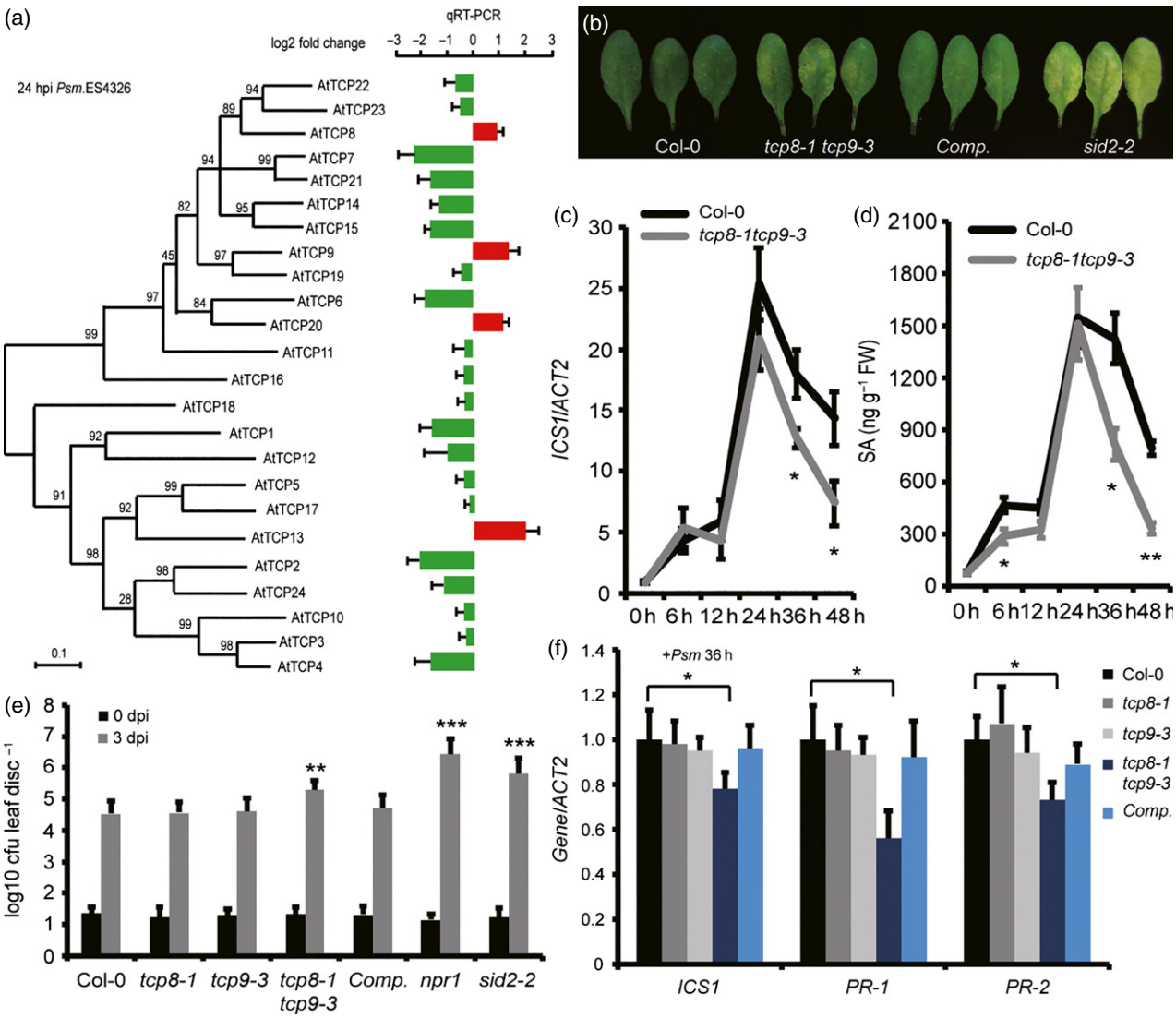

Figure 3. TCP8 and TCP9 act redundantly to regulate *ICS1* expression, and contribute to plant resistance to *Psm*.ES4326.

(a) Changes in the transcription of 24 TCP genes in response to *Psm*.ES4326 infection. Leaf samples of Col-0 were collected 24 h post-inoculation.

(b) Disease symptoms of Col–0, *tcp8–1 tcp9–3*, *Comp.* (complementation) and *sid2–2* at 3 days post-inoculation (dpi). *Comp.* refers to transgenic *tcp8–1 tcp9–3* plants harboring *TCP8* genomic DNA. Four-week-old plants were pressure-infiltrated with *Psm*.ES4326 at an OD_{600} of 0.0001.

(c) Changes in the transcript level of *ICS1* in Col–0 and *tcp8–1 tcp9–3* upon inoculation with *Psm*.ES4326. Leaf samples were collected at the indicated time points and subjected to gene expression analysis.

(d) Changes in the level of endogenous SA in Col–0 and *tcp8–1 tcp9–3* upon inoculation with *Psm*.ES4326.

Values in (c) and (d) are means and SD of measurements from three biological replicates, each comprising a mixed sample of at least six individual plants.

(e) Measurements of *Psm*.ES4326 growth in the leaves of various genotypes. Data were obtained from three independent experiments, each with 5–8 biological replicates.

(f) Transcript levels of *ICS1*, *PR–1* and *PR–2* at 36 h after *Psm*.ES4326 inoculation. Values in (e) and (f) are means and SDs. Asterisks indicate statistically significant differences compared with 0 dpi or as indicated (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Unpaired *t* tests.

In (a), (c) and (f), the transcription of related genes was quantified by quantitative RT–PCR, with *ACTIN2* as an internal control for normalizing their transcript levels. The transcript level of each gene in Col–0 was arbitrarily set to 1.

as well as *ICS1* induction (Figure 4a–c). This again suggests the involvement of TCPs in the positive regulation of *ICS1*. We subsequently performed a dual luciferase assay to analyze the activation of *ICS1* promoter activity in Arabidopsis protoplasts (Hellens *et al.*, 2005). A 1529 bp *ICS1* promoter fragment, with or without a mutation in the TCP binding site, was fused to the *LUC* gene. Protoplasts from the mature leaves of 4-week-old Col–0 plants were transiently transfected via poly(ethylene glycol)-mediated transformation, and relative LUC activities were measured accordingly. Protoplasts were prepared 24 h after *Psm*.ES4326 inoculation. A dramatic reduction in the responsiveness of the mutated *ICS1* promoter was observed, and mutations involving more nucleotides

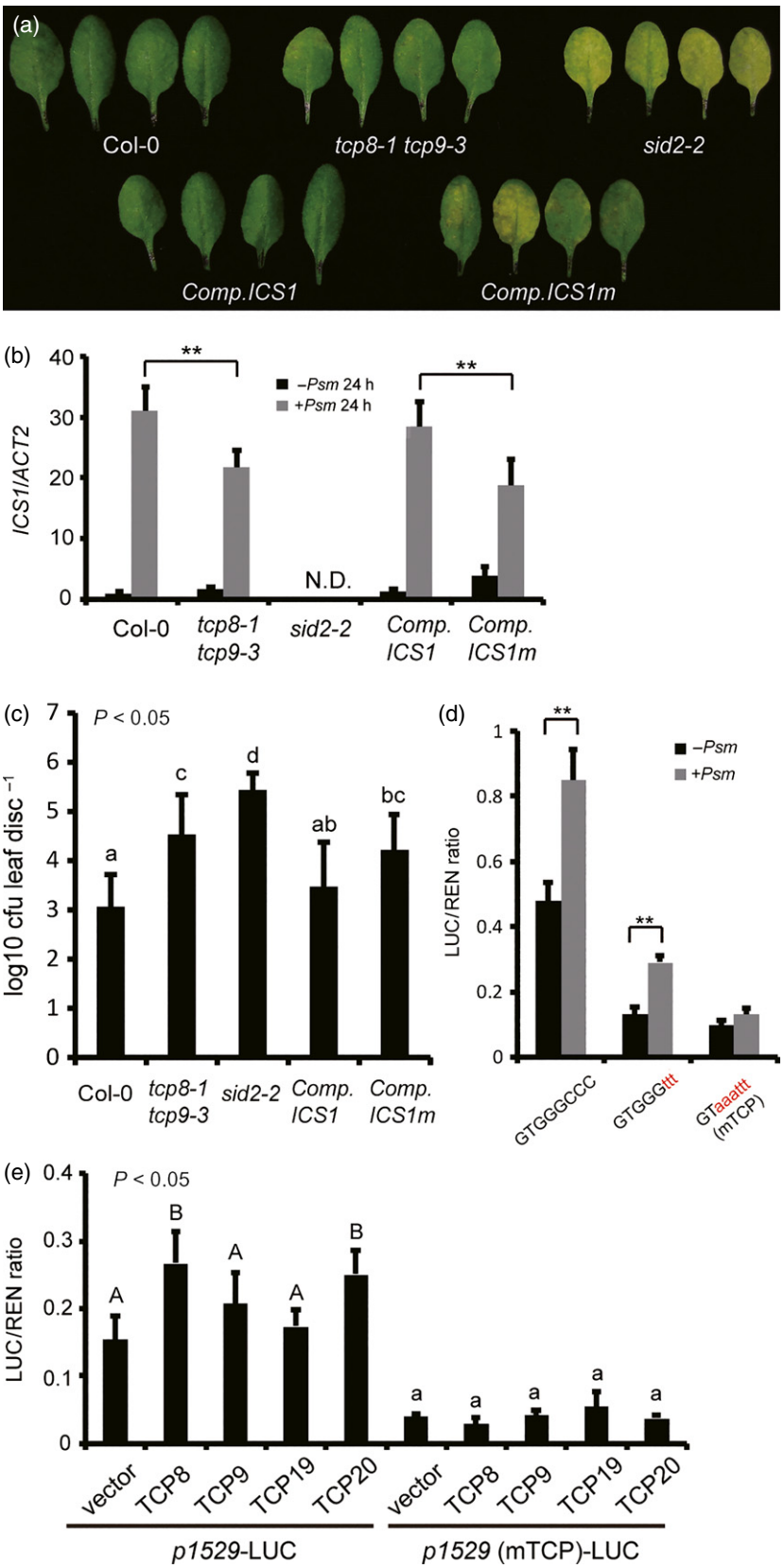

Figure 4. The TCP binding site is important for *ICS1* expression.

(a) Disease symptoms of Col–0, *tcp8–1 tcp9–3*, *sid2–2* and *Comp.*(complementation) at 3 days post-noculation. The leaves of 4-week-old plants were inoculated with *Psm*.ES4326 at an OD_{600} of 0.0001.

(b) Changes in the transcript level of *ICS1* upon inoculation of *Psm*.ES4326 in the leaves of various genotypes. Transcription of *ICS1* was quantified by quantitative RT–PCR, with *ACTIN2* as an internal control for normalizing its expression level. The transcript level of *ICS1* in untreated Col–0 was arbitrarily set to 1. Data from four independent T_1 lines were merged.

(c) Measurements of *Psm*.ES4326 growth in various genotypes at 3 dpi. Data from six independent T_1 lines were merged; different letters indicate statistically significant differences between means (unpaired *t* tests).

Comp.ICS1 refers to transgenic *sid2–2* plants harboring the *ICS1* promoter and the *ICS1* CDS; *Comp.ICS1m* refers to transgenic *sid2–2* plants harboring the *ICS1* promoter with a mutated TCP binding site and the *ICS1* CDS. The mTCP sequence is shown in (d).

(d) The importance of the TCP binding site for *ICS1* promoter activity in protoplasts. The promoter activity was expressed as the ratio of firefly luciferase activity (LUC) to 35S-driven *Renilla* luciferase activity (REN, internal control). The TCP binding sequence is shown in capitals, and the mutated nucleotides are shown in lower case (*x* axis).

(e) Over-expression of TCP genes further enhances the activity of the *ICS1* promoter (*p1529*-LUC) in protoplasts. The mTCP sequence is shown in (d).

In (d) and (e), data from three biological replicates were merged. Values are means and SDs. Different letters indicate statistically significant differences at the *P* value indicated (paired *t* tests). Asterisks indicate statistically significant differences at $P < 0.01$ (paired *t* tests).

resulted in a more significant decrease in LUC activity (Figure 4d). These results suggest that the TCP binding site is critical for up-regulating *ICS1* expression upon pathogen attack. There was a significant enhancement of LUC activity in Arabidopsis protoplasts when p*ICS1-LUC* and *TCP8*/*TCP20* were co-expressed, but no change in LUC activity was detected when p*ICS1-LUC* was co-expressed with *TCP9* or *TCP19* (Figure 4e). This suggests that TCP8 or TCP20 alone possesses a positive regulatory role in *ICS1* expression. This is consistent with the trans-activation ability of TCP8 (Figure S5b). Mutations in the consensus TCP binding site abolished the enhancement of LUC activity (Figure 4e), suggesting the pivotal role of this site in *ICS1* regulation mediated by TCP TFs. Our data collectively indicate that TCP proteins are probably involved in direct positive regulation of *ICS1* expression. However, this is a little surprising, given that the TCP binding site resides in the negative regulatory region of *ICS1* promoter.

TCP8 interacts with other TFs involved in *ICS1* regulation

In parallel with the ongoing research in our laboratory, a number of TFs that directly regulate *ICS1* expression have been reported. Of these, SARD1, CBP60g and WRKY28 are transcriptional activators (Zhang *et al.*, 2010b; van Verk *et al.*, 2011), whereas EIN3, EIL1, NAC019, NAC072 and NAC055 are transcriptional repressors (Chen *et al.*, 2009; Zheng *et al.*, 2012). These findings indicate that *ICS1* expression is regulated in a delicate and sophisticated manner in plant cells. To explore the potential relationship between TCPs and the identified TFs with respect to regulation of *ICS1* expression, a bimolecular fluorescence complementation (BiFC) assay was performed in tobacco mesophyll cells to test whether TCPs interacted with these TFs, using self-interaction of TCP8 as a positive control (Valsecchi *et al.*, 2013) (Figure S9a). Our results showed that TCP8 interacted with most of the TFs involved in *ICS1* regulation, including SARD1, NAC019 and WRKY28 (Figure 5a). Other TCPs also showed more or less interaction with these TFs, but not as strong as TCP8 (Figure 5a). However, there were no interactions between TCPs and EIN3 or EIL1 (Figure 5a and Figure S9b,c). Interestingly, strong interactions were detected among TCP8, TCP9 and TCP20 (Figure S9d, e). We then used an *in vitro* pull-down assay to further examine the interaction of TCP8 with these TFs. MBP-fused TFs and glutathione *S*–transferase (GST)-fused TCP8 were expressed in *E. coli* and incubated with amylase resin beads and glutathione Sepharose beads, respectively. The pulled-down proteins were separated by SDS–PAGE and immunoblotted using appropriate antibodies. As shown in Figure 5(b,c), similar interaction profiles were observed. These results suggest that TCP proteins and other related TFs may co-regulate the expression of *ICS1*. As both activators and repressors interact with TCP8, it is not surprising that the TCP proteins involved in *ICS1* regulation function either as activators or repressors, depending on the specific TFs that they interact with. This is consistent with a previous report suggesting that TCP20 may function as both an activator or repressor (Herve *et al.*, 2009).

DISCUSSION

It is thought that *ICS1* is under sophisticated regulation, but the underlying mechanism is far from being eluci-

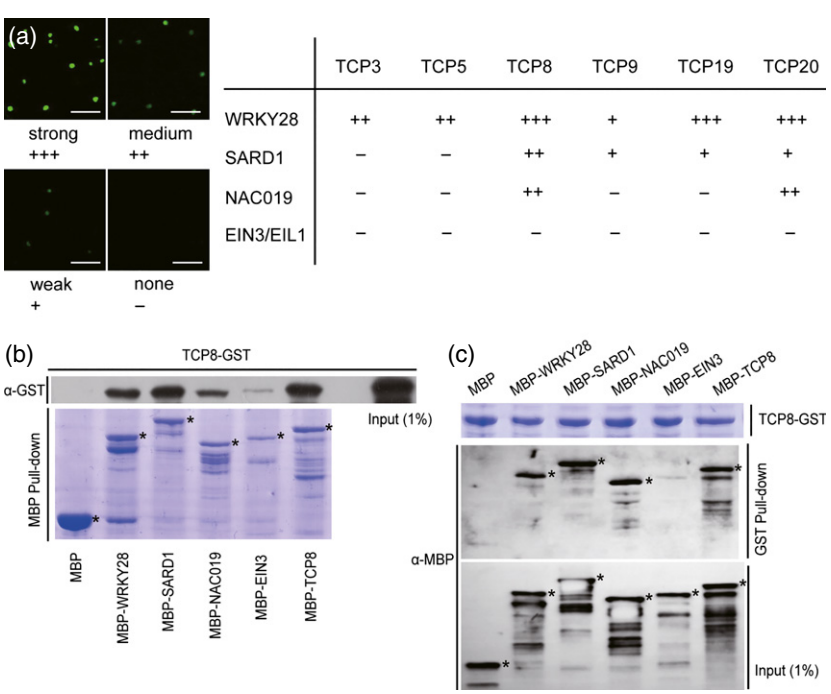

Figure 5. Interactions between TCP transcription factors and other known *ICS1* regulators.
(a) Demonstration of interactions between TCPs of interest and various transcription factors (TFs) by a bimolecular fluorescence complementation (BiFC) assay. TCPs were fused to the N–terminal part of YFP (nYFP) and TFs to be examined were fused to the C–terminal part of YFP (cYFP). Scale bar = 40 μm.
(b, c) Indication of interactions between TCP8 and various TFs by an *in vitro* pull-down assay. A mixture of GST-tagged TCP8 with MBP-tagged TFs was used for this assay. MBP protein was included as a negative control. Asterisks indicate target protein bands. In (b), MBP-tagged TFs were pulled down by amylose resin, and the association of GST–TCP8 was examined using an anti-GST antibody. In (c), GST–TCP8 was pulled down by glutathione Sepharose beads, and the association of MBP-tagged TFs was examined using an anti-MBP antibody. Experiments were repeated twice with similar results.

dated. In this study, we identified TCP8 and some other TCP TFs as putative trans-regulators of *ICS1* by yeast one-hybrid screening. The results were subsequently validated by detection of a typical TCP binding site within the promoter region. The involvement of TCP8 in the regulation of *ICS1* expression was further supported by results showing *in vitro* binding and *in vivo* targeting to the TCP binding site (Figures 1c and 2), as well as by a similar expression pattern to *ICS1* under pathogen attack (Figure 3a). By examining the regulatory effect of the TCP binding site and over-expressing *TCP8* in Arabidopsis protoplasts (Figure 4), we demonstrated that TCPs, represented by TCP8, act as positive regulators of *ICS1* expression upon pathogen inoculation. Although the regulatory role of TCP8 itself was ambiguous when examined by knocking out *TCP8*, the role of TCPs in the positive regulation of *ICS1* expression was validated, because when both *TCP8* and *TCP9* were mutated, the *ICS1* induction was significantly compromised (Figure 3c,f), and, as expected, a significant reduction in immune response was also observed upon pathogen infection. This indicates that there is a functional redundancy among TCP proteins with respect to positive regulation of *ICS1* expression, and TCP9 is one of the corresponding proteins of TCP8. The importance of TCPs in the positive regulation of *ICS1* is further supported by detection of a severe defect in *ICS1* expression in the *sid2–2* complementation and dual-luciferase assays when the TCP binding site was mutated (Figure 4b,d). However, although other TCP protein(s) may also be involved in the activation of *ICS1*, as implied by the observation that numerous TCP proteins bind to the *ICS1* promoter in yeast cells (Figure 1d), TCP8 and TCP9 may be the most important ones in terms of mediating *ICS1* induction during pathogen attack, because the *tcp8 tcp9* double mutant exhibited comparable *ICS1* induction and susceptibility to *Psm*.ES4326 inoculation to transgenic *Comp.ICS1m sid2–2* plants (Figure 4a–c).

We were initially puzzled by the identification of a TCP binding site with a positive regulatory role in a promoter region that appears to play a negative role in modulating the basal level of *ICS1*. Nevertheless, the finding may imply the existence of a sophisticated regulation mode associated with this region. We speculated that the region may possess a dual function, i.e. suppressing *ICS1* expression under normal growth conditions, but enhancing its expression upon defense induction. Our speculation is indeed supported by a report that EIN3/EIL1, which bind to the *ICS1* promoter region (between −120 and −324 bp) that covers the TCP binding site (at approximately −150 bp), negatively regulate *ICS1* expression in a constitutive manner, and removal of the EIN3 binding region results in increased *ICS1* transcription (Chen *et al.*, 2009). Given the finding that the TCPs and EIN3/EIL1 may not interact with each other (Figure 5 and Figure S9b,c), it would not be surprising if the TCPs compete directly with EIN3/EIL1 for *ICS1* promoter binding in a circumstance-dependent manner. In addition, more studies are also required to identify other *cis*- and *trans*-acting elements, if any, and to determine their combined effect on *ICS1* expression.

TCP proteins, which are plant-specific TFs, include 24 putative members in the Arabidopsis genome (Martin-Trillo and Cubas, 2010). They share a basic helix-loop-helix (bHLH) motif called the TCP domain. Based on slight differences in their TCP domains, TCP proteins may be divided into two sub-families, class I and class II. The class I proteins recognize the consensus binding sequence GGNCCCAC, whereas the class II proteins prefer the rather similar sequence GGGNCCAC (Kosugi and Ohashi, 2002). Interestingly, the TCP binding sequence GGGCCCAC in the *ICS1* promoter meets the binding requirements for both class I and class II members. This suggests a greater complexity of regulation and/or the possibility of involvement of multiple TCP proteins in the regulation of *ICS1* expression, because class I and class II members may function divergently or even antagonistically, as revealed in the regulation of other aspects of plant growth and development (Danisman *et al.*, 2012). Consistently, an enhanced level of *ICS1* basal expression was detected when the TCP binding site was mutated (Figure 4b), and a reduction in the level of *ICS1* basal expression was observed in the *tcp8 tcp9* double mutant (Figure S8b). This may indicate that negative regulation of *ICS1* basal expression may be partially accomplished by antagonistic class II TCP(s) by directly competing for the same binding site. TCP proteins are involved in the regulation of cell division, expansion and differentiation (Kosugi and Ohashi, 1997; Li *et al.*, 2005; Yuan *et al.*, 2009), as well as other aspects of growth and development (Takeda *et al.*, 2006; Tatematsu *et al.*, 2008; Pruneda-Paz *et al.*, 2009). TCPs were also shown to be targeted by diverse virulence pathogen effectors, suggesting the involvement of TCP proteins in plant–pathogen interactions (Mukhtar *et al.*, 2011; Wessling *et al.*, 2014). Recently, TCP8, TCP14 and TCP15 were reported to be involved in effector-trigged immunity by interacting with SRFR1, a negative regulator that specifically regulates effector-trigged immunity (Kim *et al.*, 2014). To our knowledge, no TCP proteins have ever been identified as involved in the direct regulation of SA synthesis and SA-related basal resistance.

TCP proteins usually form homo- or heterodimers to recognize target genes with various affinities (Kosugi and Ohashi, 2002). TCP8 is able to self-assemble in dimers, trimers and even oligomers in a concentration-dependent manner, and blocking TCP8 aggregation by interaction with TCP15 blocks TCP8 aggregation (self-assembling) and stabilizes TCP8 in monomers (Valsecchi *et al.*, 2013). Interactions of TCP proteins with TFs in other regulatory modules have been extensively described (Masuda *et al.*, 2008; Pruneda-Paz *et al.*, 2009). In this study, we demonstrated strong

interactions between TCP8 and SARD1, WRKY28 and NAC019 (Figure 5). SARD1 and its closely related protein CBP60g belong to a plant-specific ACBP60 protein family. They are recruited to the *ICS1* promoter, bind to the upstream sequence GAAATTTTGG at approximately −1217 bp upon pathogen attack, and positively regulate SA synthesis SARD1 and CBP60g redundantly regulate SA synthesis (Zhang *et al.*, 2010b; Wang *et al.*, 2011). WRKY28 activates *ICS1* expression via two binding sites at −445 bp and −460 bp, and its over-expression results in a 4.5-fold increase in *ICS1* mRNA in Arabidopsis protoplasts (van Verk *et al.*, 2011). NAC019, NAC055 and NAC072 are homologous NAC transcription factors that exert their inhibitory effect by directly repressing *ICS1* expression during pathogen infection, with NAC019 being shown to bind to several regions across the entire *ICS1* promoter (Zheng *et al.*, 2012). Although an interaction of the TCP protein TCP-interacting with CUP (TIC) with NAC family member CUPULIFORMIS (CUP) has been observed in *Antirrhinum majus* (Weir *et al.*, 2004), there are no reports on interactions between TCP proteins and CBP60 or the WRKY family members so far. Overall, our findings provide an insight into the regulatory modules of the TCP family.

SA and jasmonic acid (JA) are mutually antagonistic in regulating immune defense (Bari and Jones, 2009; Pieterse *et al.*, 2012). Recent studies have demonstrated that the class II CINCINNATA (CIN)/TCP members positively regulate JA synthesis by directly binding to the JA biosynthesis gene *LOX2*, whereas the class I TCP members, TCP9 and TCP20, exhibit an inhibitory effect on *LOX2* expression (Schommer *et al.*, 2008; Danisman *et al.*, 2012). In this study, we show that TCP8 and TCP9 positively regulate *ICS1* expression. Interestingly, in our BiFC assay, interactions were detected among TCP8, TCP9 and TCP20 (Figure S9d,e). It would be intriguing if TCP8, TCP9 and TCP20 constitute a regulatory node responsible for mutual communication between JA signaling and SA signaling by regulating their synthesis during the immune response.

In conclusion, TCP8 and TCP9 have been convincingly identified as *ICS1* regulators. Our demonstration of the involvement of TCP proteins in *ICS1* regulation, together with the interactions between TCP8 and other *ICS1* regulators, provides a new basis for exploring the sophisticated regulatory network of *ICS1* expression.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All mutants and transgenic lines used in this study were in the *Arabidopsis thaliana* ecotype Columbia–0 (Col–0) background. The T–DNA insertion mutant lines *tcp8–1* (CS875709) and *tcp9–3* (SALK_035853) were obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). The mutants were genotyped (Figure S7), and their insertion sites were confirmed by sequencing using a T–DNA left border primer (Lba1). The double mutant *tcp8–1 tcp9–3* was generated by crossing *tcp8–1* and *tcp9–3* and genotyping their F_2 progeny. All primers used for genotyping and sequencing are listed in Table S1.

Plants were grown at 22–24°C under a light intensity of approximately 100 μmol m^{-2} sec^{-1} and a 16 h light/8 h dark photoperiod.

Plasmid constructs

A 1.2 kb promoter fragment upstream of the *ICS1* translation start site (*p1200*) was amplified by PCR using primers NPICS1–1200–S and NPICS1–A (Table S1), and then cloned into the pCAMBIA1301 (GeneBank accession number AF234297) vector (GeneBank accession number AF234297) to generate the *ICS1* promoter-driven GUS expression construct *p1200*-GUS. The *p316*-GUS and *p128*-GUS constructs were generated similarly using primers NPICS1–316–S/NPICS1–A and NPICS1–128–S/NPICS1–A, respectively. For the Y1H assay, the CDS of *TCP8* was amplified using primers AD–TCP8–S and AD–TCP8–A, and cloned into the pGADT7 vector (Clontech, http://www.clontech.com/). To test TCP8 trans-activation activity in yeast cells, the *TCP8* CDS was amplified using primers BD–TCP8–S/BD–TCP8–A, and cloned into the pGBKT7 vector (Clontech). For the *TCP8* genomic complementation experiment, a DNA fragment of approximately 2.8 kb containing the TCP8 coding region as well as the 1582 bp promoter region was amplified from Col–0 by PCR using primers TCP8g–Com–S/TCP8g–Com–A and sub-cloned into the pENTR–D–topo (Invitrogen, http://www.lifetechnologies.com/cn/zh/home/brands/invitrogen.html) entry vector to produce pENTR–TCP8g. The entry clone was transferred into the destination vector pMDC204 (Curtis and Grossniklaus, 2003) by LR recombination to produce p*TCP8*-TCP8-GFP. For the *ICS1* genomic complementation experiment, a 1529 bp fragment of the *ICS1* promoter with or without a mutation in the TCP binding site was fused with the *ICS1* CDS. The fused sequence was sub-cloned into the pENTR–D–topo entry vector, and then transferred into destination vector pMDC204 to produce *Comp.ICS1* and *Comp.ICS1m*. For the dual luciferase assay, a 1529 bp fragment of the *ICS1* promoter with or without a mutation in the TCP binding site was amplified using primers TPICS1–1529–S/TPICS1–A, digested with *Xho*I/*Spe*I and cloned into the pGreenII 0800–LUC vector (Hellens *et al.*, 2005). For the BiFC assay, the CDSs of *TCP3*, *TCP5*, *TCP8*, *TCP9*, *TCP19* and *TCP20* with the stop codon removed were amplified and cloned into the pXY103 (nYFP) (Sun *et al.*, 2013) vector. The CDSs of *TCP8*, *TCP9*, *TCP20*, *SARD1*, *NAC019*, *WRKY28*, *EIN3* and *EIL1* with the stop codon removed were cloned into the pXY104 (cYFP) vector (Sun *et al.*, 2013). To produce proteins in *E. coli* BL21, the CDS of *TCP8* was cloned into the pGEX–4T–1 vector (GE Healthcare, http://www.gelifesciences.com/), while the CDSs of *TCP8*, *SARD1*, *NAC019* and *WRKY28* were cloned into the pMAL–C5G vector (New England Biolabs, https://www.neb.com/). The DNA binding region of *EIN3* (amino acids 1–314) (Zhong *et al.*, 2009) was amplified using primers pMAL–EIN3–S/pMAL–EIN3–A, and cloned into pMAL–C5G. All the primers used for plasmid construction are listed in Table S1.

Yeast one-hybrid (Y1H) screening and trans-activation activity assay

Y1H screening was performed using the MATCHMAKER system (Clontech). The bait sequence was inserted into the p*HISi–1* (GeneBank accession number U89928) and p*LacZi* (GeneBank accession number U89671) reporter vectors (GeneBank accession number U89671) with a minimal promoter. These vectors were then integrated into the genome of yeast strain YM4271 to create a target–reporter strain. An Arabidopsis cDNA library CD4–30 (purchased

from the Arabidopsis Biological Resource Center) was introduced into the target–reporter strain. Approximately 5×10^5 transformants were initially screened on plates containing SD medium lacking Trp, Leu and His (SD-TLH) supplemented with 10 mM 3–amino-1,2,4–triazole (3–AT) (HIS reporter). Colonies grown on SD-TLH were transferred to filter paper for the β–galactosidase activity test (LacZ reporter). The prey fragments of the positive colonies were identified by sequencing. For the re-transformation assay and individual TCP tests, the full-length CDSs of candidate genes and TCPs were cloned into the pGADT7 vector, and transferred into strain YM4271 harboring the integrated bait sequences. The trans-activation assay was performed using yeast strain AH109 with the *HIS* gene as a reporter.

Protoplast transformation and dual-luciferase reporter assay

Arabidopsis protoplasts were isolated from Col–0 young leaves (approximately 4 weeks old), using an enzymatic hydrolysate containing 0.35% cellulose RS (Yakult, http://www.yakult.co.jp/ypi/en/product_k.html) and 0.15% pectolyase Y–23 (Yakult), 20 mM KCl, 0.4 M mannitol, 20 mM MES and 10 mM $CaCl_2$. Plasmids were introduced into protoplasts by the poly(ethylene glycol)-mediated method (Liu and Howell, 2010). Protoplasts were spun down (100 ***g***, 4°C for 5 min), and incubated in lysis buffer. Firefly and *Renilla* luciferases were detected using a dual-luciferase assay kit (Promega, http://cn.promega.com/) and a Synergy 2 multi-mode microplate (Bio–Tek, http://www.biotek.com/) as described previously (Sun *et al.*, 2013). For pathogen induction experiments, leaves inoculated with *Psm*.ES4326 for 24 h were used for protoplast preparation.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously with minor modifications (Zhang *et al.*, 2011). Briefly, approximately 10 g of 10-day-old seedlings of p*TCP8*-TCP8-GFP, 35S-TCP8-GFP and 35S-GFP transgenic plants were collected from square Petri dishes on which they were growing vertically on nylon mesh. Tissues were suspended in 1.0% formaldehyde and fixed for 15 min under vacuum. The fixation was quenched for 10 min on ice with addition of glycine at a final concentration of 125 mM. The samples were washed four times with water (4°C for 5 min), dried with paper towels, and frozen in liquid nitrogen. Nuclei were isolated and then sonicated as described by Zhang *et al.* (2011). Tubes containing sonicated chromatin with DNA fragments of approximately 0.5–1 kb were centrifuged to remove any insoluble debris (16 000 ***g***, 4°C for 60 min), and incubated at 4°C overnight with an anti-GFP antibody (ab290, Abcam, http://www.abcam.com/). The protein–DNA complex was then immunoprecipitated by incubation with Protein A agarose beads (Upstate, Merck, http://www.merckmillipore.com/CN/en) for 6 h at 4°C with rotation. Elution and reverse cross-linking were performed as previously described (Zhang *et al.*, 2011). The purified DNA was resuspended in TE buffer, and the enrichment of DNA fragments was measured by quantitative PCR using the primers listed in Table S1. The 18S rRNA gene was used to normalize the quantitative PCR results in each ChIP sample. Fold enrichment of each region in the p*TCP8*-TCP8-GFP and 35S-TCP8-GFP transgenic lines was calculated by comparison with the control 35S-GFP transgenic line. Data were subjected to Student's *t* test for determination of the significance of differences.

Pseudomonas syringae inoculation

Psm.ES4326 was grown on plates containing King's B medium (King *et al.*, 1954) supplemented with 100 μg ml^{-1} streptomycin at 30°C for 2 days before inoculation. The growth of bacterial was measured as described previously with minor modifications (Durrant *et al.*, 2007). The *Psm*.ES4326 suspension (OD_{600} = 0.0001 unless otherwise indicated) in 10 mM $MgSO_4$ was infiltrated into the abaxial side of the 5th and 6th leaves of 4-week-old plants using a needle-less syringe. Eight plants were assayed for each genotype. Leaf discs were harvested immediately or at the indicated time points after inoculation, homogenized into fine powder, and dissolved in 10 mM $MgSO_4$ to generate serial tenfold dilutions. Rows of 10 μl aliquots from each dilution were plated on King's B medium containing 100 μg ml^{-1} streptomycin using an eight-channel multi-pipettor, and dispensed by drawing along the surface. Plates were incubated for 2 days at 30°C, and individual colonies growing on the most readable dilution ($20 < X < 100$ was desired, where X represents colony number) were counted. Statistical analysis was performed using Student's *t* test.

Salicylic acid quantification

Leaf samples inoculated with *Psm*.ES4326 were harvested at the indicated time points. Free SA was then extracted and quantified from approximately 350 mg tissue per sample using HPLC analysis as previously described (Yu *et al.*, 2010). Briefly, tissues were homogenized and sonicated twice in 3 ml of 100% methanol. After centrifugation (12 000 ***g***, 4°C for 10 min), the combined supernatants were dried with nitrogen. The residue was resuspended in 1 ml of 5% trichloroacetic acid and sonicated for 5 min. The free SA was then extracted using 4 ml of an ethylacetate/cyclohexane mixture (1:1 v/v). The organic phase containing free SA was dried with nitrogen. The dried extract was dissolved in 0.5 ml water, filtered, and analyzed by HPLC. HPLC was performed on an Agilent (http://www.agilent.com/home?cc=cn) C18 column [3.5 μm (particle size), 4.6 mm (inside diameter) × 150 mm (length)] and run at 30°C in 20 mM NaAc, pH 5.2, with a flow rate of 1 ml min^{-1}. SA was detected and quantified fluorometrically (305 nm excitation and 407 nm emission).

Protein subcellular localization and BiFC analyses

Seedlings of homozygous T_3 lines of TCP8–GFP transgenic plants were grown on half-strength MS medium. The subcellular localization of TCP8–GFP was examined under an LSM A710 laser confocal fluorescence microscope (Zeiss, http://www.zeiss.com/microscopy/en_de/home.html). For BiFC analysis, paired constructs were transiently expressed in *Nicotiana benthamiana* leaf mesophyll cells via *Agrobacterium*-mediated infiltration, and fluorescence was examined 48 h after infiltration by confocal microscopy.

EMSA

Recombinant TCP8, TCP15 and TCP19 proteins fused with MBP and a His tag were expressed in *E. coli* strain BL21, and purified using both maltose and Ni–NTA columns. EMSA was performed using a LightShift® chemiluminescent EMSA kit (Thermo Scientific, http://www.thermofisher.com/en/home.html) according to the manufacturer's instructions. The 25 bp DNA probes used in EMSA are listed in Table S1.

Pull-down assay

All proteins were expressed in *E. coli* strain BL21, and soluble proteins in 1× column buffer (20 mM Tris/HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) with 0.5% v/v Triton X–100 and 10% v/v glycerol were used for the pull-down assay. Approximately 5–10 μg of each MBP-tagged and GST-tagged protein were mixed and incubated with 30 μl of the appropriate resin beads for

4 h at 4°C. The resin beads were then washed six times with 1 ml of 1× column buffer. Fifty microliters of 1× SDS loading buffer were added to each of the tubes containing the washed beads, and recombinant proteins were separated by 10% SDS–PAGE and detected by Western blotting using either anti-GST or anti-MBP antibodies (TransGen, http://www.transgen.com.cn/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. A 1.2 kb *ICS1* promoter is responsive to *Pseudomonas syringae* inoculation.

Figure S2. Fragments *p128* and *p316* of the *ICS1* fragments show contrasting transcriptional activities.

Figure S3. Sequence alignment of *ICS* promoters from *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Capsella rubella*, *Brassica rapa* and *Thellungiella halophile*.

Figure S4. EMSA analysis for binding of TCPs to the *ICS1* promoter.

Figure S5. Nuclear localization and trans-activation activity of TCP8 in yeast cells.

Figure S6. Changes in the expression of TCP genes in response to *Psm*.ES4326 infection.

Figure S7. Characterization of *tcp8–1* and *tcp9–3*.

Figure S8. *tcp8–1* and *tcp9–3* showed no obvious attenuation in terms of resistance to *Psm*.ES4326.

Figure S9. BiFC analysis of interactions between TCPs and TFs.

Table S1. Sequences of primers used in this study.

REFERENCES

Aggarwal, P., Padmanabhan, B., Bhat, A., Sarvepalli, K., Sadhale, P.P. and Nath, U. (2011) The TCP4 transcription factor of Arabidopsis blocks cell division in yeast at G1→S transition. *Biochem. Biophys. Res. Commun.* **410**, 276–281.

Alonso-Ramirez, A., Rodriguez, D., Reyes, D., Jimenez, J.A., Nicolas, G., Lopez-Climent, M., Gomez-Cadenas, A. and Nicolas, C. (2009) Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in Arabidopsis seeds. *Plant Physiol.* **150**, 1335–1344.

Bari, R. and Jones, J. (2009) Role of plant hormones in plant defence responses. *Plant Mol. Biol.* **69**, 473–488.

Buchanan-Wollaston, V., Page, T., Harrison, E. *et al.* (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. *Plant J.* **42**, 567–585.

Catinot, J., Buchala, A., Abou-Mansour, E. and Metraux, J.P. (2008) Salicylic acid production in response to biotic and abiotic stress depends on isochorismate in *Nicotiana benthamiana*. *FEBS Lett.* **582**, 473–478.

Chen, H.M., Xue, L., Chintamanani, S. *et al.* (2009) ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in Arabidopsis. *Plant Cell*, **21**, 2527–2540.

Coquoz, J.L., Buchala, A. and Metraux, J.P. (1998) The biosynthesis of salicylic acid in potato plants. *Plant Physiol.* **117**, 1095–1101.

Curtis, M.D. and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiol.* **133**, 462–469.

Danisman, S., van der Wal, F., Dhondt, S. *et al.* (2012) Arabidopsis class I and class II TCP transcription factors regulate jasmonic acid metabolism and leaf development antagonistically. *Plant Physiol.* **159**, 1511–1523.

Delaney, T.P., Uknes, S., Vernooij, B. *et al.* (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1250.

Durrant, W.E., Wang, S. and Dong, X. (2007) Arabidopsis SNI1 and RAD51D regulate both gene transcription and DNA recombination during the defense response. *Proc. Natl Acad. Sci. USA*, **104**, 4223–4227.

Fu, Z.Q., Yan, S., Saleh, A. *et al.* (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature*, **486**, 228–232.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, **261**, 754–756.

Garcion, C., Lohmann, A., Lamodiere, E., Catinot, J., Buchala, A., Doermann, P. and Metraux, J.P. (2008) Characterization and biological function of the ISOCHORISMATE SYNTHASE2 gene of Arabidopsis. *Plant Physiol.* **147**, 1279–1287.

Heil, M., Hilpert, A., Kaiser, W. and Linsenmair, K.E. (2000) Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *J. Ecol.* **88**, 645–654.

Hellens, R.P., Allan, A.C., Friel, E.N., Bolitho, K., Grafton, K., Templeton, M.D., Karunairetnam, S., Gleave, A.P. and Laing, W.A. (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods*, **1**, 13.

Herve, C., Dabos, P., Bardet, C., Jauneau, A., Auriac, M.C., Ramboer, A., Lacout, F. and Tremousaygue, D. (2009) In vivo interference with AtTCP20 function induces severe plant growth alterations and deregulates the expression of many genes important for development. *Plant Physiol.* **149**, 1462–1477.

Horvath, E., Szalai, G. and Janda, T. (2007) Induction of abiotic stress tolerance by salicylic acid signaling. *J. Plant Growth Regul.* **26**, 290–300.

Kim, S.H., Son, G.H., Bhattacharjee, S., Kim, H.J., Nam, J.C., Nguyen, P.D., Hong, J.C. and Gassmann, W. (2014) The Arabidopsis immune adaptor SRFR1 interacts with TCP transcription factors that redundantly contribute to effector-triggered immunity. *Plant J.* **78**, 978–989.

King, E. O., Ward, M. K. and Raney, D. E. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.*, **44**, 301–307.

Kosugi, S. and Ohashi, Y. (1997) PCF1 and PCF2 specifically bind to cis elements in the rice proliferating cell nuclear antigen gene. *Plant Cell*, **9**, 1607–1619.

Kosugi, S. and Ohashi, Y. (2002) DNA binding and dimerization specificity and potential targets for the TCP protein family. *Plant J.* **30**, 337–348.

Lee, S., Kim, S.G. and Park, C.M. (2010) Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in Arabidopsis. *New Phytol.* **188**, 626–637.

Leon, J., Shulaev, V., Yalpani, N., Lawton, M.A. and Raskin, I. (1995) Benzoic acid 2-hydroxylase, a soluble oxygenase from tobacco, catalyzes salicylic acid biosynthesis. *Proc. Natl Acad. Sci. USA*, **92**, 10413–10417.

Li, C.X., Potuschak, T., Colon-Carmona, A., Gutierrez, R.A. and Doerner, P. (2005) Arabidopsis TCP20 links regulation of growth and cell division control pathways. *Proc. Natl Acad. Sci. USA*, **102**, 12978–12983.

Li, W., Ahn, I.P., Ning, Y. *et al.* (2012) The U-Box/ARM E3 ligase PUB13 regulates cell death, defense, and flowering time in Arabidopsis. *Plant Physiol.* **159**, 239–250.

Liu, J.X. and Howell, S.H. (2010) bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in Arabidopsis. *Plant Cell*, **22**, 782–796.

Malamy, J., Carr, J.P., Klessig, D.F. and Raskin, I. (1990) Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. *Science*, **250**, 1002–1004.

Martinez, C., Pons, E., Prats, G. and Leon, J. (2004) Salicylic acid regulates flowering time and links defence responses and reproductive development. *Plant J.* **37**, 209–217.

Martin-Trillo, M. and Cubas, P. (2010) TCP genes: a family snapshot ten years later. *Trends Plant Sci.* **15**, 31–39.

Masuda, H.P., Cabral, L.M., De Veylder, L., Tanurdzic, M., Engler, J.D., Geelen, D., Inze, D., Martienssen, R.A., Ferreira, P.C.G. and Hemerly, A.S. (2008) ABAP1 is a novel plant Armadillo BTB protein involved in DNA replication and transcription. *EMBO J.* **27**, 2746–2756.

Mauch, F., Mauch-Mani, B., Gaille, C., Kull, B., Haas, D. and Reimmann, C. (2001) Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. *Plant J.* **25**, 67–77.

Mauch-Mani, B. and Slusarenko, A.J. (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to *Peronospora parasitica*. *Plant Cell*, **8**, 203–212.

Metraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W. and Inverardi, B. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science*, **250**, 1004–1006.

Morris, K., Mackerness, S.A.H., Page, T., John, C.F., Murphy, A.M., Carr, J.P. and Buchanan-Wollaston, V. (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J.* **23**, 677–685.

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

Mukhtar, M.S., Carvunis, A.R., Dreze, M. *et al.* (2011) Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science*, **333**, 596–601.

Pajerowska-Mukhtar, K.M., Wang, W., Tada, Y., Oka, N., Tucker, C.L., Fonseca, J.P. and Dong, X. (2012) The HSF-like transcription factor TBF1 is a major molecular switch for plant growth-to-defense transition. *Curr. Biol.* **22**, 103–112.

Pieterse, C.M., Van der Does, D., Zamioudis, C., Leon-Reyes, A. and Van Wees, S.C. (2012) Hormonal modulation of plant immunity. *Annu. Rev. Cell Dev. Biol.* **28**, 489–521.

Pruneda-Paz, J.L., Breton, G., Para, A. and Kay, S.A. (2009) A functional genomics approach reveals CHE as a component of the Arabidopsis circadian clock. *Science*, **323**, 1481–1485.

Rajjou, L., Belghazi, M., Huguet, R., Robin, C., Moreau, A., Job, C. and Job, D. (2006) Proteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. *Plant Physiol.* **141**, 910–923.

Ribnicky, D.M., Shulaev, V.V. and Raskin, I.I. (1998) Intermediates of salicylic acid biosynthesis in tobacco. *Plant Physiol.* **118**, 565–572.

Schommer, C., Palatnik, J.F., Aggarwal, P., Chetelat, A., Cubas, P., Farmer, E.E., Nath, U. and Weigel, D. (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. *PLoS Biol.* **6**, 1991–2001.

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., Geisler, M., Mauch, F., Nawrath, C. and Metraux, J.P. (2013) Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiol.* **162**, 1815–1821.

Su, Z., Ma, X., Guo, H., Sukiran, N.L., Guo, B., Assmann, S.M. and Ma, H. (2013) Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis. *Plant Cell*, **25**, 3785–3807.

Sun, L., Yang, Z.T., Song, Z.T., Wang, M.J., Lu, S.J. and Liu, J.X. (2013) The plant-specific transcription factor gene NAC103 is induced by bZIP60 through a new cis-regulatory element to modulate the unfolded protein response in Arabidopsis. *Plant J.* **76**, 274–286.

Takeda, T., Amano, K., Ohto, M.A., Nakamura, K., Sato, S., Kato, T., Tabata, S. and Ueguchi, C. (2006) RNA interference of the Arabidopsis putative transcription factor TCP16 gene results in abortion of early pollen development. *Plant Mol. Biol.* **61**, 165–177.

Tatematsu, K., Nakabayashi, K., Kamiya, Y. and Nambara, E. (2008) Transcription factor AtTCP14 regulates embryonic growth potential during seed germination in *Arabidopsis thaliana*. *Plant J.* **53**, 42–52.

Tedman-Jones, J.D., Lei, R., Jay, F., Fabro, G., Li, X.M., Reiter, W.D., Brearley, C. and Jones, J.D.G. (2008) Characterization of Arabidopsis mur3 mutations that result in constitutive activation of defence in petioles, but not leaves. *Plant J.* **56**, 691–703.

Traw, M.B. and Bergelson, J. (2003) Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in Arabidopsis. *Plant Physiol.* **133**, 1367–1375.

Tsuda, K., Sato, M., Stoddard, T., Glazebrook, J. and Katagiri, F. (2009) Network properties of robust immunity in plants. *PLoS Genet.* **5**, e1000772.

Uppalapati, S.R., Ishiga, Y., Wangdi, T., Kunkel, B.N., Anand, A., Mysore, K.S. and Bender, C.L. (2007) The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with *Pseudomonas syringae* pv. *tomato* DC3000. *Mol. Plant Microbe Interact.* **20**, 955–965.

Valsecchi, I., Guittard-Crilat, E., Maldiney, R., Habricot, Y., Lignon, S., Lebrun, R., Miginiac, E., Ruelland, E., Jeannette, E. and Lebreton, S. (2013) The intrinsically disordered C-terminal region of *Arabidopsis thaliana* TCP8 transcription factor acts both as a transactivation and self-assembly domain. *Mol. BioSyst.* **9**, 2282–2295.

Verberne, M.C., Verpoorte, R., Bol, J.F., Mercado-Blanco, J. and Linthorst, H.J.M. (2000) Overproduction of salicylic acid in plants by bacterial transgenes enhances pathogen resistance. *Nat. Biotechnol.* **18**, 779–783.

van Verk, M.C., Bol, J.F. and Linthorst, H.J.M. (2011) WRKY transcription factors involved in activation of SA biosynthesis genes. *BMC Plant Biol.* **11**, 89–100.

Vos, I.A., Pieterse, C.M.J. and van Wees, S.C.M. (2013) Costs and benefits of hormone-regulated plant defences. *Plant. Pathol.* **62**, 43–55.

Wang, D., Pajerowska-Mukhtar, K., Culler, A.H. and Dong, X. (2007) Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. *Curr. Biol.* **17**, 1784–1790.

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen, L.V., Katagiri, F. and Glazebrook, J. (2011) CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling. *Plant J.* **67**, 1029–1041.

Weir, I., Lu, J., Cook, H., Causier, B., Schwarz-Sommer, Z. and Davies, B. (2004) CUPULIFORMIS establishes lateral organ boundaries in Antirrhinum. *Development*, **131**, 915–922.

Wessling, R., Epple, P., Altmann, S. *et al.* (2014) Convergent targeting of a common host protein-network by pathogen effectors from three kingdoms of life. *Cell Host Microbe*, **16**, 364–375.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**, 562–565.

Yu, J., Gao, J.O., Wang, X.Y., Wei, Q.A., Yang, L.F., Qiu, K. and Kuai, B.K. (2010) The pathway and regulation of salicylic acid biosynthesis in probenazole-treated Arabidopsis. *J. Plant Biol.* **53**, 417–424.

Yuan, Z., Gao, S., Xue, D.W., Luo, D., Li, L.T., Ding, S.Y., Yao, X., Wilson, Z.A., Qian, Q. and Zhang, D.B. (2009) RETARDED PALEA1 controls palea development and floral zygomorphy in rice. *Plant Physiol.* **149**, 235–244.

Zhang, X.D., Chen, S.X. and Mou, Z.L. (2010a) Nuclear localization of NPR1 is required for regulation of salicylate tolerance, isochorismate synthase 1 expression and salicylate accumulation in Arabidopsis. *J. Plant Physiol.* **167**, 144–148.

Zhang, Y.X., Xu, S.H., Ding, P.T. *et al.* (2010b) Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proc. Natl Acad. Sci. USA*, **107**, 18220–18225.

Zhang, Z.L., Ogawa, M., Fleet, C.M., Zentella, R., Hu, J., Heo, J.O., Lim, J., Kamiya, Y., Yamaguchi, S. and Sun, T.P. (2011) Scarecrow-like 3 promotes gibberellin signaling by antagonizing master growth repressor DELLA in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **108**, 2160–2165.

Zhang, K., Halitschke, R., Yin, C., Liu, C.J. and Gan, S.S. (2013) Salicylic acid 3-hydroxylase regulates Arabidopsis leaf longevity by mediating salicylic acid catabolism. *Proc. Natl Acad. Sci. USA*, **110**, 14807–14812.

Zheng, X.Y., Spivey, N.W., Zeng, W., Liu, P.P., Fu, Z.Q., Klessig, D.F., He, S.Y. and Dong, X. (2012) Coronatine promotes *Pseudomonas syringae* virulence in plants by activating a signaling cascade that inhibits salicylic acid accumulation. *Cell Host Microbe*, **11**, 587–596.

Zhong, S., Zhao, M., Shi, T., Shi, H., An, F., Zhao, Q. and Guo, H. (2009) EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of Arabidopsis seedlings. *Proc. Natl Acad. Sci. USA*, **106**, 21431–21436.