ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity

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Recognition of pathogens by host plants leads to rapid transcriptional reprogramming and activation of defence responses. The expression of many defence regulators is induced in this process, but the mechanisms of how they are controlled transcriptionally are largely unknown. Here we use chromatin immunoprecipitation sequencing to show that the transcription factors SARD1 and CBP60g bind to the promoter regions of a large number of genes encoding key regulators of plant immunity. Among them are positive regulators of systemic immunity and signalling components for effector-triggered immunity and PAMP-triggered immunity, which is consistent with the critical roles of SARD1 and CBP60g in these processes. In addition, SARD1 and CBP60g target a number of genes encoding negative regulators of plant immunity, suggesting that they are also involved in negative feedback regulation of defence responses. Based on these findings we propose that SARD1 and CBP60g function as master regulators of plant immune responses.

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Plants use a multilayered defence system to combat microbial pathogens. At the front line, pattern recognition receptors on the plasma membrane recognize conserved features of microbes, collectively known as microbe-associated molecular patterns or pathogen-associated molecular patterns (PAMPs), to activate PAMP-triggered immunity (PTI)\textsuperscript{1}. Most PAMP receptors belong to the receptor-like kinase and the receptor-like protein families. A second line of plant defence called effector-triggered immunity (ETI) relies on resistance (R) proteins that detect effector proteins secreted by pathogens to inhibit PTI (ref. 2). The majority of plant R proteins belong to the intracellular nucleotide-binding site (NB) leucine-rich repeats (LRR) protein family. Recognition of pathogens and activation of local defence responses further induce a secondary immune response in the distal part of plants termed systemic acquired resistance (SAR)\textsuperscript{3}.

Salicylic acid (SA) is a signal molecule that plays key roles in local defence and SAR (ref. 4). SALICYLIC ACID INDUCTION-DEFICIENT 2 (SID2) and ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) are required for pathogen-induced SA accumulation\textsuperscript{5,6}. Mutations in SID2 or EDS5 block the accumulation of SA, resulting in enhanced susceptibility to pathogens and loss of SAR (refs 5–7). SID2 encodes Isocorismate Synthase 1 (ICS1), which is a key enzyme in pathogen-induced SA synthesis\textsuperscript{8}. EDS5 encodes a transporter involved in exporting SA from chloroplast to cytoplasm\textsuperscript{8,9}. Activation of defence gene expression and resistance pathogenesis by SA depends on the downstream component NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1)\textsuperscript{10}. Recent studies showed that NPR1 and its paralogs, NPR3 and NPR4, bind to SA and may function as SA receptors\textsuperscript{11,12}.

Several genes encoding enzymes implicated in the synthesis of secondary metabolites have also been identified to be essential for SAR. Among them, FLAVIN-DEPENDENT MONOXYGENASE 1 (FMO1) encodes a putative monooxygenase\textsuperscript{13–15}, AGD2-LIKE DEFENSE RESPONSE PROTEIN1 (ALD1) encodes an aminotransferase\textsuperscript{16}, and avrPphB SUSCEPTIBLE3 (PBS3) encodes a member of the firefly luciferase superfamily\textsuperscript{17–19}. In fmo1, ald1 and pbs3 mutants, SA is severely compromised\textsuperscript{15,20,21}. ALD1 is involved in the synthesis of piperoc acid, which contributes to the induction of SAR (ref. 22), while the chemicals synthesized by FMO1 and PBS3 remain to be determined.

Two pathogen-induced transcription factors, SAR DEFICIENT 1 (SARD1) and CAM-BINDING PROTEIN 60-LIKE G (CBP60g), regulate the expression of ICS1 and are required for pathogen induction of SA synthesis\textsuperscript{23–25}. Following pathogen infection, SARD1 and CBP60g are recruited to the promoter of ICS1 (ref. 24). In the sard1 cbp60g double mutant, induction of ICS1 expression and SA synthesis is blocked\textsuperscript{24,25}. SARD1 and CBP60g belong to the same protein family but are regulated differently, suggesting that they function in parallel pathways to activate ICS1 expression\textsuperscript{23,24}. CBP60g, but not SARD1, can bind calmodulin. On the other hand, overexpression of SARD1, but not CBP60g, leads to constitutive activation of defence responses.

Arabidopsis SNC2 encodes a receptor-like protein that is required for resistance against pathogenic bacteria Pseudomonas syringae pv tomato (P.s.t) DC3000 and non-pathogenic bacteria P. syringae pv tomato DC3000 hrcC (refs 26,27). A gain-of-function mutation in snc2-1D leads to constitutive activation of both SA-dependent and SA-independent defence pathways\textsuperscript{26}. The snc2-1D mutant has small stature, accumulates high levels of salicylic acid, constitutively expresses PATHOGENESIS-RELATED (PR) genes, and exhibits enhanced pathogen resistance. From a suppressor screen of snc2-1D npr1-1, WRKY DNA-BINDING PROTEIN 70 (WRKY70) was identified as an essential regulator of the SA-independent pathway downstream of snc2-1D (ref. 26).

Here we report that SARD1 and CBP60g regulate not only the expression of ICS1 and SA synthesis, but also the expression of WRKY70 and the SA-independent defence pathway in snc2-1D. Chromatin immunoprecipitation (ChIP) analysis revealed that a large number of plant defence regulators including WRKY70 are direct binding targets of SARD1 and CBP60g suggesting that SARD1 and CBP60g function as master regulators of plant defence responses.

**Results**

**SARD1 and CBP60g are required for autoimmunity in snc2-1D.** To determine whether the increased SA synthesis in snc2-1D mutant plants is dependent on SARD1 and CBP60g, we crossed sard1-1 and cbp60g-1 into snc2-1D to obtain the sard1-1 snc2-1D and cbp60g-1 snc2-1D double mutants and the sard1-1 cbp60g-1 snc2-1D triple mutant. Quantitative reverse transcription PCR (RT–PCR) analysis showed that the expression of ICS1 in snc2-1D is much higher than in wild type, but the increased expression of ICS1 is blocked in the sard1-1 cbp60g-1 snc2-1D triple mutant (Fig. 1a). Consistent with the expression levels of ICS1, increased accumulation of SA in snc2-1D is also suppressed in the triple mutant (Fig. 1b).

The sard1-1 snc2-1D and cbp60g-1 snc2-1D double mutants have similar morphology as snc2-1D and are only slightly bigger than snc2-1D (Fig. 1c). Surprisingly, the mutant morphology of snc2-1D is almost completely suppressed in the sard1-1 cbp60g-1 snc2-1D triple mutant (Fig. 1d). Quantitative RT–PCR analysis showed that the expression levels of defence marker genes PR1 and PR2 are slightly lower in the double mutants but are markedly reduced in the triple mutant compared with snc2-1D (Fig. 1d,e). In addition, the enhanced resistance to Hyaloperonospora arabidopsidis Noco2 in snc2-1D is partially reduced in the double mutants and almost completely lost in the triple mutant (Fig. 1f). As blocking SA accumulation by eds5-3 has very little effect on the morphology, PR2 expression and resistance to H. arabidopsidis Noco2 in snc2-1D (ref. 26), these data suggest that SARD1 and CBP60g also regulate SA-independent pathways in snc2-1D.

**SARD1 and CBP60g regulate the expression of WRKY70.** In sard1 cbp60g mutant plants expressing the SARD1-1A fusion protein under its native promoter, pathogen-induced ICS1 expression was restored to similar level as in the cbp60g single mutant, suggesting that SARD1-1A functions similarly as wild-type SARD1 protein (Supplementary Fig. 1). To identify genes targeted by SARD1, ChIP was carried out on transgenic plants expressing a SARD1-1A fusion protein under its own promoter using an anti-HA antibody. The immuno-precipitated DNA was sequenced by Illumina sequencing. Analysis of the ChIP-seq data showed a × 20 genome coverage.

Sequence coverage at each position on the genome was plotted to identify peaks in the Arabidopsis genome. Analysis of peaks in the genic region showed that most sequence peaks are located in the 1.5 kb region upstream of the translation start site, which includes the 5'-UTRs and promoter regions. After removing genes that showed similar sequence peaks in the negative control, peaks with heights of 90 or greater were found in the introns of 84 genes, the 3'-UTRs of 60 genes and the 1.5 kb region upstream of the translation start sites of 1,902 genes. We focused our analysis on the group containing peaks with heights of 90 or greater in the 1.5 kb region upstream of the translation start sites (Supplementary Data 1), because it contains many genes encoding known regulators of plant defence that are strongly
induced by pathogen infection (Table 1). Distribution of sequence reads in the promoter and coding regions of these known defence regulators are shown in Supplementary Fig. 2.

One of the candidate target genes of SARD1 identified by ChIP-seq is WRKY70 (Table 1), which is known to regulate SA-independent defence responses in snc2-1D (ref. 26). Quantitative PCR analysis of the DNA immunoprecipitated by the anti-HA antibody confirmed that WRKY70 is a binding target of SARD1 (Fig. 2a). In sard1 cbp60g mutant plants expressing the CBP60g-HA fusion protein under its native promoter, pathogen-induced ICS1 expression was restored to similar level as in the sard1 single mutant, suggesting that CBP60g-HA functions similarly as wild-type protein (Supplementary Fig. 1). To determine whether WRKY70 is also a binding target of CBP60g, we carried out ChIP-PCR experiments on transgenic plants expressing a CBP60g-HA fusion protein under its own promoter using the anti-HA antibody. As shown in Fig. 2b, CBP60g is also targeted to the promoter region of WRKY70.

Next we analysed the expression of WRKY70 in snc2-1D, sard1-1 snc2-1D, cbp60g-1 snc2-1D and sard1-1 cbp60g-1 snc2-1D mutant plants. As shown in Fig. 2c, WRKY70 is expressed at a considerably higher level in snc2-1D than in wild type. The expression of WRKY70 is slightly lower in cbp60g-1 snc2-1D and clearly reduced in sard1-1 snc2-1D compared with snc2-D. However, it is further reduced to below wild-type level in the sard1-1 cbp60g-1 snc2-1D triple mutant (Fig. 2c). These data suggest that SARD1 and CBP60g have overlapping functions in regulating the expression of WRKY70 and that reduced expression of WRKY70 is at least partly responsible for the suppression of the snc2-1D-mediated SA-independent constitutive defence responses in the sard1-1 cbp60g-1 snc2-1D triple mutant.

SARD1 and CBP60g regulate the expression of EDS5 and NPR1. EDS5 is involved in pathogen-induced SA synthesis5,7. Analysis of the SARD1 ChIP-seq data revealed that EDS5 is a potential target gene of SARD1 as well (Table 1). A peak with a
height of 110 was identified ~700 bp upstream of the translation start site of EDS5. ChIP-PCR experiments confirmed that SARD1 is targeted to the promoter region of EDS5 (Fig. 3a). Further ChIP-PCR analysis showed that CBP60g also binds to the promoter region of EDS5 (Fig. 3b). To determine whether SARD1 and CBP60g are required for the induction of EDS5 by *Pseudomonas syringae* pv. *maculicola* (*P.s.m.*) ES4326, we compared the expression levels of EDS5 in wild type and *sard1-1 cbp60g-1* plants. As shown in Fig. 3c, induction of EDS5 by *P.s.m.* ES4326 is greatly reduced in the *sard1-1 cbp60g-1* double mutant (Fig. 3c). These data suggest that SARD1 and CBP60g directly regulate the expression of EDS5.

Another candidate target gene of SARD1 identified by ChIP-seq is *NPR1*, which encodes a putative SA receptor. A peak with a height of 163 was identified ~100 bp upstream of the translation start site of *NPR1* (Table 1). Binding of SARD1 to the promoter region of *NPR1* was confirmed by ChIP-PCR (Fig. 3d). As shown in Fig. 3e, CBP60g also targeted to the promoter region of *NPR1*. Analysis of the expression levels of *NPR1* in wild type and *sard1-1 cbp60g-1* plants showed that induction of *NPR1* by *P.s.m.* ES4326 is compromised in the *sard1-1 cbp60g-1* double mutant (Fig. 3f). These data suggest that SARD1 and CBP60g also regulate pathogen-induced expression of *NPR1*.

Multiple SAR regulators are targets of SARD1 and CBP60g. In addition to EDS5 and *NPR1*, three other genes required for SAR, *FMO1*, *ALD1* and *PBS3*, were identified as candidate target genes of SARD1 from the ChIP-seq data. The height of the peaks identified in the promoter regions of *FMO1*, *ALD1* and *PBS3* are 99, 138 and 199, respectively (Table 1). Binding of SARD1 to the promoters of these three genes was confirmed by ChIP-PCR experiments (Fig. 4a). Further ChIP-PCR analysis showed that CBP60g also binds to the promoters of these genes (Fig. 4b). Consistent with data from previous gene expression studies, we also observed dramatic reduction in bacteria-induced expression of *FMO1*, *ALD1* and *PBS3* in the *sard1-1 cbp60g-1* double mutant (Fig. 4c). These data suggest that SARD1 and CBP60g directly regulate the expression of *FMO1*, *ALD1* and *PBS3* in plant defence responses.

**Table 1 | Known defence regulators identified as candidate target genes of SARD1 by ChIP-seq.**

| AGI number   | Protein name                          | Peak height |
|--------------|---------------------------------------|-------------|
| AT3G56400    | WRKY DNA-BINDING PROTEIN 70 (WRKY70) | 214         |
| AT1G74710    | ISOCHORISATE SYNTHASE 1 (ICS1)        | 125         |
| AT4G39030    | ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5) | 110         |
| AT1G64280    | NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) | 163         |
| AT1G19250    | FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) | 99          |
| AT2G13810    | AGD2-LIKE DEFENCE RESPONSE PROTEIN 1 (ALD1) | 138         |
| AT5G31320    | avrPphB SUSCEPTIBLE 3 (PBS3)          | 199         |
| AT3G48090    | ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) | 258         |
| AT3G52090    | PHYTOALEXIN DEFICIENT 4 (PAD4)        | 137         |
| AT1G33560    | ACTIVATED DISEASE RESISTANCE 1 (ADRI) | 117         |
| AT4G33300    | ADRI-LIKE 1 (ADRI-L1)                 | 324         |
| AT5G04720    | ADRI-LIKE 2 (ADRI-L2)                 | 230         |
| AT4G33430    | BRII-ASSOCIATED RECEPTOR KINASE 1 (BAK1) | 134         |
| AT2G13790    | BAK1-LIKE 1 (BK1)                     | 190         |
| AT4G34460    | ARABIDOPSIS G PROTEIN β-SUBUNIT 1 (AGBI) | 200         |
| AT2G39660    | BOTRYTIS-INDUCED KINASE 1 (BKI)       | 99          |
| AT4G08500    | MAPK/ERK KINASE KINASE 1 (MEKK1)      | 135         |
| AT1G31660    | MITOGEN-ACTIVATED PROTEIN KINASE 4 (MPK4) | 141         |
| AT3G45640    | MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) | 264         |
| AT4G09570    | CALCIMUM-DEPENDENT PROTEIN KINASE 4 (CPK4) | 104         |
| AT3G46510    | PLANT U-BOX 13 (PUB13)                | 207         |
| AT1G80840    | WRKY DNA-BINDING PROTEIN 40 (WRKY40)  | 97          |
| AT2G25000    | WRKY DNA-BINDING PROTEIN 60 (WRKY60)  | 138         |
| AT2G04450    | NUCLEOSIDE DIPHOSPHATE LINKED TO SOME MOIETY X 6 (NUDT6) | 107         |
| AT4G12720    | NUCLEOSIDE DIPHOSPHATE LINKED TO SOME MOIETY X 7 (NUDT7) | 169         |
| AT1G1310     | MILDEW RESISTANCE LOCUS O 2 (MLO2)   | 100         |
| AT5G69000    | BONZAI 1 (BON1)                       | 151         |
| AT3G61970    | BON ASSOCIATION PROTEIN 1 (BAP1)      | 215         |
| AT2G45760    | BON ASSOCIATION PROTEIN 2 (BAP2)      | 314         |

AGI, Arabidopsis Genome Initiative; ChIP, chromatin immunoprecipitation; SARD1, SAR DEFICIENT1.

SARD1 and CBP60g target positive regulators of ETI. ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) encode positive regulators of defence responses activated by TIR-NB-LRR R proteins. *NDR1* is required for defence responses activated by CC-NB-LRR R proteins, but not *NDR1*, were identified as candidate target genes of SARD1 by ChIP-seq (Table 1). The height of the peaks identified in the promoter regions of *EDS1* and *PAD4* are 258 and 137, respectively. ChIP-PCR experiments showed that SARD1 was targeted to the promoter regions of *EDS1* and *PAD4*, but not *NDR1* (Fig. 5a). In addition, CBP60g is also targeted to the promoters of *EDS1* and *PAD4*, but not *NDR1* (Fig. 5b). Quantitative RT–PCR was subsequently carried out to determine whether induction of the expression of *EDS1* and *PAD4* by bacterial infections is dependent on SARD1 and CBP60g. As shown in Fig. 5c, induction of *EDS1* and *PAD4* by *P.s.m.* ES4326 is markedly reduced in the *sard1-1 cbp60g-1* double mutant. These data suggest that induction of *EDS1* and *PAD4* following pathogen infection is directly regulated by SARD1 and CBP60g.

**ADRI** and **ADRI-L1** and **ADRI-L2** encode three closely related CC-NB-LRR proteins required for immunity mediated by TIR-NB-LRR R proteins RPP2 and RPP4 (ref. 35). They were also identified as candidate target genes of SARD1 by ChIP-seq (Table 1). The heights of the peaks identified in the promoter regions of *ADRI*, *ADRI-L1* and *ADRI-L2* are 117, 324 and 230,
SARD1 and CBP60g are target negative regulators of defence. Analysis of the SARD1 ChIP-seq data also identified a number of negative regulators of plant immunity including PUB13, WRKY40, WRKY60, NUDT6, NUDT7, MLO2, BON1, BAP1 and BAP2 as candidate target genes of SARD1 (Table 1). Binding of SARD1 to the promoter regions of PUB13, WRKY40, WRKY60, NUDT6, NUDT7, MLO2, BON1, BAP1 and BAP2 was confirmed by ChIP-PCR analysis (Fig. 7a). In addition, CBP60g was also found to target the promoter regions of these nine genes (Fig. 7b). Quantitative RT–PCR analysis showed that the expression of PUB13, WRKY40, WRKY60, NUDT6, NUDT7, MLO2, BON1, BAP1 and BAP2 is all induced by P.s.m. ES4326 and the induction is either reduced or blocked in the sard1-1 cbp60g-1 double mutant (Fig. 7c). These data suggest that SARD1 and CBP60g regulate the expression of these negative regulators of plant immunity during plant defence.

SARD1 regulates gene expression through the GAAATTT element. Previously, we showed that SARD1 and CBP60g bind preferentially to the oligonucleotide probe GAAATTTGG (ref. 24). Bioinformatics analysis showed that the GAAATTT motif within this probe is over-represented in the promoters of the genes with SARD1 and CBP60g-dependent expression. Analysis of the 1,902 candidate target genes of SARD1 in Supplementary Data 1 showed that the GAAATTT motif is also over-represented in the promoter regions of these genes (P<10−15, Fisher’s exact test). This motif is over-represented in the promoter regions of 29 confirmed target genes of SARD1 and CBP60g listed in Table 1 (P<0.005, Fisher’s exact test) as well. However, not every gene in this group contains this motif in their promoter region. It is likely SARD1 and CBP60g can also bind to certain variants of the GAAATTT motif. Interestingly, a closely related sequence motif, G(A/T)AAAT(T/G), was identified as a conserved motif (P<10−25, Fisher’s exact test) among the sequence peaks of genes in Supplementary Data 1 using the motif discovery algorithm DREME.

To test whether SARD1 activates its target gene expression through the GAAATTT motif, we made a construct expressing the luciferase reporter gene under the control of a 56 bp fragment from the ChIP-Seq peak region in the promoter of ICS1, which contains a GAAATTT and a related GAAATT motif (Fig. 8a). Two additional constructs containing mutations in these two motifs were also created to determine whether they are required for activation of reporter gene expression by the 56 bp fragment. These reporter gene constructs were transformed into Arabidopsis protoplasts to examine the luciferase reporter expression levels. As shown in Fig. 8b, all three constructs expressed similar levels of luciferase as the original NOS101-Luc vector, suggesting that the 56 bp promoter fragment cannot activate luciferase expression on its own in protoplast transient assays. However, when the

expression of BAK1, BKK1, AGB1, BIK1, MEEK1, MKK4, MPK3 and CPK4 is induced P.s.m. ES4326 and the induction is reduced in the sard1-1 cbp60g-1 double mutant, suggesting that SARD1 and CBP60g may directly regulate their expression in plant defence responses.
**Figure 3** | **EDS5 and NPR1** are direct binding targets of SARD1 and CBP60g. (a,b) Recruitment of SARD1-HA (a) and CBP60g-HA (b) to EDS5 promoter after *P. syringae* ES4326 infection as determined by ChIP-PCR. ChIP was performed as described in Fig. 2. Real-time PCR was carried out using primers specific to EDS5 promoter. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of IgG controls, which are set as one. Bars represent means ± s.d. (n = 3). (c) Induction of EDS5 expression in wild type and sard1-1 cpb60g-1 by *P. syringae* ES4326. Leaves of 25-day-old plants were infiltrated with *P. syringae* ES4326 (OD600 = 0.001) or 10 mM MgCl2 (mock) 12 h before collection for RT–PCR analysis. Bars represent means ± s.d. (n = 3). (d,e) Recruitment of SARD1-HA (d) and CBP60g-HA (e) to the promoter of NPR1 after treatment with *P. syringae* ES4326. ChIP and data analysis were carried out similarly as in a and b. Bars represent means ± s.d. (n = 3). (f) Induction of NPR1 expression by *P. syringae* ES4326 in wild-type and sard1-1 cpb60g-1 double mutant plants. Samples were collected 12 h after infiltration with *P. syringae* ES4326 (OD600 = 0.001) or 10 mM MgCl2 (mock). Bars represent means ± s.d. (n = 3).

**Figure 4** | **SARD1 and CBP60g target genes essential for SAR.** (a,b) Binding of SARD1-HA (a) and CBP60g-HA (b) to the promoter regions of FMO1, ALD1 and PBS3 following infection by *P. syringae* ES4326 as determined by ChIP-PCR. ChIP was performed as described in Fig. 2. Real-time PCR was carried out using gene-specific primers. ChIP results are presented as fold changes by dividing signals from ChIP with the anti-HA antibody by those of the IgG control, which are set as one. Bars represent means ± s.d. (n = 3). (c) Induction of the expression of FMO1, ALD1 and PBS3 by *P. syringae* ES4326 infection. Samples were collected 12 h after inoculation with *P. syringae* ES4326 (OD600 = 0.001) or 10 mM MgCl2 (mock). Expression levels were normalized with ACTIN1. Bars represent means ± s.d. (n = 3).
SARD1 and CBP60g function in coordinating the induction of SAR regulators during plant defence.

Several defence regulators that function upstream of SA synthesis are also regulated by SARD1 and CBP60g. Both PAD4 and EDS1 are required for pathogen-induced SA synthesis. PAD4 and EDS1 are targeted to the promoter regions of ADR1-L1 and ADR1-L2 following infection by P.s.m. ES4326 as determined by ChIP-qPCR. ChIP and data analysis were carried out similarly as in a and b. Bars represent means ± s.d. (n = 3). qPCR, quantitative PCR.

Discussion
SA functions as a key signalling molecule in SAR. SARD1 and CBP60g have previously been shown to regulate pathogen-induced SA synthesis. In this study, we showed that, in addition to ICS1, the expression of another regulator of SA synthesis, EDS5, is also likely controlled by SARD1 and CBP60g.

NPR1, a gene required for the perception of SA by plants, is a target of SARD1 and CBP60g as well. Moreover, SARD1 and CBP60g also regulate pathogen-induced expression of several other genes required for SAR. Both SARD1 and CBP60g are targeted to the promoter regions of FMO1, ALD1 and PBS3 and induction of these genes by P.s.m. ES4326 is markedly reduced in the sard1 cbp60g double mutant. These data suggest that SARD1 and CBP60g function in coordinating the induction of SAR regulators during plant defence.

Seventy-five defence regulators that function upstream of SA synthesis are also regulated by SARD1 and CBP60g. Both PAD4 and EDS1 are required for pathogen-induced SA synthesis. In addition, ADR1, ADR1-L1 and ADR1-L2, three helper R genes required for pathogen-induced SA synthesis, are also targets of SARD1 and CBP60g. Regulation of the induction of PAD4, EDS1, ADR1, ADR1-L1 and ADR1-L2 by SARD1 and CBP60g may play critical roles in promoting SA synthesis during pathogen infection.
We also found that SARD1 and CBP60g function downstream of the receptor-like protein SNC2 to regulate both SA-dependent and SA-independent defence pathways. SARD1 and CBP60g are required for the increased expression of ICS1 and SA synthesis in snc2-1D. Regulation of the SA-independent defence pathway by SARD1 and CBP60g appears to be at least partly through their control of WRKY70 expression, a key regulator of the SA-independent defence responses in snc2-1D (ref. 26), as both SARD1 and CBP60g are targeted to the promoter of WRKY70 and are required for the induction of WRKY70 in snc2-1D.

Furthermore, a large number of genes encoding regulatory components of PTI are binding targets of SARD1 and CBP60g and require SARD1 and CBP60g for induction by bacterial infection. Among them, BAK1 and BKK1 serve as co-receptors of FLS2 and EF-TU RECEPTOR (EFR) (refs 36–38). BIK1 and AGB1 function downstream of multiple PAMP receptors to regulate ROS production and defence against pathogen infection39–45. MEKK1, MKK4 and MPK3 are components of MAP kinase cascades downstream of PAMP receptors47,51–54. CPK4 was identified as a calcium dependent protein kinase downstream of FLS2 (ref. 48). The critical role of SARD1 and
CBP60g in PTI was further confirmed by the attenuation of flg22-induced pathogen resistance in the sard1 cbp60g double mutant.

In addition to upregulation of positive regulators, negative regulators are often induced during plant defence as well. Induction of negative regulators is critical for feedback inhibition of defence responses to prevent uncontrolled activation, which may lead to autoimmunity. We showed that a number of negative regulators of plant immunity including PUB13, WRKY40, WRKY60, NUDT6, NUDT7, MLO2, BON1, BAP1 and BAP2 are also targets of SARD1 and CBP60g. Among them, PUB13 is a U-box/ARM E3 ubiquitin ligase that regulates cell death as well as degradation of FLS2 after flagellin induction. WRKY40 and WRKY60 function redundantly with their close homologue, WRKY18, to repress basal defence. NUDT6 and NUDT7 are two Nudix domain-containing proteins that negatively regulate EDS1-dependent immune responses. MLO2 functions as a negative regulator of immunity mediated by the TIR-NB-LRR R protein SNC1 (ref. 62); BAP1 and BAP2 encode two C2 domain-containing proteins that negatively regulate programmed cell death. All these genes are induced following infection by P.s.m. ES4326 and their induction requires SARD1 and CBP60g, suggesting that SARD1 and CBP60g also play an important role in the negative feedback regulation of plant defence.

Bioinformatics analysis has previously been used to analyze genes that are co-expressed with a group of SARD1/CBP60g-dependent genes. Four genes including AGP5, At5g25760, CML46 and CML47 that form a small cluster with SARD1 and ICS1 were identified as candidate target genes of SARD1 and CBP60g. These genes are also identified as binding targets of SARD1 in our ChIP-seq data (Supplementary Data 1). EDS1 and PAD4 were also found to cluster with ICS1 in the co-expression analysis. They were placed upstream of SARD1 and CBP60g. Interestingly, both EDS1 and PAD4 have been shown to be targets of SARD1 and CBP60g in our ChIP studies. The commonly used defence marker genes PR1 and PR2 were also found in one of the clusters co-expressed with SARD1/CBP60g-dependent genes. However, both of them were not identified as binding targets of SARD1 in our ChIP-seq data, suggesting that they are not directly regulated by SARD1 and CBP60g. It is likely that genes co-expressed with SARD1/CBP60g-dependent genes include...
genes that are either directly or indirectly regulated by SARD1 and CBP60g.

In summary, a large number of genes encoding key regulators of plant immunity are direct binding targets of SARD1 and CBP60g and their expression is modulated by SARD1 and CBP60g during plant defense. This is consistent with the functions of these two transcription factors in PTI, ETI and SAR. Based on this data we suggest that SARD1 and CBP60g orchestrate the induction of plant defense regulators in plant immunity (Fig. 9).

Methods

Plant materials and growth conditions. Arabidopsis sard1-1, cbp60g-1, sard1-1 cbp60g-1 and sn2-1D npr1-1 mutants and SARD1-HA and CBP60g-HA transgenic plants were described previously. The sn2-1D single mutant was identified from the F2 population of a cross between Col-0 and sn2-1D npr1-1. sard1-1 sn2-1D, cbp60g-1 sn2-1D and sard1 cbp60g sn2-1D mutants were isolated from the F2 population of a cross between sard1 cbp60g-1 and sn2-1D npr1-1. Primers used for genotyping are listed in Supplementary Table 1. Plants were grown under long day conditions (16 h light per 8 h dark cycle) at 23°C unless otherwise specified.

Mutant analysis. To analyze gene expression in sn2-1D, sard1-1 sn2-1D, cbp60g-1 sn2-1D and sard1 cbp60g sn2-1D, 30 mg of leaves were collected from week-old soil-grown plants for RNA isolation. To analyze gene expression after P.s.m. ES4326 infection, leaves of 25-day-old plants grown under short day conditions (16 h light per 12 h dark cycle) at 23°C were used.

Pathogen infection. For ChIp experiments, two to three fully expanded leaves of 25-day-old plants grown under short day condition were infiltrated with P.s.m. ES4326 (OD600 = 0.001). The inoculated leaves were collected after 24 h. About 4 g of leaf tissue was cross-linked in 75 ml of 1% formaldehyde solution plus 0.01% Silwet L-77 under vacuum for 20 min. Glycine (2 M) was added to a final concentration of 0.125 M and the sample was vacuumed for an additional 5 min to stop cross-linking. The tissue was rinsed three times with 60 ml of cold ddH2O and dried with blotted paper. The nuclei were prepared as previously described and re-suspended in 300 μl of nuclei lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 0.1 M PMSF, 1×P). The nuclei suspensions were subsequently sonicated to shear the DNA to an average size of 0.3–1 kb.

Figure 9 | Proposed scheme for regulation of plant defence responses by SARD1 and CBP60g. Following pathogen infection, SARD1 and CBP60g are activated. Subsequently, the expression of a large number of their target genes is turned on. Upregulation of positive regulators of PTI, ETI and SAR and increased SA synthesis lead to enhanced plant immunity against pathogens. Meanwhile, negative regulators of plant immunity are turned on to attenuate plant defense responses.
at ~20 °C as ‘input’. ChIP dilution buffer (3 mL 1% Triton X-10, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl) was then added to the 15 mL tube. For pre-clearing, 100 μL of Protein A agarose beads was added with the ChIP dilution buffer was added to the chromatin samples and kept at 4 °C for 1 h with rotation. The beads were pelleted at 2,400 g for 2 min and the supernatants were divided equally into two samples. A volume of 5 μL (0.4 mg mL−1) of anti-HA antibody (Roche) was added to one sample for immunoprecipitation and immunoglobulin G was added to the other sample as control. The samples were incubated overnight at 4 °C with gentle agitation. Subsequently, 100 μL of Protein A agarose beads with ChIP dilution buffer was added to each sample and kept at 4 °C for 2 h with gentle agitation.

The Protein A beads were then pelleted by centrifugation at 2,400 g for 10 s at 4 °C. The beads were washed with low salt wash buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0), high salt wash buffer (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0, 0.5% NP-40, 1% LAC, 1% Sodium deoxycholate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and TE buffer sequentially. For each buffer, a quick wash by spinning at 2,400 g for 10 s and a second wash with 5 min agitation were performed. A volume of 1 mL of buffer was used in each wash.

After the final wash, the samples were pelleted for an additional 2 min at 2,400 g to remove the supernatant thoroughly. To elute the immune complexes, 250 μL of Elution Buffer (1% SDS, 0.1 M NaHCO3) was added to the beads. The samples were vortexed briefly and incubated at 65 °C for 15 min with gentle agitation. After spinning at 3,800 g for 2 min, the supernatant was carefully transferred to a fresh tube. The pellet was eluted one more time with 250 μL of Elution Buffer and the two eluates were combined (a total of ~500 μL). At the same time, 500 μL of Elution Buffer was added to the input samples to determine unconjugated input DNA. A volume of 1 μL of 10 mM MgCl2− DNA-free RNase A was added to each sample. After incubation at 37 °C for 1 h, 1 μL of 0.5 mM EDTA, 20 μL 1 M Tris-HCl (pH 6.5) and 2 μL of 10 mg mL−1 protease K were added to each sample. The samples were incubated at 45 °C for 1 h and extracted with the same volume of phenol:chloroform:isoamyl alcohol (25:24:1v/v) twice. DNA was then precipitated with glycogen and incubating at room temperature for 30 min. DNA was pelleted by centrifugation.

For ChIP-sequencing, DNA sequencing libraries were prepared from chromatin immunoprecipitated DNA using a DNA library preparation kit (E6000s, New England Biolabs) according to the manufacturer’s instructions and sequenced using an Illumina Genome Analyzer. Tissue from untreated SARD1-HA transgenic plants for further use.

For ChIP sequencing, DNA was quantified by real-time PCR using gene-specific primers. The primers used to amplify the promoter regions of the target genes are listed in Supplementary Table 1. Real-time PCR was performed in 96-well format using Bio-Rad CFX connect Real-Time PCR systems and the SYBR Premix Ex Taq II (TAKARA).

Promoter activity assay. The NOS101-Luciferase reporter vector was created by modifying 5′Green0229 to include a firefly luciferase gene driven by a basal promoter of the nopaline synthase gene (−101 to +4, designated NOS101). The wild type and mutant versions of the 56 bp promoter fragment of ICS1 were synthesized and inserted upstream of the NOS101 basal promoter in the reporter vector. Promoter activity assays were performed by expressing the reporter constructs with the 35S-NOS101 construct or empty vector in Arabidopsis protoplasts. A 35S-driven Renilla luciferase reporter was included in the assays as a normalization control. Promoter activity assays were performed by expressing the reporter constructs with the 35S-SARD1 construct or empty vector in Arabidopsis wild type and mutant versions of the 56 bp promoter fragment of NOS101-Luciferase. A volume of 5 μL (10 ng) of ICS1 promoter was added to the reporter vector. Promoter activity assays were performed by expressing the reporter constructs with the 35S-SARD1 construct or empty vector in Arabidopsis wild type and mutant versions of the 56 bp promoter fragment of NOS101-Luciferase. A volume of 5 μL (10 ng) of ICS1 promoter was added to the reporter vector.

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Author contributions
Conceived and designed the experiments: T.S., Y.X.Z. and Y.L.Z. Performed the experiments: H.S., S.D., C.T., K.H., Y.Y.X., X.Y.Z., S.S., Y.C., Y.Y.Y., Z.W., Y.L.Z. and J.S.P. Analyzed the data: T.S., Y.X.Z., Y.L.Z. and Z.W. Contributed reagents/materials/analysis tools: J.S.P., T.S., Y.X.Z., Y.L.Z. Wrote the paper: T.S. and Y.L.Z.

Additional information
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Competing financial interests: The authors declare no competing financial interests.
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