TECHNICAL ADVANCE

ATAC-Seq Reveals the Landscape of Open Chromatin and *cis*-Regulatory Elements in the *Phytophthora sojae* Genome

Zhichao Zhang,^{1,2,3} Long Lin,^{1,2,3} Han Chen,^{1,2,3} Wenwu Ye,^{1,2,3,†} Suomeng Dong,^{1,2,3} Xiaobo Zheng,^{1,2,3} and Yuanchao Wang^{1,2,3}

¹ Department of Plant Pathology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

² Key Laboratory of Plant Immunity, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China

³ Key Laboratory of Integrated Management of Crop Diseases and Pests (Ministry of Education), Nanjing, Jiangsu 210095, China

Accepted 12 January 2022.

Nucleosome-free open chromatin often harbors transcription factor (TF)-binding sites that are associated with active *cis*-regulatory elements. However, analysis of open chromatin regions has rarely been applied to oomycete or fungal plant pathogens. In this study, we performed the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) to identify open chromatin and *cis*-regulatory elements in *Phytophthora sojae* at the mycelial stage. We identified 10,389 peaks representing nucleosome-free regions (NFRs). The peaks were enriched in gene-promoter regions and associated with 40% of *P. sojae* genes; transcription levels were higher for genes with multiple peaks than genes with a single peak and were higher for genes with a single peak than genes without peak. Chromatin accessibility was positively correlated with gene transcription level. Through motif discovery based on NFR peaks in core promoter regions, 25 candidate *cis*-regulatory motifs with evidence of TF-binding footprints were identified. These motifs exhibited various preferences for location in the promoter region and associations with the transcription level of their target genes, which included some putative pathogenicity-related genes. As the first study revealing the landscape of open chromatin and the correlation between chromatin accessibility and gene transcription level in oomycetes, the results provide a technical reference and data resources for future studies on the regulatory mechanisms of gene transcription.

Keywords: ATAC-seq, *cis*-regulatory element, oomycete, open chromatin, promoter, transcription factor

Chromatin structure is an important factor for transcriptional regulation in eukaryotic organisms. Open chromatin in the

promoter, enhancer, and other gene regulatory regions allows for binding of transcription factors (TFs), which regulate gene transcription (Thurman et al. 2012). With recent advancements in high-throughput sequencing, a series of omics technologies have been developed for identifying open chromatin, e.g., formaldehyde-assisted isolation of regulatory elements (Giresi et al. 2007), micrococcal nuclease digestion with deep sequencing (Kent et al. 2011), methylated DNA immunoprecipitation sequencing (Kelly et al. 2012), DNase I-hypersensitive site sequencing (DNase-seq) (Thurman et al. 2012), and assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) (Buenrostro et al. 2013). Compared with other open chromatin sequencing technologies, ATAC-seq is faster and more efficient. It can be used to identify regions of open and nucleosome-bound chromatin and to infer the binding positions of DNA/TF-binding proteins through footprinting analysis. For these reasons, ATAC-seq has been widely used in recent years for large-scale identification of open chromatin in mammals and plants (Bao et al. 2015; Lu et al. 2017; Murano et al. 2018; Pálffy et al. 2020; Starks et al. 2019); however, this method has only been applied to a few fungi, including *Ustilagoideae virens*, *Spraxis latifolia*, and *Aspergillus* spp. (Chen et al. 2021; Huang et al. 2021; Yang et al. 2019), and there was no report in oomycetes.

Phytophthora sojae is a filamentous oomycete plant pathogen that causes soybean stem and root rot, causing serious yield reductions in soybean production worldwide (Kamoun et al. 2015; Tyler et al. 2006). Precise gene transcriptional regulation is essential to the development and infection processes of *P. sojae*. For example, transcription of genes encoding RxLR effectors, which target various functional processes of plant defense responses, is programmed globally (Wang et al. 2011). In the field population of *P. sojae*, differences in transcription of the avirulence genes *PsAvr1a* and *PsAvr3a* result in gain or loss of virulence (Qutob et al. 2009). *PsAvr3b* was engineered using mutated promoters, which led to differences in transcription levels and plant disease compatibility in *P. sojae* (Ochola et al. 2020). Silencing of *PsGPA1*, which encodes a G-protein alpha subunit in the cellular signaling pathway, does not impair hyphal growth or sporulation but severely affects zoospore behavior (Hua et al. 2008). Transcription of *PsSAK1*, a gene encoding a stress-activated mitogen-activated protein kinase, is upregulated in zoospores, cysts, and during early infection; silencing of *PsSAK1* severely hinders zoospore development and pathogenicity (Li et al. 2010). Several genes of the Myb TF family are

†Corresponding author: W. Ye; yeww@njau.edu.cn

Funding: This work was supported by grants from the National Natural Science Foundation of China (31772140 and 31721004).

*The e-Xtra logo stands for “electronic extra” and indicates there are supplementary figures and tables published online.

The author(s) declare no conflict of interest.

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differentially transcribed in response to the silencing of *PsSAKI*, while silencing of *PsMYB1* affects zoospore development (Zhang et al. 2012).

TFs play important roles in oomycete biological processes through regulation of gene transcription at various stages. In *P. infestans*, some Myb genes are upregulated at the sporangium and zoospore stages and, thus, may be related to spore development (Xiang and Judelson 2010); *PiMADS*, a MADS-box TF-encoding gene, is necessary for asexual sporulation (Leesutthiphonchai and Judelson 2018); overexpression of the CBF/NFY gene *PITG_10861* results in increased synthesis of α hormone (Wang et al. 2020); and the bZIP TF PiBZP1 is a key regulator in zoospore-mediated infection (Blanco and Judelson 2005). In both *P. sojae* and *P. litchii*, the bZIP TF BZP32 is involved in cyst germination, the oxidative stress response, and pathogenicity (Kong et al. 2020; Sheng et al. 2021). In *P. sojae*, the heat-shock TF PsHSF1 helps pathogens manage oxidative stress by reducing the accumulation of plant-produced reactive oxygen species (Sheng et al. 2015), while the MADS-box TF PsMAD1 is involved in zoosporogenesis and pathogenesis (Lin et al. 2018b). Recently, the “plant-specific” RWP-RK TF family was found in oomycetes, and some genes showed specific conserved transcription patterns during development and pathogenesis (Yin et al. 2020).

Given the major roles of TFs in transcriptional regulation, identification of TF-binding sites (TFBS) is crucial to elucidating their functional mechanisms. The first systematic TFBS discovery analysis in oomycetes utilized the genome sequences of *P. infestans* and two related *Phytophthora* species (*P. ramorum* and *P. sojae*) along with in planta gene transcription data and predicted 19 conserved DNA motifs (Seidl et al. 2012). Then, genome-wide prediction of promoter motifs in *P. infestans* revealed 103 motifs, and stage-specific transcription patterns were validated for some motifs, using the *GUS* reporter gene (Roy et al. 2013a). The absence of a TATA box and presence of novel core promoter elements in *P. infestans* and other oomycetes were also reported (Roy et al. 2013b). However, all of these candidate TFBS were initially identified based on bioinformatics-based prediction using artificially defined regions as putative promoter sequences. Because the promoter region is variable in length and some gene models may be incorrect, the accuracy of the promoter sequences used for motif discovery was not perfect.

In this study, we confirmed that ATAC-seq was feasible for identification of open chromatin and TFBS in the *P. sojae* genome. We identified 10,389 peaks representing nucleosome-free open chromatin regions. These peaks were enriched in gene promoter regions, and the chromatin accessibility of these peaks showed a positive correlation with the transcription level of their target genes. Motif discovery analysis revealed 25 TF-binding footprint-supported *cis*-regulatory elements in core promoter regions. Location preference and association with gene transcription level were also investigated for these motifs.

RESULTS

Identification of the nucleosome-free open chromatin regions.

To identify open chromatin and *cis*-regulatory elements in the *P. sojae* genome (Fig. 1A), we generated approximately 20 million sequence reads through ATAC-seq, using DNA from mycelia for library construction. After read processing, read mapping, and alignment processing, 4,728,346 high-quality reads (17.0% of clean reads) were obtained for subsequent analysis (Fig. 1B). The distribution of sequence fragment size showed obvious periodicity, with three major regions

indicating a nucleosome-free region (NFR) (<100 bp), a mononucleosome protected region (MNR) (150 to 250 bp), and a di-nucleosome protected region (>250 bp) (Fig. 2A). The distributions of the NFR and MNR fragments were enriched in the region upstream of the start codon (ATG), whereas the signal for MNR fragments showed two peaks surrounding the single peak of NFR fragments (Fig. 2B). Using the NFR fragments, we identified 10,389 NFR peaks (Fig. 1B; Supplementary Table S1). Almost 70% and over 60% of the peaks, respectively, were enriched within 1 and 0.5 kb upstream of the ATG (Fig. 2C); thus, we defined 0.5 kb upstream of the ATG as the core promoter region for further analysis. Promoter-flanking genomic regions contained fewer chromatin accessible peaks, suggesting that such regions might be bound and protected by nucleosomes (Fig. 2B and C). These results indicate that the ATAC-seq assay in *P. sojae* was successful.

Gene transcription level is correlated with chromatin accessibility.

Among 19,230 annotated *P. sojae* genes, 7,652 (40%) genes were associated with NFR peaks, including 5,724 genes associated with one peak, and 1,406 and 522 genes associated with two and more than two peaks, respectively (Fig. 2D). Through analysis of the relationship between the transcription level of target genes and the number of peaks, we found that genes associated with one or more peaks exhibited significantly higher transcription levels than genes without a peak (Fig. 3A), indicating that the chromatin-accessible regions were involved in transcriptional activation of their target genes. In addition, transcription levels were significantly higher for genes associated with two or more peaks than genes associated with a single peak (Fig. 3A).

According to the number of Tn5 transposase cut sites in peaks, chromatin accessibility could be evaluated. Among the genes associated with one or more peaks, the proportion of those with detectable transcription increased gradually as chromatin accessibility increased, with Pearson's correlation coefficient $R = 0.86$ ($P < 0.05$) (Supplementary Fig. S1). Through analysis of the relationship between chromatin accessibility of peaks and transcription level of target genes of peaks, we found a positive correlation, with $R = 0.33$ ($P < 0.01$) (Fig. 3B). These results suggest that chromatin accessibility in the promoter region is positively correlated with the transcription level of target genes in *P. sojae*.

Gene transcription is controlled by the interplay of regulatory events at promoters and distal regulatory elements called enhancers. We analyzed the genes that contain one peak inside (I-peak) and one peak outside (O-peak) the core promoter regions and found that the transcription levels of these genes were significantly higher than the genes that appear to contain a single I-peak or a single O-peak (Fig. 3C). The single I-peaks and single O-peaks both showed lower chromatin accessibility than the I-peaks that have an additional O-peak (Fig. 3C). These results imply that the O-peaks might contain enhancers, to increase chromatin accessibility of the corresponding I-peaks, as well as transcription level of the target genes.

Identification of the footprint-supported *cis*-regulatory elements.

To assess potential TFBSs or *cis*-regulatory elements, we performed motif discovery analysis using 6,248 NFR peaks identified in the core promoter region (Fig. 1B). Using the DREME tool in MEME Suite (Bailey et al. 2015), we identified 56 candidate motifs of 5 to 8 nt in length. Through the FIMO search program, we then reproduced the distributions of 48 motifs in the NFR regions and filtered out eight redundant motifs. The 48 motifs

were designated ATAC motif 1 to 48 (AM-1 to AM-48); 65% of these motifs ($n = 31$) were similar to motifs previously predicted in *P. infestans*, based on genome-wide promoter sequence analysis (Roy et al. 2013a; Seidl et al. 2012) (Table 1).

ATAC-seq can be used for analysis of TF-binding footprints, as TFs bind to DNA to protect it from Tn5 transposase cleavage (Fig. 1A). Among the 48 motifs analyzed, 25 motifs exhibited strong footprint characteristics (Fig. 4A and B; Supplementary

Fig. S2), which were considered as candidate *cis*-regulatory elements. Most of these motifs (22 motifs, 88%) were similar to motifs previously reported in *P. infestans* (Table 1).

Motif location preference and association with gene transcription.

Most of the 25 footprint-supported motifs in the core promoter were located within 150 bp upstream of the start codon, with a

Fig. 1. Biological principle and data analysis flow chart of ATAC-seq in this study. **A**, Cleavage sites of Tn5 transposase in open chromatin regions, the sequence reads obtained in this study, and a nucleosome-free peak with a transcription factor (TF)-binding footprint. **B**, Data analysis flow chart and corresponding results are shown at each step.

frequency of 58%. Different motifs showed differing location preferences, with four, 11, and 10 motifs concentrated in the ranges of -150 to -100 bp, -100 to -50 bp, and -50 to -1 bp, respectively (Fig. 5A). The NFR peaks containing different motifs exhibited differences in chromatin accessibility, and genes associated with different motifs had differing transcription levels. A strong positive correlation relationship was observed between the chromatin accessibility of NFR peaks located in motifs and transcription levels of motif-associated genes ($R = 0.76$, $P < 0.01$) (Fig. 5B and

C). We also observed that the NFR peaks and genes containing motifs near the start codon had higher chromatin accessibility and transcription levels, respectively (Fig. 5A, B, and C).

A total of 3,236 *P. sojae* genes contained one or more footprint-supported motifs in the core promoter region, and 711 genes were predicted to function as pathogenicity-related genes, including genes encoding secreted proteins (SPs), effectors (EFs), carbohydrate-active enzymes, oxidoreductases, transporters, protein kinases (PKs), and TFs (Supplementary Table S2). Probably

Fig. 2. Characteristics of sequence fragments and peaks obtained from ATAC-seq analysis. **A**, Distribution of ATAC-seq fragment sizes. NFR = nucleosome-free region, MNR = mononucleosome protected region. **B**, Distribution of nucleosome-free fragments and mononucleosome fragments surrounding the gene start codon. **C**, Distribution of nucleosome-free peaks surrounding the gene start codon. **D**, Statistics of genes associated with different numbers of peaks.

Fig. 3. Correlations of peak number and chromatin accessibility with gene transcription level. **A**, Comparison of gene transcription levels according to peak number. **B**, Correlation analysis between gene transcription level and chromatin accessibility. The Pearson correlation coefficient (R) is shown, with the asterisk indicating $P < 0.01$. RNA-seq data from *Phytophthora sojae* at the mycelial stage were used for analysis. **C**, Comparison of gene transcription levels and chromatin accessibility among three categories of genes: 1+0, 1+1, and 0+1, based on the number of peaks inside the core promoter (first digit) and outside the core promoter (final digit).

because ATAC-seq was performed at the *P. sojae* mycelial stage, motif-associated genes showed relatively high average transcription levels at this stage (Fig. 5C). Comparing the proportion of genes containing one or more motifs among pathogenicity-related gene families, we found that the PK gene family accounted for the largest proportion, while the SP and EF families made up smaller proportions (Fig. 5D); it is well-known that many genes in the SP and EF families are specifically induced during infection.

DISCUSSION

ATAC-seq has been widely used to identify open chromatin in animals and plants (Davie et al. 2015; Lu et al. 2017; Maher

et al. 2018; Starks et al. 2019), but probably due to the difficulty of application in microorganisms, few studies have used this method in filamentous plant pathogens. Contamination of DNA from bacteria and organelles (e.g., mitochondria) is a common problem, which can result in a low mapping rate of reads. To identify the source of contaminants for our data, we performed k-mer analysis using Kraken2 (Wood and Salzberg 2014) and found that 16% of the clean reads were mapped to bacteria and 83% of the clean reads had no hits. We actually performed two ATAC-seq assays with and without adding antibiotics for culturing *P. sojae* mycelia, respectively, and found that adding antibiotics is useful to improve the read mapping rate. Further efforts are still needed to identify the causes of contamination and reduce the contamination.

Table 1. Motifs predicted from the nucleosome-free region peaks at core promoter region

Motif ID ^a	Sequence pattern	Motifs in publication 1 ^b	Motifs in publication 2 ^c
AM-1 (FP)	[GA]CCAATC[GA]	M40: CAATCAG	M4: CCAAT
AM-2 (FP)	[GA]CTTCAA[CG]	M57: CTTT[AG]AC; M58: CTTCAAC	M2: ACTTCAAC; M15: [ATG][CA]TTCAACCA
AM-3 (FP)	ATGG[CAG]TAC	M36: ATGGCTAC	—
AM-4	ATACAAA	—	M3: ATACAAANNCAAGTC
AM-5 (FP)	TAC[TC]G[TA]A	M4: [ACG]TAC[ACT]GTA; M87: T[AG]C[AT]GTAC	—
AM-6 (FP)	GTG[GCT]ACCA	M83: GTGC[AT]GCA	—
AM-7 (FP)	CG[TC]ACTTA	M53: CTCAC TTC	—
AM-8 (FP)	T>GTGT[GA]	—	M18: T[ACG]GTGTGG[TC]
AM-9 (FP)	GCTGCTG[CG]	M75: GCTGC[AT]G	—
AM-10 (FP)	[GA]CCAATG[GA]	M40: CAATCAG	—
AM-11	CGCCGC[CG]	M47: CGCCACC	—
AM-12 (FP)	A[ATG]AGTGA	—	—
AM-13 (FP)	C[TC][TC]CCTC	M54: CTCCTTC; M60: CTTCT[TC]C	—
AM-14	CCAATC[CA]	M40: CAATCAG	—
AM-15	ACTTG[GA][TC]	M25: ACT[CT]G[AGC]AG	—
AM-16 (FP)	[CG]AAG[TA]GA	M3: [ACG]AAGT[ACG]G	—
AM-17	CCTA[CG]CCC	—	—
AM-18	GCCAG[TC]C	—	—
AM-19 (FP)	TACCGGT[TA]	M88: T[AG]CCGG[TC]A; M94: TACCGGTA	M9: GTACCGGTA
AM-20 (FP)	TACATGTA	M93: TACATGTA	M1: TACATGTA[CT]
AM-21 (FP)	CGCCAT[CG]G	—	—
AM-22	GACG[ACT]CGA	M24: ACGCCGG	—
AM-23	CTTCC[ACT]GC	—	—
AM-24 (FP)	C[ATG]CCACCA	M5: [AT]CCACCA	—
AM-25	GAGACCG[TA]	—	—
AM-26	AAAAAG[TA]	M14: AAAAGAAG	—
AM-27	AAAACCT	—	—
AM-28 (FP)	CGCCAC[GA][TC]	M47: CGCCACC	—
AM-29	ATCTTG[GA]	—	—
AM-30	CCATCAC[TC]	M41: CATC[ACG]A[CT]G	—
AM-31 (FP)	GAGCGAG[CG]	—	—
AM-32	TACTACTA	M80: GTACTAC	—
AM-33	ATAGAG[GA]G	M30: AGAGAGAG	—
AM-34 (FP)	CCAAGCAG	M19: AAGCA[AG]A	—
AM-35	GACG[ATG]GGC	M18: AACGGGGT	—
AM-36 (FP)	CCGAG[TC]C	—	—
AM-37	CC[ATG]TTTC	—	—
AM-38	CGTC[CA]GTC	—	—
AM-39	ACCGACC[CAG]	—	—
AM-40 (FP)	GCAGCG[CA]C	M70: GCA[AG]TGC	—
AM-41	TCTGAT[TA]	—	—
AM-42 (FP)	TGGCT[ATG]CA	M98: TG[CG]CTG[CG]C	—
AM-43	AAGATCGC	—	—
AM-44 (FP)	GC[TC]GGCC	—	—
AM-45 (FP)	CG[GCT]CGCAG	M48: CGCCGAAG	—
AM-46	TATT[TA]ATA	M89: T[AT]TTAATA	M7: TA[CT]TATTAATA
AM-47 (FP)	TGGAC[CAG]AA	—	—
AM-48	ATATTT[GA]G	M37: ATT[CT]TTA	—

^a FP indicates the footprint-supported motifs.

^b Roy et al. (2013a) has been designated publication 1, 103 predicted motifs.

^c Seidl et al. (2012) has been designated publication 2, 22 predicted motifs.

According to the periodic distribution of fragment sizes, the major parts of sequence fragments obtained from ATAC-seq could be readily divided into NFR and MNR fragments. MNR is a mononucleosome-binding region and less accessible to TF binding, while NFR is a nucleosome-free region and a TF would probably bind; thus we focused on NFR for TF-binding motif discovery. We found that the NFRs were most enriched near the transcription initiation site, which is similar to the previous reports (Buenrostro et al. 2013; Lu et al. 2017). Although the RNA-seq data were not generated alongside the ATAC data for *P. sojae* at the mycelial stage, there was a positive correlation between chromatin accessibility and the transcription level of target genes of peaks, consistent with those reported in mammals (Ampuja et al. 2017; Pastor et al. 2014; Starks et al. 2019). The above evidence suggested that ATAC-seq can be applied to *P. sojae* and may also be applicable to more oomycete and fungal pathogens.

The main benefit of ATAC-seq is the ability to identify TF footprints similar to DNase-seq, as the cleavage preference of Tn5 transposase is low (Li et al. 2019; Yan et al. 2020). In this study, we used HINT-ATAC software to correct for Tn5 cleavage preference (Li et al. 2019). Through footprinting analysis of 48 motifs enriched by DREME, we observed that 25 motifs exhibited apparent footprint characteristics. The 25 footprint-supported motifs identified here may be helpful for accurately

identifying regulatory regions and factors in *P. sojae* (Neph et al. 2012; Sung et al. 2016). Low sequencing depth or low native chromatin accessibility affect the detection of footprints, which may be one reason for the footprints of other motifs not being observed (Quach and Furey 2017). In addition, because TF binding is an instantaneous and transient status, some motif sites are difficult to be captured in Tn5 cleavage experiments (Gusmao et al. 2016; Sung et al. 2014). We also found that the distal peaks additional to the core promoter peaks may contain enhancers. Additional evidence of enhancers may be obtained through combined analysis with other methods, such as epigenetic assays for H3K4me1 and H3K27ac and highest-throughput chromosome conformation capture (Hi-C) assay (Lin et al. 2018a; Rada-Iglesias et al. 2011), and further experimental verification.

Using the Tomtom tool in MEME Suite (Bailey et al. 2015), we found that several motifs had matches in the database of known motifs (Supplementary Table S3). For example, AM-1 had a match to nuclear transcription factor Y subunit alpha (NFYA). NFYA is a key subunit of the nuclear TF trimer complex, which specifically binds to DNA sequences and functions as a regulatory subunit. NFY forms a highly conserved TF that binds to CCAAT motifs in the promoter regions of many genes. In *Fusarium graminearum*, CCAAT-binding TFs play important roles in development, DNA damage response, virulence, and toxin production (Kim et al. 2020). Recent research has

Fig. 4. Transcription factor (TF)-binding footprint analysis. **A**, Examples of motifs with (red) and without (gray) apparent footprint characteristics based on Tn5 integration sites. Typical TF-binding footprint profile exhibits specific signal fluctuations (usually weakening) at the motif center. **B**, Distribution of Tn5 transposase integration number surrounding the centers of 48 motifs. Motifs with apparent footprint profiles, designated highly reliable motifs, are highlighted with red labels.

demonstrated that overexpression of a TF related to CBF/NFY in *P. infestans* increases the synthesis of α hormone (Wang et al. 2020); thus, NFY may also play an important regulatory role in *P. sojae*. AM-2 has an apparent footprint but unknown annotation in databases. However, AM-2 was previously predicted in *P. infestans* (Table 1) and revealed as footprint-supported motifs in *P. sojae* in this study, suggesting that this motif may be conserved to *Phytophthora* species.

We have observed TF-binding footprints at the promoter region of some pathogenicity-related genes. Further ATAC-seq analysis would be useful to reveal the dynamics of open chromatin regions and chromatin accessibility of these genes and to identify the *cis*-regulatory elements associated with their specific transcription at the infection stage. We actually also conducted ATAC-seq assays for *P. sojae* at its infection stage; however, likely due to contamination of DNA from plant tissues and environmental microorganisms such as bacteria, the read-mapping rate was low and the background noise was intense. Therefore, the ATAC-seq data from this stage was not used for further analysis.

In conclusion, our results show that ATAC-seq can be effectively applied to *P. sojae* at the mycelial stage and can be used to identify *cis*-regulatory elements that are footprint-supported, providing candidates for experimental validation of protein-DNA interactions.

MATERIALS AND METHODS

Sample preparation, library construction, and sequencing.

To avoid bacterial contamination, ampicillin (50 μ g/ml) and rifampicin (50 μ g/ml) were added to *P. sojae* (strain P6497)

culture medium. After culturing *P. sojae* on a V8 plate at 25°C in the dark for 3 days, five 3 × 3 mm pieces of mycelial block were incubated, in a culture dish containing V8 liquid medium, in the dark for 3 days, and then, the mycelia were collected. ATAC-seq was performed as previously described (Wu et al. 2016). Mycelia (0.1 g) was ground with liquid nitrogen, was transferred to 1.5-ml precooled NPB (20 mM HEPES, 40 mM NaCl, 90 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.2 mM spermine, and 1× Roche complete protease inhibitors), and was lysed for 15 min, and then, filtering the mixture through a double-layer of Miracloth, collecting the liquid in a new centrifuge tube, and centrifuging at 6,000 × g for 10 min at 4°C. The supernatant was removed, the pellet was resuspended with 1 ml of precooled NEB2 (0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1% Triton X-100, and 1× Roche complete protease inhibitors), and the supernatant was transferred to a 1.5-ml centrifuge tube and was centrifuged at 12,000 × g for 10 min at 4°C. The supernatant was removed and the pellet was resuspended with 300 μ l of NEB3 (95% (vol/vol) percoll, 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 5 mM β -ME, and 1× Roche complete protease inhibitors). Precooled NEB3 (300 μ l) was added to a new 1.5-ml centrifuge tube, the supernatant from the previous step was then carefully added to the centrifuge tube, centrifuging at 2,200 × g, 4°C for 10 min. The supernatant was removed and the pellet was resuspended with 1 ml of precooled NPB. Isolated nuclei were stained with 0.04% trypan blue and were counted using a microscope. Supernatant with 50,000 nuclei from the previous step was added to a new 1.5-ml centrifuge tube, was centrifuged at 500 × g, 4°C for 10 min, and the

Fig. 5. Characteristics of the 25 footprint-supported motifs. **A**, Location distribution of motifs in the region 0.5 kb upstream of the gene start codon. **B**, The number of nucleosome-free region (NFR) peaks containing the indicated motifs is shown to the left of column plots showing the average chromatin accessibility of the NFR peaks. **C**, Average transcription levels of genes associated with the indicated motifs. RNA-seq data from *Phytophthora sojae* at four stages, including mycelia (MY), zoospores (ZO), germinated cysts (GC), and 3 h postinoculation on susceptible soybean leaves (IF3h) are shown. **D**, Heat map with labels indicating the numbers of motif-associated genes in the indicated gene families (SP = secreted protein, EF = effector, CA = carbohydrate-active enzyme, OX = oxidoreductase-related protein, TP = transporter, PK = protein kinase, TF = transcription factor). Upper column plots summarize the proportions of members associated with highly reliable motifs in each gene family.

supernatant was removed. The pellet was mixed with transposition reaction mixture and was incubated for 30 min at 37°C. The transposition reaction mixture contained 25 µl of target DNA (2× reaction buffer), 2.5 µl of TDE1 (Nextera Tn5 transposase), and 22.5 µl of nuclease-free H₂O. After the transposition reaction, the Qiagen MinElute PCR purification kit was used immediately to purify DNA. Sequencing library construction was conducted by Igenebook Biotechnology Co., Ltd. (Wuhan, China) using the HiSeq X Ten platform and 150-bp paired-end sequencing.

Data processing.

Raw reads were filtered with Trim Galore software (version 0.6.6) to remove low-quality reads and adaptor sequences. Clean reads were mapped to the reference genome of *P. sojae* (v1.1) using Bowtie2 software (version 2.2.9) (Langmead and Salzberg 2012). Contaminants were evaluated by Kraken2 (version 2.1.2) (Wood and Salzberg 2014). PCR duplicated reads were removed using GATK software (version 4.1) (McKenna et al. 2010). Using SAMtools software (version 1.3, -f 2 -q 30), only properly paired alignments with alignment quality ≥30 were retained. Then, the reads were shifted +4 bp and -5 bp for the positive and negative strand, respectively, using deepTools software (version 3.3, alignment -ATAC shift) (Ramírez et al. 2014). Reads were separated into NFR (<100 bp) and MNR (150 to 250 bp), based on fragment size, using a custom shell script. The NFR fragments were used to identify open peaks using MACS2 software (version 2.1.4, -f BAMPE -keep-dup all -cutoff-analysis) (Zhang et al. 2008). A fragment size distribution plot was generated with the GATK software. The NFR and MNR fragment distribution around the start codon ATG was plotted with the R package soGGi (version 3.13). ChIPseeker software (version 3.11) was used to assign the center of each peak to the nearest or overlapping gene (Yu et al. 2015).

Correlation between ATAC-seq and RNA-seq results.

The correlation between chromatin accessibility and the transcription level of target genes was visualized as a density contour plot using the R package ggplot2 and the function stat_density2d(). Because Tn5 transposase cuts two DNA strands with 9-bp overhangs and the adjusted position represents the center point between these cuts, this position is recorded as the cut site representing the chromatin accessibility event. Chromatin accessibility was evaluated based on the number of Tn5 transposase cut sites in peaks, which were calculated by pyDNase software (version 0.2.4, dnase_cut_counter.py -A) and logarithm (base 2) normalized (Piper et al. 2015). RNA-seq data for *P. sojae* at four stages, including mycelia, zoospores, germinated cysts, and 3 h postinoculation on susceptible soybean leaves, were reported previously (BioProject accession number in GenBank: PRJNA426510) (Wang et al. 2018). RNA-seq results were calculated as fragments per kilobase per million mapped fragments. The Pearson correlation coefficient was calculated using R packages with the cor.test() function.

Motif discovery and footprint analysis.

Motif enrichment analysis was performed using the MEME Suite software (version 5.3; including DREME, FIMO, and Tomtom) (Bailey et al. 2015). Peaks in the core promoter region (500 to 1 bp upstream of ATG) were used as the input file of DREME for motif discovery with minimum and maximum widths of 5 and 8, respectively. FIMO was used to determine the positions of motifs and matches were filtered using default parameters (*P* value < 1E-4). FIMO automatically filtered several low-credibility motifs and 48 motifs were retained. Tomtom was used to compare the motifs against a database of known motifs with default parameters.

HINT-ATAC corrected for strand-specific Tn5 cleavage bias and was used to predict all footprint sites for NFR peaks according to a typical footprint feature (peak-dip-peak). The footprint profiles were generated for each of the 48 motifs using HINT-ATAC, with the configuration file customized by modifying the RGT data folder.

Data and code availability.

The ATAC-seq data have been deposited in GenBank (BioProject accession number: PRJNA761250). ATAC-seq workflow and custom scripts are available in the GitHub repository.

AUTHOR-RECOMMENDED INTERNET RESOURCE

GitHub repository: https://github.com/biogeeker/atac_analysis

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