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Genetic and Epigenetic Regulations of EDS1-Mediated Plant Immunity and Identification of Bacterial Type III Effectors Targeting *De Novo* DNA Methyltransferase DRM2 in *Arabidopsis*

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GENETIC AND EPIGENETIC REGULATIONS OF EDS1-MEDIATED PLANT IMMUNITYAND IDENTIFICATION OF BACTERIAL TYPE III EFFECTORS TARGETING *DE NOVO* DNA METHYLTRANSFERASE DRM2 IN *ARABIDOPSIS*

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ABSTRACT

In *Arabidopsis thaliana*, Enhanced Disease Susceptibility1 (EDS1) acts as an indispensable hub in plant immunity and is mainly responsible for plant basal defense, systemic acquired resistance (SAR), increase of endogenous content of salicylic acid (SA) and effector-triggered immunity (ETI). Epigenetic regulators play crucial roles in multiple important cellular processes. As one catalytic core of Polycomb Repressive Complex (PRC2), CURLY LEAF (CLF/SDG1) protein promotes H3K27me3 deposition at specific loci for genes repression. In this work, I found that EDS1 interacted with epigenetic regulators SDG1, SDG9, SDG15, SDG22 and SDG35 in yeast two-hybrid assays. The growth of the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst*.DC3000) was obviously higher in one *clf* mutant (*clf-28*), proposing that CLF has the property of a positive regulator in plant immunity. Confirmation of association of CLF with EDS1 in *vivo* by BiFC assays indicates the EDS1-CLF protein complex is only located in the nucleus. I also demonstrated that protein levels of EDS1 were severely decreased in *clf-28* mutant compared with Col-0 although the relative mRNA level of *EDS1* in *clf-28* resembled that in Col-0. Apart from epigenetic regulators, mediator member CDK8 was also shown to interact with EDS1 *in vitro* and *in planta.* Previous studies demonstrated that CDK8 positively regulates plant defense by upregulating the transcription of SA-associated plant defense genes. Here, I also clarified that CDK8 positively regulates EDS1 protein accumulation.

In plants, Domains Rearranged Methyltransferases 2 (DRM2) is essential for construction of *de novo* methylation and maintenance of non-CG DNA methylation. Previous research showed that the *drm1-2 drm2-2 cmt3-11* (*ddc*) mutant, which abolished the RNA-directed DNA methylation (RdDM) pathway, had the resistant phenotype after inoculation of the bacteria *Pst* DC3000 compared with Col-0. On other side of the shield, many gram-negative bacterial pathogens inject effectors into their host's cells, inducing effector-triggered susceptibility (ETS) or effector-triggered immunity (ETI) in the host immune system. Here, I tested genome-wide pairwise associations of type III effectors with DRM2 and found that HopO1-2, HopAS1, HopAJ2, and HopAG1 interacts with DRM2 in Y2H assays. I have also found that that transgenic *Arabidopsis* plants overexpressing HopO1-2 and HopAS1 altered abundance on DNA methylation at DRM2 target loci *AtSN1* and *MEA-ISR*. This work will lay the foundation for further exploration of the relationship between DNA methylation and plant immunity.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Type III effectors

Plants, animals, and humans suffer from diseases caused by bacterial pathogens. Many gram-negative bacterial pathogens including *Salmonella, Xanthomonas*, *Pseudomonas*, *Chlamydia*, *Escherichia*, *Shigella*, and *Yersinia* etc. are able to secrete effectors into host's cells to suppress host innate immunity using a type III secretion system (T3SS) (Troisfontaines & Cornelis, 2005). Interestingly, some toxic effectors mimic eukaryotic proteins with enzymatic activities in structure and function blocking their host normal metabolism (Coburn et al., 2007). Effector HopAB2 secreted by *Pseudomonas syringae* pv. *tomato (Pst)* DC3000 acts as 26S proteasome to target and degrade NON-EXPRESSER OF PR GENE 1 (NPR1), the most important component in the salicylic acid (SA) -induced immune pathway (Chen et al., 2017). Effector HopU1 that required for full virulence in *Arabidopsis thaliana* from DC3000, functions as a mono-ADP-ribosyl transferase, to restrain normal functions of RNA-binding proteins (Fu et al., 2007). Two kinases RPM1-INDUCED PROTEIN KINASE(RIPK) and Botrytis -induced kinase 1 (BIK1) were targeted and added uridine 5′-monophosphate by a uridylyl transferase effector AvrAC from *Xanthomonas campestris* pathovar *campestris*, subsequently blocking transmission of immune signals (Feng et al., 2012). Therefore, exploration of functions on Type III effectors involved in repressing host immunity should be a hotspot in existing and future research.

Plant immunity and Enhanced Disease Susceptibility1 (EDS1)

Facing challenges from various pathogens, plants have developed two layers of immune responses (Nürnberger et al., 2004). Firstly, conserved molecules in plant pathogens were distinguished by receptors on plant cell membrane independently to trigger restriction of pathogens' growth, which is called pathogen-associated molecular patterns (PAMPs) -triggered immunity (PTI) (Jones & Dangl, 2006). Accordingly, effectors produced by pathogens move into plant cells to target and repress those conserved components in PTI to render plants more susceptible to diseases again (Jones & Dangl, 2006). Plants also develop a unique layer of immune response, namely effector-triggered immunity (ETI), which is when internal receptors leucine-rich repeat (NB-LRR) resistance proteins (R proteins) are combined with specific effectors directly or indirectly in protoplast (Wu et al., 2014). ETI endows plants with a stronger immune response along with accumulation of localized programmed cell death (Coll et al., 2011). Trigger of PTI or ETI confers board antibacterial properties on plants ranging from local infected tissue to distal uninfected parts. This resistance is called systemic acquired resistance (SAR) (Fu & Dong, 2013).

Increase of endogenous content of salicylic acid (SA) serves as an essential index with antibacterial properties in the responses to PTI, ETI and SAR in plants (Fu & Dong, 2013). Many positive and negative regulators play important roles in SA synthesis, accumulation, and plant defense responses (Nürnberger et al., 2004). The lipase-like protein EDS1 functions as a central hub in SA-mediated plant immunity by promoting SA biosynthesis (Heidrich et al., 2011). In addition, the SA receptor NONEXPRESSER OF PR GENES1 (NPR1) can form immune complexes with TGA transcription factors to

promote the transcription of *PR* genes encoding anti-microbial proteins (Ding et al.,2018), while others SA receptors NONEXPRESSER OF PR GENES3 (NPR3) and NONEXPRESSER OF PR GENES4 (NPR4) act as adaptor proteins of CUL3□based E3 ligase to accelerate degradation of NPR1 and EDS1 via the 26S proteasome, thus making plants more susceptible to diseases (Fu et al., 2012; Chang et al., 2019). Transcriptional repressor Suppressor of NPR1 Inducible 1 (SNI1) downregulates *PR1* induction by inhibiting the activation of chromatin remodeling (Kim et al., 2012), and transmembrane nucleoporin Constitutive Expresser of PR (CPR5) suppresses plant immunity responses by inhibiting programmed cell death (Gu et al.,2016).

EDS1 protein is indispensable for plant basal defense, ETI, SA accumulation and SAR (Heidrich et al., 2011). There is no SAR in different *eds1* mutants in *Arabidopsis* and thus enhanced susceptibility to virulent pathogens *Hyaloperonospora arabidopsidis (Hpa)* and *DC3000* and to avirulent strain *DC3000* carrying the avirulent gene *AvrRps4*, which induces TIR-NB-LRR resistance protein RPS4-dependent ETI (Parker et al., 1996). Transcriptional levels of SA biosynthesis genes *isochorismate synthase 1 (ICS1)* and *enhanced disease susceptibility5 (EDS5)* and marker genes *PATHOGENESIS-RELATED (PR)* of SA-induced defense pathway was significantly decreased (Parker et al., 1996). EDS1 could form diverse complexes by association with a great deal of partners participated in plant immune responses (Bhattacharjee et al., 2011). EDS1 combines with PHYTOALEXIN DEFICIENT4 (PAD4) and SENESCENCE ASSOCIATED GENE101 (SAG101), which is prerequisite for ETI mediated by different R proteins (Wagner et al., 2013). Besides, EDS1 regulates reprogramming of immunity- related genes at transcriptional level (Bhandari et al.,2019.).

Despite many years of research on EDS1, it is still unknown how EDS1 activates plant immunity by transcriptional regulation.

DNA methylation and Domains Rearranged Methyltransferases 2 (DRM2)

Epigenetic information, which can control target genes expression spatially and temporally, regulates the development and stress responses of eukaryotic organisms (Corso-Díaz et al., 2018). It mainly includes DNA methylation, histone modifications, regulation by non-coding RNA types, and ATP-dependent chromatin remodeling (Corso-Díaz et al., 2018). The most prevalent DNA methylation happens when one methyl group of S-adenosine-L-methionine in donor cells is transferred to the fifth carbon atom of pyrimidines within target genes by the action of DNA methyl transferase. This methylation results in the formation of 5-methyl cytosine (m5C) (Sánchez-Romero et al., 2015). As one of several important conserved epigenetic modifications, DNA methylation adjusts gene transcription and chromosomal stability by silencing repetitive sequences / transposable elements (TE) (Weber & Schübeler, 2007). In addition, promoters and enhancers regions in expressed genes are also lightly and heavily methylated, respectively (Schultz et al., 2015). DNA methyltransferase 3 (DNMT3) and DNMT1 are in charge of establishment of the *de novo* methylation and maintenance of DNA methylation respectively in CG context, the most abundant pattern in animals (Bird, 2002). Overall patterns on DNA methylation change during development and are associated with environmental changes. Previous studies showed that during diverse developmental stages in animals, such as in preimplantation embryos, genome-wide loss of m5C has been observed (Sasaki & Matsui, 2008). Environmental stresses are

responsible for aberrant patterns in mammals, dropping a hint many diseases, such as neurological disorders and tumorigenesis (Jin & Liu, 2018).

In plants, DNA methylation often occurs in symmetric sequences of CG, CHG, and asymmetric sequence of CHH (H=A, C, or T), separately accounting for 24%, 6.7% and 1.7% respectively in model plant *Arabidopsis* at normal conditions (Vanyushin & Ashapkin,2011). The formation and maintenance of DNA methylation in plants are various with those in animals. Plant different DNA methyltransferases contribute to regulation on specific contexts shown in Table 1.1 (Wendte & Schmitz, 2018; Zhang et al., 2018). Among them, Domains Rearranged Methyltransferases 2 (DRM2), and its homolog DRM1 are necessary for *de novo* DNA methylation in all types though the RNA-directed DNA methylation (RdDM) pathway (Cao & Jacobsen, 2002).

Table 1.1. Enzymes refer to *de novo* DNA methylation and maintenance of DNA methylation in plants.

Background of different DNA methylations	De novo methylation	Maintenance of DNA methylation
CG	RNA-directed DNA methylation (RdDM) (DRM2/DRM1)	METHYLTRANSFERASE1 (MET1)
CHG	RNA-directed DNA methylation (RdDM)	CHROMOMETHYLASE2 and 3 (CMT2 and CMT3)
CHH	RNA-directed DNA Methylation (RdDM)	CMT2 or RdDM

All *de novo* methylation is established by RNA-directed DNA methylation (RdDM), which DRM1 and DRM2 catalyze this pathway. Besides, MET1 maintains DNA methylation under CG background; CMT2 and CMT3 maintain DNA methylation under CHG background. CMT2 or RdDM pathway maintain DNA methylation under CHH background. (Wendte & Schmitz, 2018; Zhang et al., 2018)

In *Arabidopsis,* model of DRM2's regulation on its target gene loci is attributed to RdDM pathway (Zhong, 2016). RdDM pathway begins with when RNA polymerase IV is recruited to target loci and produces single-stranded RNA (ssRNA) transcripts, which then are replicated into double- strand RNA (dsRNA) within the function of enzyme RNA-directed RNA polymerase 2 (Law & Jacobsen, 2010). The dsRNA is further processed into 24nt short interfering RNAs (siRNAs) and then one strand is loaded into an Argonaute4 (AGO4) (Zhong et al, 2014). Next this complex recruit DRM2 to add methyl groups to specific target loci (Henderson et al., 2010). A methyltransferase (MTase) domain and ubiquitin-associated (UBA) domains are significant for DRM2 driving its biological functions (Raasi et al., 2005). UBA domains are crucial for binding to specific loci and MTase domain could be dimerized, which is important for preservation of DRM2 enzymatic activity (Zhong et al., 2014).

Unlike DRM1 and CMT3 which are only highly expressed in floral organs, DRM2 plays a vital role throughout the life cycle of plants and is highly expressed in different organs, including root, cotyledons, young leaf, expanded leaf, and flowers (Forgione et al., 2019). DRM2 is modified at both the post-transcriptional and post-translational levels (Denis et al., 2011). The transcriptional level of *DRM2* was shown to be downregulated by the microRNA miR-773a, reducing the whole DNA methylation level in plant genome (Jha& Shankar, 2011). Recently, COP9 SIGNALOSOME INTERACTING F-BOX KELCH1 (CFK1) was identified as a physical interactor of DRM2 and facilitated DRM2 ubiquitination and degradation, thus altering DNA methylation abundance (Chen et al, 2020). Another DRM2-interactor was U2AF56 Associated Protein 56 (UAP56a/b), which

also changes DNA methylation patterns and affects plant development and formation of reproductive tissues (Azevedo et al., 2019).

Histone Methylations and CURLY LEAF(CLF) Protein

Another important aspect of epigenetic regulation is histone modifications in eukaryotic cell nucleus, some of which activate gene transcription (e.g., acetylation, ubiquitination, phosphorylation, etc.), while others inhibit gene transcription (e.g., deacetylation, biotin, SUMO, etc.) (Strahl & Allis, 2000). Histone methylation often participated in silencing transposable element (TE) to keep genome stable (Strahl & Allis, 2000).

Histone methylation is widely studied and its methylated sites are very variable. The establishment of histone methylation marks is produced by histone lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs) (Liu et al., 2010). HKMTs and PRMTs function as 'writers' to introduce methylation information at lysine or arginine sites in histones (Johnson et al., 2004). Then, 'readers', mainly consisted of protein, bind particularly to those methylation marks resulting in specific biological outcomes or phenotypes (Zhang et al., 2007). Owing to dynamic histone methylation, 'erasers', namely the histone demethylases (HDMs), are mainly in charge of deletion of the marks (Jenuwein & Allis, 2001). HDMs include the FAD-dependent lysine specific demethylase (KDM1/LSD1) family and Jumonji C (JmjC) domain demethylase (Liu et al., 2010). The regulation of histone methylation by the 'writers', 'readers' and 'erasers' are most well characterized in eukaryotes. In *Arabidopsis*, histone methylation usually happens to H3 and H4 core histones in

nucleosome (Pontvianne et al., 2010). The main methylation sites on the H3 at lysine and arginine residues were Lys4 (K4), Lys9 (K9), Lys27 (K27), Lys36 (K36) and Arg17 (R17), and methylation of H4 is mainly on Lys20 (K20) and Arg3 (R3) (Pontvianne et al., 2010). Most histone methylations are in the mono (me)-, di(me2) -, or tri(me3)-methylated forms in *Arabidopsis*. Existing studies have shown that H3K4 methylation and H3K36 methylation contribute to upregulating gene transcription, but H3K9 methylation and H3K27 methylation refer to the occurrence of gene repression (Qiao & Fan, 2011). The H3K27me3 mark accounts for about 17% of protein-coding genes methylated in *Arabidopsis,* which is seem to be the major gene silencing mode (Naumann et al., 2005; Zhang et al., 2007).

By establishing a state of compacted chromatin, Polycomb Group (PcG) proteins act as transcriptional repressors (Francis et al., 2004). There are two important PcG members: Polycomb Repressive Complex 1 (PRC1) and PRC2, which catalyze the histone H2A lysine monoubiquitination and H3K27me3 modification respectively (Förderer et al., 2016). The enzymatic site of PRC2 is encoded by three genes, which are referred as *CURLY LEAF* (*CLF/SDG1*), *SWINGER*(*SWN*), *MEDEA*(*MEA*) (Mozgova & Hennig, 2015). Mutations in *CLF* result in obvious curled leaves, early flowering and partial homeotic transformation of the sepals and petals to carpels and stamens, correspondingly (Goodrich et al., 1997; Schubert et al.,2006). Those abnormal phenotypes could result from increased expression of *AGAMOUS(AG), AGAMOUS-LIKE19(AGL19), FLOWERING LOCUST(FT)* and *FLOWERING LOCUS C (FLC)* and corresponding decreased H3K27me3 marks in *clf* mutants (Jiang et al., 2008). Some proteins could interact and modify CLF to alter H3K27me3 patterns in *Arabidopsis*. The

temporal combination between CLF and mediator Nuclear Factor-Y subunit C drastically reduces the H3K27me3 pattern within the promoter of *FT* to delay flowering time (Liu et al., 2018). Telomere-repeat-binding factors (TRBs) recruit CLF binding to telobox-related motifs to enhance H3K27me3 deposition (Zhou et al., 2018). An F-box E3 ligase UPWARD CURLY LEAF1 (UCL1) targets and degrades CLF protein to decrease CLF-mediated H3K27me3 levels (Jeong et al.,2011).

CYCLIN-DEPENDENT KINASE 8 (CDK8)

Mediator is a conserved large protein complex which can regulate transcription by building a molecular bridge between RNA polymerase II (pol II) and transcription factor (TF) bound at an enhancer element (Bourbon, 2008). The mediator complex is composed of kinase Cyclin-Dependent Kinase 8 (CDK8) module, Tail module, Middle module and Head module (Chadick and Asturias, 2005). Among them, the core mediator complex consists of the last three members (Chadick and Asturias, 2005).

CDK8 boasts dual roles in adjusting gene transcription. On one hand, CDK8 module prevents transcription by hindering the combination between RNA pol II and core mediator (Elmlund et al., 2006). Besides, CDK8 functions as an activator to promote genes expression (Tsai et al.,2013). In animals, CDK8 promotes the growth of different types of cancers by regulating development-associated genes (Liang et al., 2018). In plants, apart from controlling plant development, CDK8 also positively regulates the occurrence of plant defense (Chen et al., 2019). Increase of endogenous salicylic acid (SA) levels and the transcription of SA biosynthesis gene *ICS1* rely on CDK8 (Huang et

al., 2019). Moreover, CDK8 recruits SA-receptor NPR1 to its own promoter and to the *PR1* gene promoter to upregulate those genes' expression (Chen et al., 2019).

The relationship between epigenetics and plant immunity

Epigenetic regulators were also reported to control plant immunity (Ramirez-Prado et al., 2018). One eukaryotic anti-viral mechanism is to promote the methylation of virus DNA that is integrated into the host genome (Brough et al., 1992). Recent results revealed virus-encoded V2 protein in *Tomato yellow leaf curl virus* could suppress methylation of the viral DNA in plants by targeting AGO4, which can cause the tomatoes to become more susceptible to diseases (Wang et al., 2020). In addition, DNA methylation levels in promoters of disease-related genes was often decreased after pathogen infection (Tirnaz & Batley, 2019). E.g., the rice mutant (*line-2*) had hypomethylated regions at the promoter of *Xa21G*, one *Xanthonmonas oryzae pv.oryzae* resistance gene. When faced with pathogens, a resistance phenotype and higher *Xa21G* expression levels had been shown compared with the wild type (Peng & Zhang, 2009). The *ddc* (*drm1-2, drm2-2, cmt3-11*) mutant, which abolished the RdDM pathway in *Arabidopsis*, showed more resistance to the bacteria *P. syringae* (Dowen et al., 2012). But it still remains to explore that how type III effectors in bacteria affect plant DNA methylation.

Immunity-related regulation of histone modifications is more complicated than DNA methylation. The interaction between HDA9 and WRKY53 decreases the expression of *Nod-Like Receptor (NLR)* genes and negatively contributes to plant immunity (Chen et al., 2016). The *hda19* mutants promote endogenous SA increase and

enhance resistance after the *Pst DC3000* inoculation (Choi et al., 2012; Latrasse et al., 2017). The protein complex HAC1–NPR1–TGA2/5 induced by SA promotes histone acetylation of the *PR1* promoter and expression of *PR1* (Jin et al., 2018). *CAROTENOID ISOMERASE2(CCR2)* and *ECERIFERUM3(CER3)* were necessary for plant defense, and their H3K4 and H3K36 methylations were established by SDG8 and SDG25 cooperatively, indicating that those two epigenetic regulators positively contribute to plant immunity though HLM (Lee et al., 2016). Therefore, to identify new relationship between histone modifications and plant defense should be of great significance.

CHAPTER 2

MATERIALS AND METHODS

Plant Materials

All plants, including *Nicotiana benthamiana* and *Arabidopsis thaliana,* were grown on greenhouse of constant temperature (22°C) with the cycle of 12-h light/12-h dark. Mutants *clf*-28 (*SALK_139371*), *drm2-2* (*SALK_150863*) were got from Arabidopsis Biological Resource Center (ABRC). The single deletion mutant *eds1-2* (Aarts et al., 1998) and triple mutant *drm1drm2cmt3* (*ddc*) (Yu et al. 2013) were described before. Transgenic lines were constructed through the floral dipping method which was mediated by *Agrobacterium tumefaciens* (strain GV3101) transformation. The *attB*-attached gene-specific primers were designed shown in Table 2.1. Coding sequences (CDS) of *DRM2*, *CLF, EDS1, HopO1-2, HopAJ2, HopAS1* and *HopAG1* were firstly cloned into entry vector pDONR207 (Clontech) by BP reaction according to the protocol of BP Clonase II Enzyme Mix (Invitrogen). *CLF (SDG1)* next was linked into the N-terminal GFP-tag fusion destination vector pWGF2K by LR reaction according to the protocol of LR Clonase II Enzyme Mix (Invitrogen). The recombined plasmid pWGF2K *35S: GFP-CLF* was introduced into Col-0 and *eds1-2* mutant respectively. Similarly, full-length effectors' coding sequences were also introduced into C-terminal hemagglutinin (HA)-tag fusion Destination vector pLN462 and then were transformed into Col-0.

Yeast Two-Hybrid (Y2H) Screening /Assays

Recombined plasmids were transferred into the *Saccharomyces cerevisiae* strains AH109 and Y187; the coding sequences of epigenetic regulators (mainly including histone methyltransferase/ di-methyltransferase, histone acetyltransferase/di-acetyltransferase) were introduced into the vector pGADT7 and then recombined plasmids were transferred into the strain AH109 individually. The coding sequences of mediator CDK8 cloned into pGBKT7 was introduced to the strain Y187. The empty vectors and pGADT7-GUS/ pGBKT7-GUS vectors served as negative controls. All procedures related to operations of molecular biology of yeast were subject to Yeast Protocols Handbook (Clontech).

Similarly, to identify which type III effectors could target DRM2, *DRM2* cloned into pGBKT7 was introduced to the *S. cerevisiae* strain Y187. The coding sequences of type III effectors in bacteria *Pseudomonas syringae* were introduced into pGADT7 and then recombined plasmids were transferred into the strain AH109 respectively. After mating and screening positive strains at DDO, yeast zygotes suspensions were dropped on control DDO and quadruple dropout (QDO, -Leu/-Trp/-Ade/-His) media at a concentration of OD_{600} = 1, 0.1, and 0.01.

Protein Extraction and Western Blot

After harvesting *Arabidopsis* with specific treatments individually, fresh leaves were ground into power using a 2010 Geno/Grinder (SPEX) for 2 min at 1200 RPM. Total proteins were incubated with 200 ul protein extraction buffer (PEB) (Chang et al., 2019) on ice for 1 hour. Crude samples were subsequently separated by absorbing the

supernatant after centrifugation with 20,000 g for 15 min three times at 4°C. After determining all protein concentration by a spectrophotometer (Eppendorf) at 595 nm wavelength and denaturing protein samples at 95°C for 10 mins or 72°C for 20 mins. The same quality total protein (30–60 μg) with specific plants were isolated on precast 4%–12% ExpressPlus PAGE Gels (GenScript) at electrophoresis from 80V for 20 mins to 150V for 1 hour, and subsequently were transferred onto nitrocellulose membranes (GE Healthcare). Different primary antibodies, followed by corresponding secondary antibodies, are applied to detect specific proteins according to the tag of fusion proteins (Chang et al., 2019). Finally, exposed photographic films were reported to display the position of proteins by an SRX-101A Medical Film Processor (Konica). Ponceau S solution (Chang et al., 2019) was used to stain every total protein to verify equal protein loading.

Instant transformation Assay in *Nicotiana benthamiana*

Recombined plasmids *35S: HA-CLF, 35S:HA-CDK8, 35S:HA-EV, 35S:EDS1-GFP, 35S:EV-GFP, pSITE-nEYFP, pSITE-nEYFP-CLF, pSITE-nEYFP-PAD4, pSITE-cEYFP,* and *pSITE-cEYFP-EDS1* and p19 were transferred into the *A. tumefaciens* strain GV3101 individually at first. Positive strains were picked up from YEB agar culture with corresponding antibiotics at 28°C after 2-3 days. After colony PCR confirmation, right bacterial cell was transferred into YEB liquid culture with corresponding antibiotics and 10 ul 100mM acetosyringone at 28°C overnight. After gathering cells by high-speed centrifugation, the pellet was resuspended again by Agro-infiltration buffer (Chang et al., 2019) and washed twice. The back leaves of 2-week-old *Nicotiana* were infiltrated gently by bacterial mixture at OD_{600} = 0.5 for next assays.

Co-Immunoprecipitation (Co-IP)

A. tumefaciens strain GV3101 mixture carrying *35S:EDS1-GFP* or *35S: EV-GFP* and *35S: HA-CLF* or *35S: HA-CDK8* and P19 was infiltrated into back leaves of 2-week-old *Nicotiana*. After harvesting samples with specific treatments individually, fresh leaves were ground into power using a 2010 Geno/Grinder (SPEX) for 2 min at 1200 RPM in liquid nitrogen. After harvesting leaves, 2 g samples in liquid nitrogen were crushed into powder exploiting the combination of mortars and pestles. Total proteins were incubated with 2ml protein extraction buffer (PEB) (Chang et al., 2019) on ice for 1 hour. Crude samples were subsequently separated by absorbing the supernatant after centrifugation with 18,000 g for 18 mins three times at 4°C. Then the supernatant crude extracts (5-10mg) with specific treatment were mixed with 15 ul of GFP-Trap_MA (ChromoTek) beads with mild shaking at cold room for 8h and those left extracts were boiled with 5 × sample buffer as input, which were stored in -20°C. After washing samples coupled with beads four times, proteins were isolated from the conjugated beads by making full use of boiling with elution buffer (Chang et al., 2019) for 8 mins. The bound EDS1-GFP/GFP or possible interactors HA-CLF and HA-CDK8 proteins were determined by immunoblot with anti-GFP antibody or anti-HA antibody respectively.

Bimolecular Fluorescence Complementation Assays (BiFC Assays)

A. tumefaciens strain GV3101 mixture carrying *pSITE-nEYFP* or *pSITE-nEYFP-CLF* or *pSITE-nEYFP-PAD4* and *pSITE-cEYFP* or *pSITE-cEYFP-EDS1* and P19 was infiltrated into young back leaves of of 2-week-old *Nicotiana*. Then, the injected plants were covered with black box to avoid light in one day. Laser scanning confocal

microscopy (Carl Zeiss LSM 700) was applied to detect fluorescence EYFP signals in different samples after 3 days.

Protein Purification

GST proteins and recombinant proteins GST-CLF and HisMBP-EDS1 were heterologous expressed in *E. coli* OverExpress^{TM} C41 (DE3) strain (Lucigen). Right strains were cultured in liquid LB at 37 °C until OD_{600} reaches 0.5~0.8 and recombined proteins were induced with 0.5 mM IPTG at 16°C for 12h. Sediments were gathered after centrifuging for 10 mins at 6,000 g at 4 °C and resuspended in the extracting solution (100mM Tris–HCl [pH 7.5], 100 mM NaCl, 1mM EDTA [pH 8.0], 0.5% NP40, 1mM DTT, 1 mM PMSF, 1× protease inhibitor cocktail, 50 μM MG115, 1 ml/ml Benzonase Nuclease, 1 ml/ml DNase, 10mg/ml lysozyme) on ice for 30 mins. The samples were rapidly crushed by ultrasonic homogenizers (Takara) with the system of "50% power, 10s on and 10s off" until supernatant was clear. The crude extracts were incubated with glutathione agarose (Sigma-Aldrich) or Ni-NTA agarose (QIAGEN). GST proteins and recombinant proteins GST-CLF and HisMBP-EDS1 were eluted by adding 50 mM glutathione and 0.5 M imidazole after washing bound proteins three times according to protocols, respectively.

GST Pull-Down Assays

10ul GST beads were incubated appropriately with the purified 5 ug GST proteins and GST-CLF proteins at 4 °C for 1 hour respectively. GST/GST-CLF protein immobilized beads was combined with 20 ug HisMBP-EDS1 fusion protein in 1ml binding buffer (Chen et al., 2019). TSNS300 washing buffer (Chen et al., 2017) was used

to wash beads for 3 times. After washing samples coupled with beads four times, proteins were isolated from the conjugated beads by making full use of boiling with elution buffer (Chang et al., 2019) for 8 mins. The subsequent electrophoresis and western blot were described above.

Quantitative Real-Time PCR

Plant total RNA with different kinds of treatment samples was extracted by utilizing the reagent RNAzol RT (Sigma-Aldrich). RNase-free DNase I (Thermo Fisher Scientific) was applied to reduce contaminated DNA at room temperature during the process of RNA extraction. 2 μg of total RNA was picked up to synthetize first-strand cDNA by using qScript cDNA SuperMix (Quanta). Ultrapure water was further applied to dilute every cDNA 10-fold. Next, 2ul cDNA template and 2X SYBR Green PCR Master Mix (Applied Biosystems) was brought into PCR reaction mixtures where the final volume is equal to 20 μl of each system. PCR was carried out by using 96-well microplates (Axygen) with the equipment of a 7300 Real-Time PCR System (Applied Biosystems). The gene *UBIQUITIN5(UBQ5)* was served as an internal control in these experiments. $2^{\triangle Ct = Ct\ (internal) - Ct\ (Sample)}$ was used to calculate each reaction value in the system. All the designed primers are presented in Table 2.1.

Chop-PCR

Genomic DNA was extracted from 0.1g ground plant leaves of Col-0, *ddc*, HopO1-2-HA/Co-0, HopAJ2-HA/Co-0, HopAS1-HA/Co-0, HopAG1-HA/Co-0. After grinding the tissues into a fine powder, 400 ul CTAB buffer (Chen et al., 2020) was transferred into the powder and samples were placed at 62°C for 40 mins. 400 ul

chloroform: isoamyl alcohol (24:1) was also transferred into every sample. After vertexing gently and centrifuging at high speed, I next siphoned 350 ul liquid phase mixing with 700 ul 100% isopropanol. The pellets isolated from centrifuging mixtures were washed three times by using 500 ul 70% ethanol and then were dissolved in ddH_2O to get genomic DNA from different plants. Next, 1ug purified DNA was digested by McrBC enzyme (NEB M0272) utilizing the protocol and the mock was mixed with ddH_2O in the same volume. All samples were placed at 37°C incubators for 4 hours, then heat inactivated at 65°C for 30 mins. 2 ul of liquid samples were regarded as templates for the qPCR reaction. All designed primers for this assay were listed in Table 2.1.

Pathogen Infection

As for pathogen infection assays, bacterial strain DC3000 was selected on KB agar medium with appreciate antibiotics. 4-6 weeks-old plants were given enough water in order to open stomata before inoculation. Bacterial colonies from the plate and wash twice times with 10mM $MgCl_2$ and resuspended in 10mM $MgCl_2$ to appreciate concentration with OD_{600} = 0.001. The pathogen resuspended solution was injected into back of *Arabidopsis* leaves by pressure-infiltration. Plants were covered by lids with high humidity in normal chamber. After 3 days, three leaf discs were ground with 500 µl of 10 mM $MgCl_2$ utilizing a 2010 Geno/Grinder. Each sample was serially diluted with 10 mM $MgCl_2$ (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}), and 20 µl was transferred onto KB plates with Rifampicin. Plates were incubated for 40-60 hours at 28°C until the colonies became visible and bacterial colony-forming units (cfu) were calculated.

Table 2.1 Primers used in experiments.

Primer Name	Primer Sequence (5'-3')
GW_F (attB1)	ggggACAAGTTTGTACAAAAAAGCAGGCTTC
GW_R (attB2)	gggcACCACTTTGTACAAGAAAGCTGGGTC
DRM2_GW_F	GW_F_ ATGTGGGATGAAACTGTAGCC
DRM2_GW_R	GW_R_ TCAACGGTGCTTGCTCCT
EDS1_GW_F	GW_F_ ATGGGCTTGACACTTTGG
EDS1_GW_R	GW_R_ GGTATCTGTTATTTCATCCATCATATAGTC
CLF_GW_F	GW_F_ ATGGCGTCAGAAGCTTC
CLF_GW_R	GW_R_ AGCAAGCTTCTTGGGTCTAC
HopAG1_GW_F	GW_F_ ATGAACCCTATAACACACAGCTTTAGTC
HopAG1_GW_R	GW_R_ TTCAGACCTTCCTTAACTACCCGCA
HopAJ1_GW_F	GW_F_ ATGCCTTCTCGTTTTACCC
HopAJ1_GW_R	GW_R_ CTGCCCCACAGGC
HopAS1_GW_F	GW_F_ ATGACCTTAAGAATCAATACTC
HopAS1_GW_R	GW_R_ AGAAAACTCGGCTTTCTGTTC
HopO1-2_GW_F	GW_F_ ATGAATATCAGTCCTGTATC
HopO1-2_GW_R	GW_R_CTCGTCTGAATTATCTG
UBQ5_RT_F	TCTCCGTGGTGGTGCTAAG
UBQ5_RT_R	GAACCTTTCCAGATCCATCG
EDS1_RT_F	AGCCATACGAGGAAGTTGAGGTAAGA
EDS1_RT_R	CTCCACCACCTAAGGTTCAGGTATCT
AtSN1_F	ACTTAATTAGCACTCAAATTAAACAAAATAAGT
AtSN1_R	TTTAAACATAAGAAGAAGTTCCTTTTTCATCTAC
MEA-ISR_F	TGGAATCGCGAACGACTATTGC
MEA-ISR_R	TGGATCCCGCCATTTAACCG

CHAPTER 3

GENETIC AND EPIGENETIC REGULATIONS OF EDS1-MEDIATED PLANT IMMUNITY

Screening of interactions between EDS1 and epigenetic regulators

Because transcriptional regulation of EDS1 on immunity-related genes is a prerequisite for plant defense and how EDS1 activates plant immunity by transcriptional reprogramming is still not well understood (García et al., 2010), it is urgent for us to investigate whether epigenetic regulators, especially those involved in histone modifications and DNA methylation, participate in EDS1-mediated transcriptional regulation. To screen potential interactors of EDS1, I used yeast strains Y187 expressing BD-EDS1 protein as a bait. I confirmed that there is no effect on yeast growth rate between colonies expressing BD-EDS1 and colonies expressing BD-EV (empty vector). Furthermore, colonies expressing BD-EDS1 did not turn blue when grown on SD-Trp + X-α-Gal agar medium, indicating that the bait protein did not activate the expression of reporter genes autonomously in Y187 (data not shown).

Genes encoding epigenetic regulators were cloned into the pGADT7 empty plasmid and then transferred into the yeast strain AH109 as preys. After confirming that there was no yeast self-activation (data not shown), colonies expressing AD- epigenetic proteins were mated with colonies expressing the bait individually. Yeast zygotes with

successful mating were grown on double dropout (DDO-Leu-/Trp) agar plates. Diploid yeast cell cultures were plated on DDO-Leu/-Trp and triple dropout (TDO, -Leu/-Trp/-His) agar medium with 1mM 3-AT to screen for interactions of epigenetic regulators with EDS1. Diploid yeast cells expressing BD-EDS1 with AD-PAD4, AD-SDG1, AD-SDG9, AD-SDG15, AD-SDG22, and AD-SDG35 grew well on TDO-LWH plates with 1mM 3-AT, while cells expressing BD-EDS1 with AD-HDA6 and AD-HDA7 showed less growth (Figure 3.1). The other mated yeast cells could not grow in this condition. These results suggest that SDG1, SDG9, SDG15, SDG22 and SDG35 may be touched upon EDS1-mediated plant defensive pathways.

clf-28 mutant was more susceptible to bacterial pathogen *Pst DC3000*

I next investigated which EDS1-interacting epigenetic regulators are involved in plant immunity. *clf-28* (SALK_139371), a mutant allele of *SDG1*, showed increased susceptibility to *Pst* DC3000 as compared with wildtype (Figure 3.2A). After inoculating the plants with this type of plant pathogen, leaves of *eds1-2* and *clf-28* mutants, but not Col-0, showed noticeably chlorotic (Figure 3.2 A). The growth of the bacterial pathogen *Pst* DC3000 was dramatically higher in *clf-28* mutants when compared with Col-0 plants but not as high as *eds1-2* mutants (Figure 3.2 B). These results propose that CLF (SDG1) boasts the characteristic of a positive regulator in plant defense.

Confirmation of interaction between CLF and EDS1 in *vitro*

In order to avoid false positives in Y2H screening by mating, I performed Y2H assays again by co-transforming pGADT7-CLF and pGBKT7-EDS1 into the same yeast AH109 strain and using empty vectors as negative controls. Similarly, only yeast cells

expressing AD-CLF and BD-EDS1 grew well on TDO-Leu/-Trp/-His with 3mM 3-AT plates, so their interaction was further confirmed by an independent Y2H assay in Figure 3.3 (A).

I also carried out pull down assays to determine whether CLF interacts with EDS1 directly *in vitro*. Firstly, I found glutathione-S-transferase (GST)-tagged CLF and HisMBP- tagged EDS1 could be induced correctly and individually in *Escherichia coli* C41 cell lysates after 0.4mM IPTG treatment (data not shown). Next the purified GST-CLF and GST proteins linked with glutathione Sepharose beads were used for incubating with cell lysates containing the HisMBP-EDS1 respectively. After washing three times by high salt solutions to remove unspecific binding proteins, the HisMBP-EDS1 proteins were efficiently pulled down by GST-CLF shown in Figure 3.3 (B).

EDS1 Co-immunoprecipitated with CLF in *Nicotiana benthamiana*.

Next, to investigate the association between EDS1 and CLF *in vivo*, I carried out co-immunoprecipitation (Co-IP) assays in *Nicotiana*. N-terminal HA-tag CLF proteins (HA-CLF) and C-terminal GFP-tag EDS1 proteins (EDS1-GFP) or GFP protein alone were co-expressed in *N. benthamiana* by infiltration with *Agrobacterium tumefaciens*. Results showed that HA-CLF proteins were efficiently co-immunoprecipitated with EDS1-GFP proteins instead of GFP protein shown in Figure 3.4, indicating that EDS1 proteins were associated with CLF proteins *in vivo*.

EDS1-CLF complex located in nucleus.

In order to determine detailed cellular location of the CLF-EDS1 complex, I performed BiFC assays. Recombined GV3101 strains holding combined plasmids

expressing fusion proteins of the N-terminal enhanced yellow fluorescent protein with CLF (nEYFP-CLF) or PAD4 (nEYFP-PAD4), which served as a positive control, and the C-terminal EYFP with EDS1 (cEYFP-EDS1) were co-infiltrated into delicate leaves of *Nicotiana*. In Figure 3.5, our data showed that unlike the EDS1-PAD4 complex, which located in the cytoplasm and the nucleus, the EDS1-CLF complex was only present in the nucleus, suggesting that EDS1-CLF complex is likely to refer to transcriptional regulation of genes.

CLF regulates EDS1 protein accumulation.

To probe how CLF protein positively regulates plant immunity by associating with EDS1 proteins, I investigated EDS1 transcriptional levels and protein levels in *clf-28* mutant. To our surprise, the mRNA level of *EDS1* in *clf-28* mutant was similar in Col-0 at normal conditions in Figure 3.6 (A), while I found that there existing decreased EDS1 protein levels in *clf-28* mutant as compared with Col-0 in Figure 3.6 (B), indicating that CLF was required for EDS1 protein accumulation.

Construction of overexpressing GFP-CLF transgenic plants.

Next, in order to clarify EDS1-mediated immune pathway is crucial for resistant phenotype of CLF, recombined plasmid *35S: GFP-CLF* was transformed into *Agrobacterium tumefaciens*, which was used for constructing transgenic plants under the background of *eds1-2* mutant or WT Col-0. Potential positive seedlings were selected on the ½ MS medium with kanamycin. Total protein extracts of those plants were obtained to further detect GFP-CLF expression by western blots. As shown in Figure 3.7, I found T1 transgenic lines #3, #6, #8, #9 in the *eds1-2* mutant background and T1 transgenic

lines *3, *4, *6, *9 in Col-0 background could express GFP-CLF well. We will use those T2 transgenic lines to detect single copy insertion of 3:1 survival ratio on the ½ MS medium with kanamycin, and homozygous T3 will be used for the future experiment.

EDS1 interacts with CDK8 *in vitro* and *in vivo*.

Apart from epigenetic regulators, the mediator complex also controls gene expression (Bourbon, 2008). To find out whether EDS1 promotes plant immune gene transcription through combining with the mediator complex, we tested the association of EDS1 with CDK8 by yeast two-hybrid (Y2H) assays. We know that most mediator members are able to recruit transcriptional activators or repressors binding to their targets to control gene expression. Therefore, I used BD-CDK8 as a bait and AD-EDS1 as a prey. Figure 3.8 (A) stated clearly an obvious interaction between EDS1 and CDK8 at TDO-LWH with 3mM 3-AT plates. To determine whether EDS1 and CDK8 could interact *in planta*, I transiently expressed HA-tagged CDK8 proteins with GFP-tagged EDS1 or GFP *in Nicotiana* plants and carried out co-immunoprecipitation (Co-IP) assays. HA-CDK8 protein was clearly co-immunoprecipitated by EDS1-GFP, proposing that the EDS1 and CDK8 interacted specifically *in vivo* shown in Figure 3.8 (B).

CDK8 regulates EDS1 protein accumulation.

Next, in order to describe the significance of CDK8 in EDS1-mediated plant immunity, I used western blots to detect EDS1 protein levels in *cdk8* mutants and *cdk8* mutants overexpressing CDK8-MYC or the kinase dead CDK8KD-MYC. Interestingly,

EDS1 protein levels were obviously decreased in two homozygous mutants *cdk8-1* (SALK_138675) and *cdk8-4* (GABI_564F11) but significantly increased in two 35S:CDK8-MYC and two 35S:CDK8KD-MYC positive seedlings, implying that CDK8 is necessary for EDS1 protein accumulation certainly and CDK8 positively regulates EDS1 protein independent of its kinase activity (Figure 3.9).

Discussion:

Although EDS1 functions as a hub in SAR, plant basal defense, TIR-NLR mediated ETI (Rietz et al., 2011) and previous study also showed that EDS1 proteins in the nucleus were required for plant immunity (García et al., 2010), how EDS1 affects expression of immune-related genes in the nucleus is not known. In this study, I have demonstrated that EDS1 directly interacted with epigenetic regulator CLF(SDG1) *in vivo* and *in planta* (Figure 3.3, 3.4, 3.5) and CLF also acts as a positive regulator in plant defense according to the result after inoculation of bacterial *Pst DC3000* (Figure 3.2). Thus, to further analyze how EDS1-CLF complex positively contribute to plant defense should be of great interest.

Previously, our lab has reported that the SA receptors NPR3 and NPR4 were regarded as adaptors for cullin 3 E3 ligase to promote EDS1 degradation (Chang et al., 2019). Combined with my recent preliminary results that EDS1 protein levels were significantly decreased in *clf-28* mutant while the transcriptional level of *EDS1* in *clf-28* mutant was the same as in WT Col-0 (Figure 3.6), I speculate that CLF protects EDS1 from NPR3 and NPR4-mediated protein degradation. Future experiments must be conducted to determine whether CLF reduces the interaction between EDS1 and NPR3/4.

On the other hand, previous research showed that EDS1 repressed the expression of *defense, ' no death' gene 1* (*DND1*) and transcription factor *MYB49,* which regulate plant defense negatively (García et al., 2010). H3K27me3 marks established by CLF, which constitutes catalytic core of Polycomb Repressive Complex (PRC2), refer to the course of gene repression (Zhou et al., 2018). Results obtained from BiFC assays suggest that EDS1-CLF complex was co-localized in the nucleus (Figure 3.5). Based on those exciting data, I hypothesize that EDS1 could suppress transcription of *DND1* and *MYB49* by associating with CLF to alter H3K27me3 marks on promoters of those two genes. Further experiments are needed to determine whether EDS1 is involved in the establishment of H3K27me3 marks by recruiting CLF protein and binding to the promoters of *DND1* and *MYB49*.

Apart from epigenetic regulators, the interaction between mediator CDK8 and EDS1 (Figure 3.8) also indicated that EDS1 participated in transcriptional reprogramming of genes. CDK8 functions as transcriptional co-activator to upregulate plant immunity signals by activating transcription of *ICS1, NPR1* and *PR1*, etc (Huang et al., 2019; Chen et al., 2019) and EDS1 also promotes expression of those genes (García et al., 2010). Those results further implied that EDS1 could also act as a transcriptional co-activator to accumulate transcription of plant immune genes. The result that CDK8 regulated EDS1 accumulation (Figure 3.9) also suggest that CDK8 could also promote transcriptional activation of EDS1.

This work expanded the functions of EDS1 in plant defense. There is a high possibility that EDS1 has dual functions in gene expression. EDS1-CDK8 complex could upregulate transcription of plant defense genes, while EDS1-CLF complex could

downregulate transcription of plant susceptibility genes at the epigenetic level. Identification of dual functions of EDS1 will pave a new avenue to research plant immunity and enrich the molecular theory of transcriptional regulation.

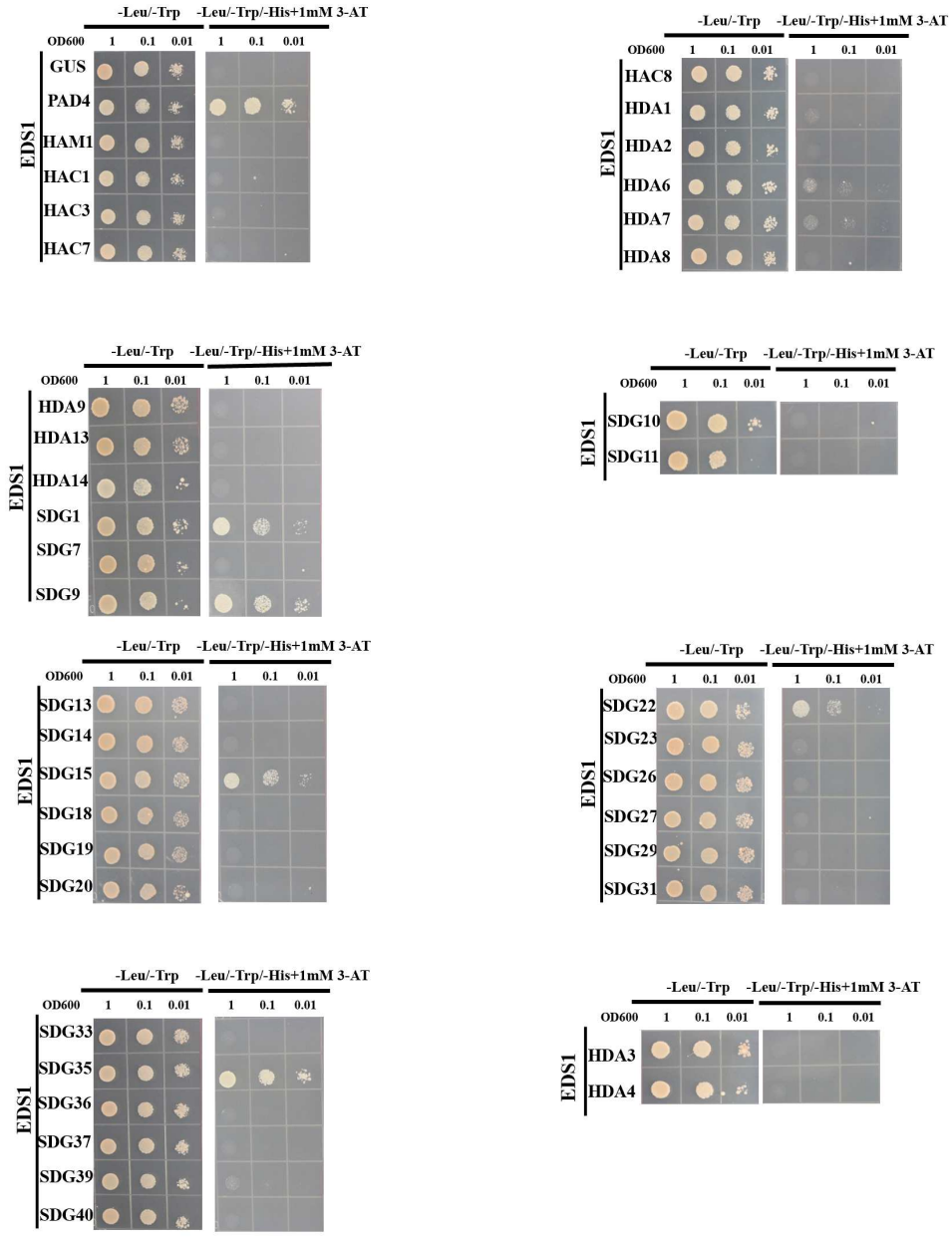

Figure 3.1 Screening of EDS1's epigenetic interactors by Y2H assays.

Yeast cells containing AD-epigenetic regulators were mated with yeast cells containing BD-EDS1 pair wisely. The mated culture was dropped on control plates DDO-Leu/-Trp (DDO-LW) and TDO-Leu/-Trp/-His (TDO-LWH) with 1mM 3–amino-1,2,4–triazole (3-AT) at OD_{600} =1.0,0.1 and 0.01 individually. Colonies containing AD-GUS and BD-EDS1 was served as a negative control. Yeast colonies containing AD-PAD4 and BD-EDS1 was served as a positive control. Photos were shoot after 3 days. These experiments were performed more than four times with consistent results.

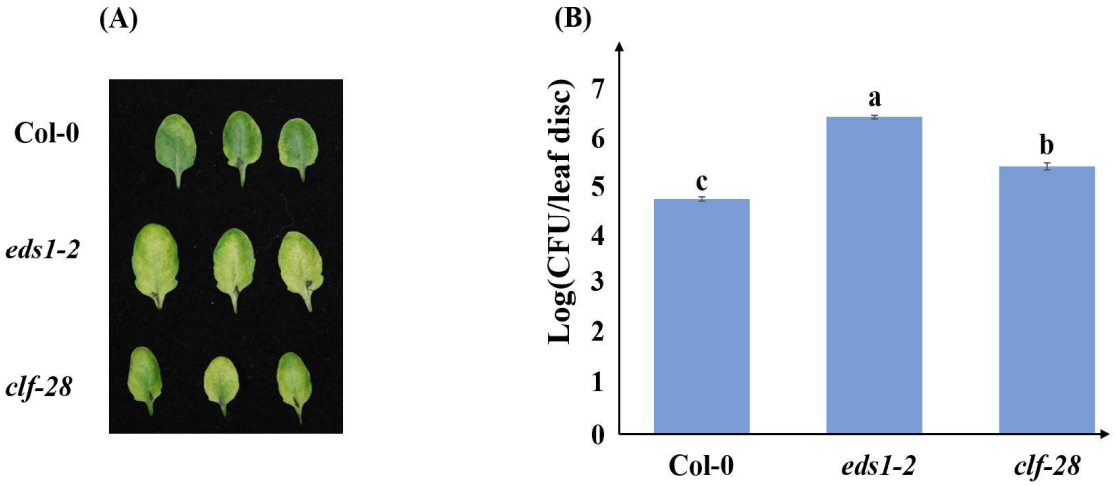

Figure 3.2 *clf-28* mutants show enhanced susceptibility to the *Pst* DC3000.

(A) The phenotype of *Arabidopsis clf-28* mutants after bacterial pathogen *P. syringae* inoculation. Healthy leaves were inoculated by *Pst DC3000* using a 1ml needless syringe. Photos were shoot after 3 days. (B) CFU detection after *P. syringae* inoculation. The *eds1-2* or *clf-28* shows a more susceptible phenotype after treatment of *P. syringae*. *Arabidopsis* Col-0, *clf-28* and *eds1-2* mutants were inoculated with *Pst* DC3000 using a 1ml needless syringe. Mean value ± s. d.(n=3). Specific lowercase letters on bars represent significant differences detected by Student's t-test ($p < 0.05$). These experiments were carried out more than four times according to consistent results.

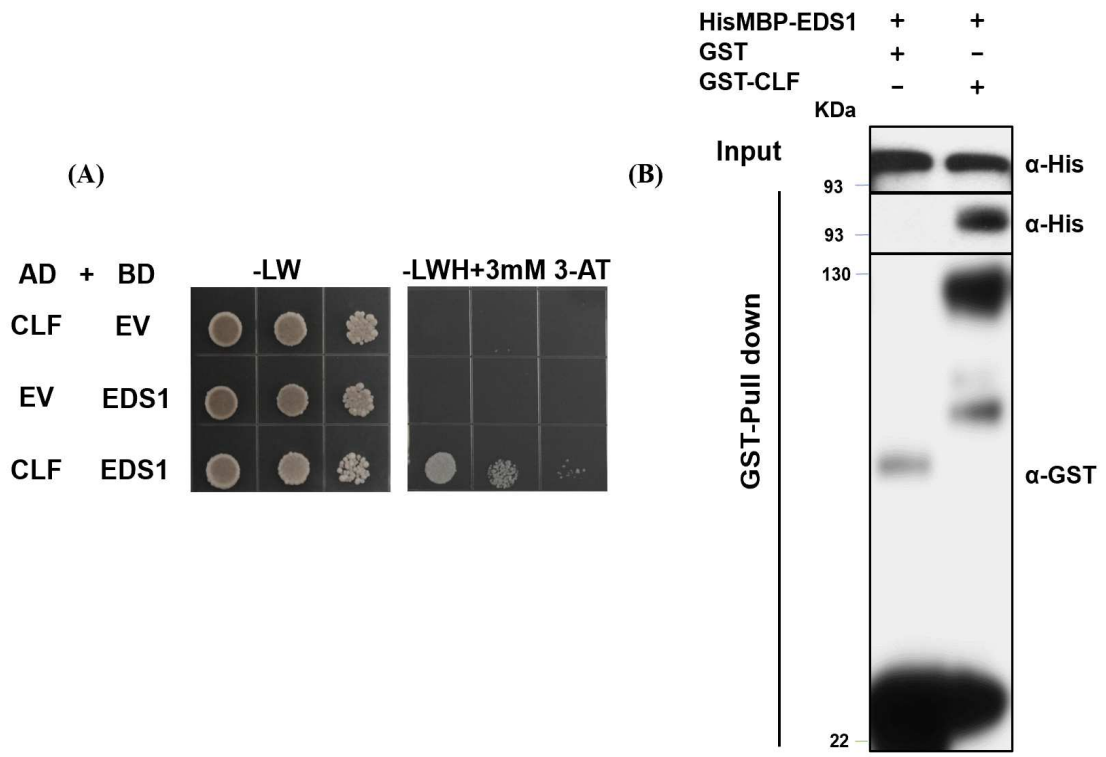

Figure 3.3 *In vitro* interaction between CLF and EDS1.

(A) EDS1 interacts with CLF in Y2H assays**.** Yeast zygotes was dropped on control plates DDO-Leu/-Trp (DDO-LW) and TDO-Leu/-Trp/-His (TDO-LWH) with 3mM 3–amino-1,2,4–triazole (3-AT) at OD_{600}=1.0,0.1 and 0.01 individually. EV means empty vector. (B) pull-down experiment on association of HisMBP-EDS1 with GST-CLF. The sediment of HisMBP-EDS1 with glutathione beads bound GST-CLF /GST was discovered by western blots according to a-His antibody before (input) and after washing. GST proteins served as negative controls. Protein sizes are indicated at the left. These experiments were performed more than four times according to consistent results.

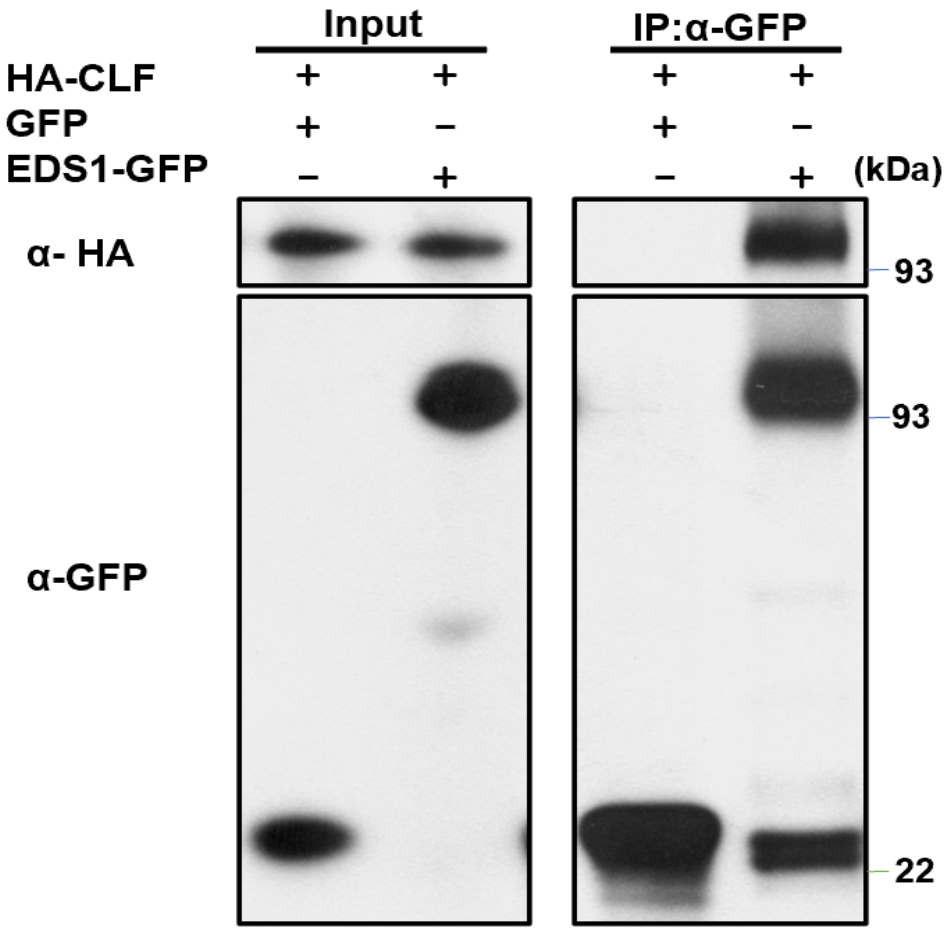

Figure 3.4 Co-immunoprecipitation (Co-IP) assays of EDS1 and CLF.

HA-CLF and EDS1-GFP or GFP were instantaneously co-expressed in *N. benthamiana* leaves mediated by *Agrobacterium*. Total protein after 50 mM MG115 additive was carried out Co-IP individually. Western blots were used for analyzing immunoprecipitated (IP) proteins and input proteins according to anti-HA and anti-GFP antibodies respectively. GFP proteins were served as negative controls. Protein sizes are indicated at the right. These experiments were performed more than four times with consistent results.

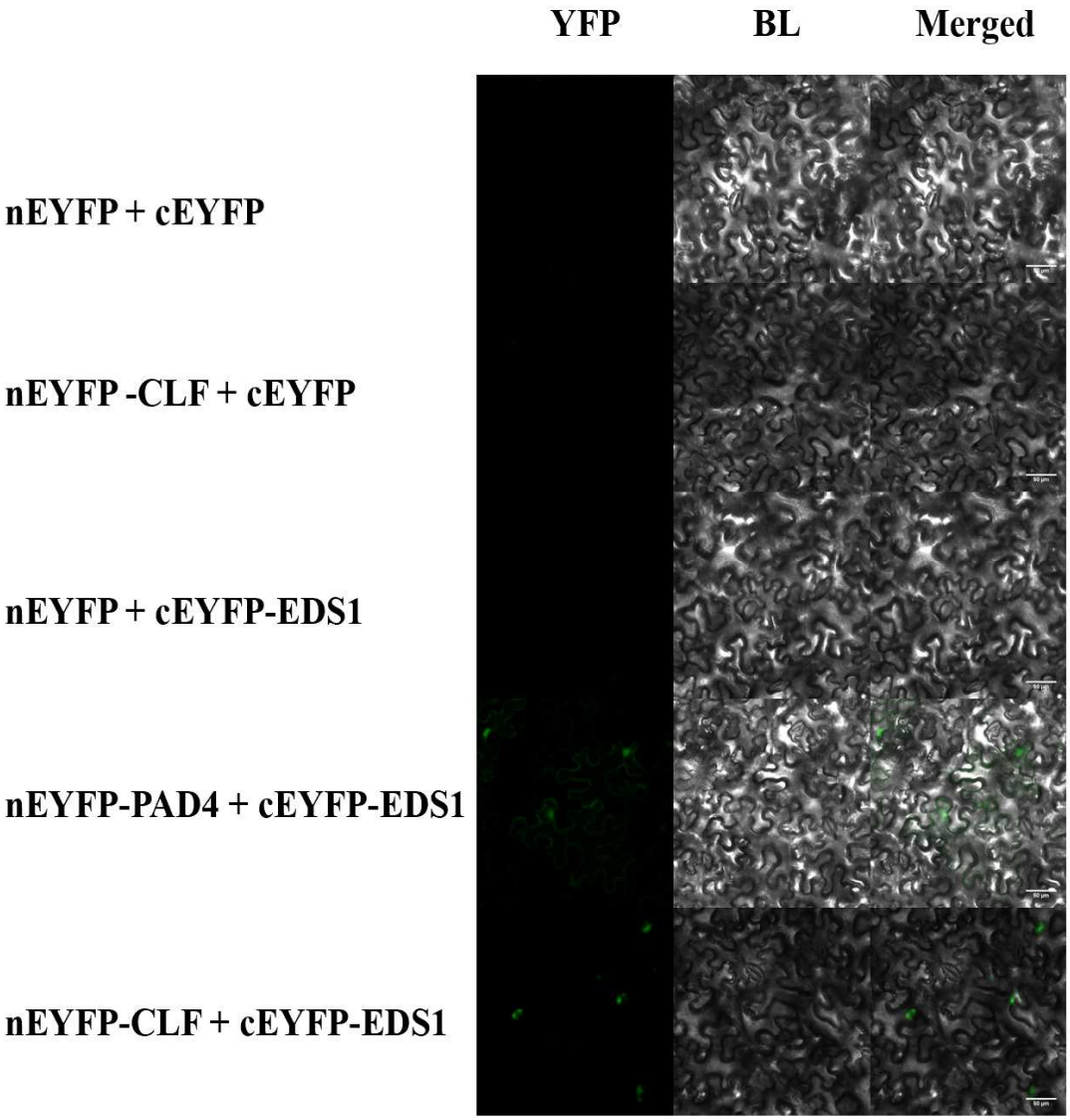

Figure 3.5 Co-localization between CLF and EDS1 detected by Bimolecular Fluorescence Complementation (BiFC).

N. benthamiana was co-transformed with corresponding constructs. All photos were shoot by Laser scanning confocal microscopy (LSCM) to detect YFP signals with 2 days later than infiltration. Bars, 50 μm. YFP, Yellow Fluorescent Protein. BL, bright field. These experiments were performed more than four times with consistent results.

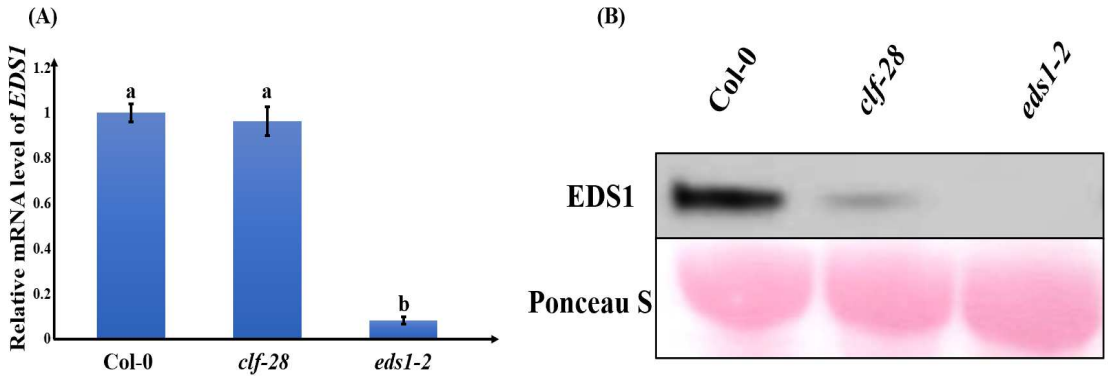

Figure 3.6 CLF increases the content of EDS1 at post-translational level.

(A) The mRNA level of *EDS1* in *clf-28* mutant resembled that in Col-0. UBQ5 was acted as an internal control. Values represent the mean ± SE. Specific lowercase letters on bars stand for diverse differences calculated by Student's t-test ($p < 0.05$). (B) EDS1 antibody was used to detect EDS1 protein levels in Col-0, *clf-28* and *eds1-2* mutants. Ponceau S stained Rubisco protein was the internal reference of a total protein. These experiments were carried out more than four times with consistent results.

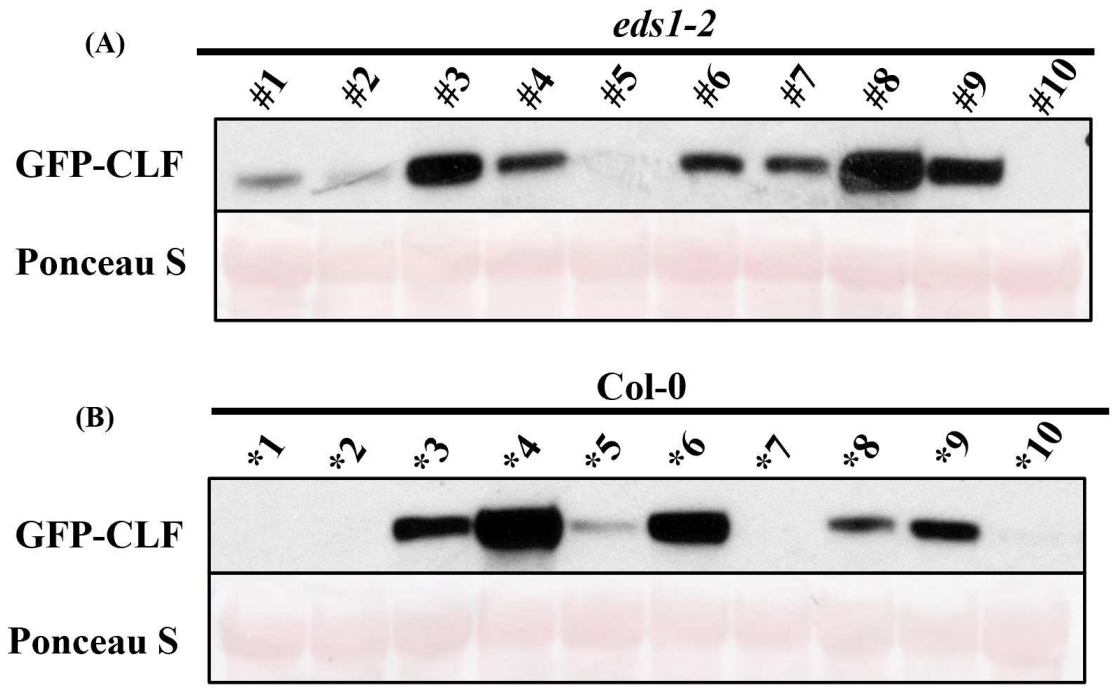

Figure 3.7 Construction of overexpressing GFP-CLF transgenic plants.

(A) GFP-CLF protein levels in *eds1-2* mutant background. Total protein extracts of ten potential positive seedlings after 50mM MG115 additive were subjected to western blot detection. Ponceau S□stained Rubisco protein was the internal reference of a total protein (B) GFP-CLF protein levels in WT Col-0 background. Total protein extracts of ten potential positive seedlings after 50mM MG115 treatment were subjected to western blot detection. Ponceau S□stained Rubisco protein was the internal reference of a total protein. These experiments were carried out more than four times according to similar results.

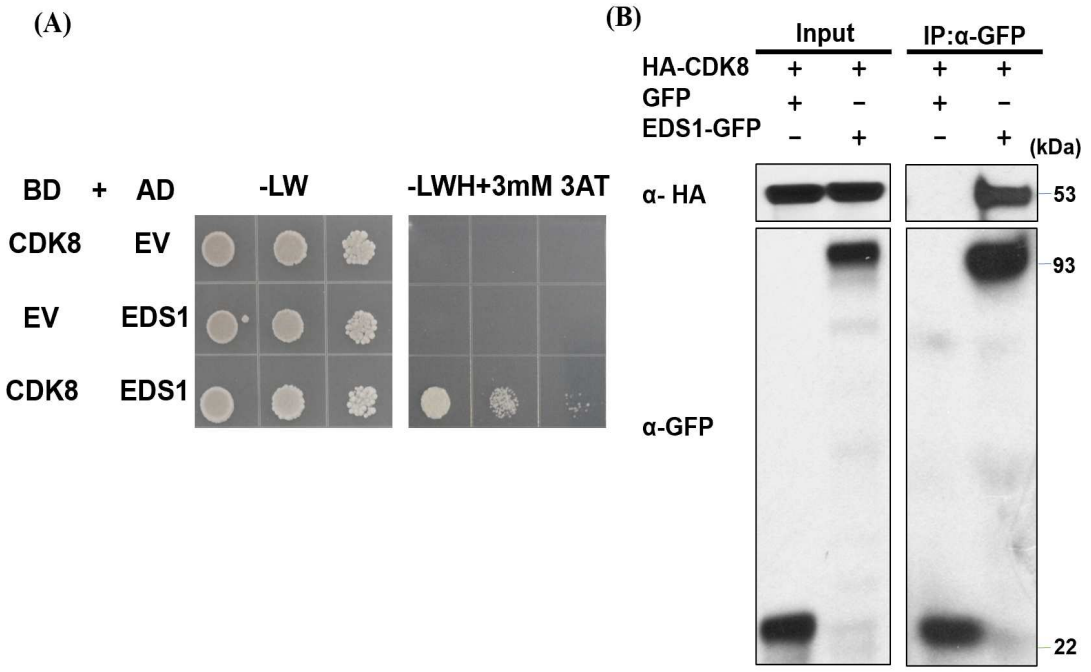

Figure 3.8. EDS1 interacts with CDK8.

(A) EDS1 interacts with CDK8 in Y2H assays. Yeast zygotes was dropped on control plates DDO-Leu/-Trp (DDO-LW) and TDO-Leu/-Trp/-His (TDO-LWH) with 3mM 3–amino-1,2,4–triazole (3-AT) at OD_{600}=1.0,0.1 and 0.01 individually. EV means empty vector. (B) EDS1-CDK8 interaction in Co-immunoprecipitation assays *in planta*. HA-CDK8 with EDS1-GFP or GFP were instantaneously co-expressed in *N. benthamiana* leaves mediated by *Agrobacterium*. Total protein extracts after 50 mM MG115 treatment were immunoprecipitated with GFP trap. The HA-CDK8 protein associated with EDS1-GFP or GFP was analyzed according to anti-HA antibodies and EDS1-GFP or GFP was also analyzed by immunoblotting according to anti-GFP antibodies. These assays were performed more than three times with consistent results. Protein sizes are indicated at the right.

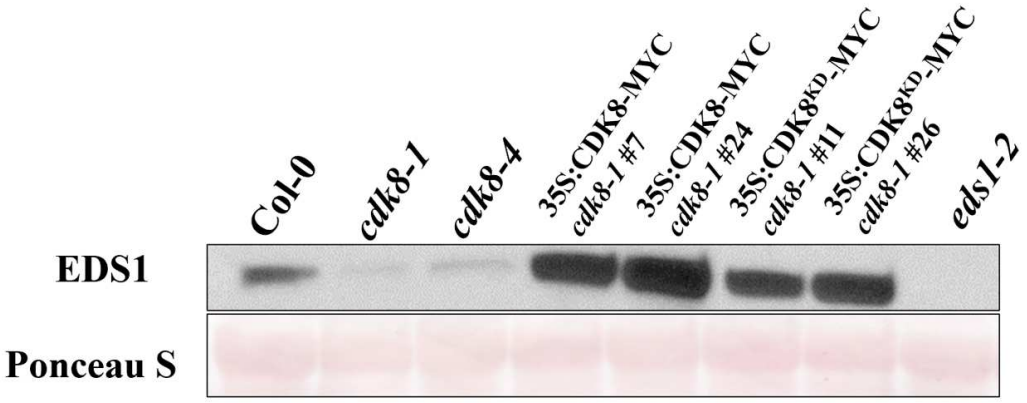

Figure 3.9 CDK8 regulates EDS1 protein accumulation.

EDS1 protein levels in Col-0, *cdk8-1* and *cdk8-4* mutants, *35S:CDK8-MYC*/*cdk8-1* #7 and #24 and *35S:CDK8^{KD}-MYC*/*cdk8-1* #11 and #26 transgenic plants. Ponceau S stained Rubisco protein was the internal reference of a total protein. KD: kinase dead. These assays were carried out more than three times according to consistent results.

CHAPTER 4

IDENTIFICATION OF BACTERIAL TYPE III EFFECTORS TARGETING *DE NOVO* DNA METHYLTRANSFERASE DRM2 IN *ARABIDOPSIS*

Screening of interactions between DRM2 and type III effectors in *Pst DC3000.*

Based on previous research data, the *ddc* (*drm1-2, drm2-2, cmt3-11*) mutants increased immunity to the bacteria *P. syringae* (Dowen et al., 2012). I want to detect whether some effectors in *P. syringae* could target DRM2 to alter phenotypes of plants in plant immunity. Similarly, I also used yeast strains Y187 expressing BD-DRM2 protein as a bait. Most of effectors in *Pst* DC3000 were cloned into pGADT7 and those recombined plasmids were further transferred into the yeast strain AH109 one by one as preys. After colonies expressing AD- effector proteins were used to mate with colonies expressing BD-DRM2, the successful mating colonies were grown on double dropout (DD, -Leu-/Trp) plates. Next, those diploid yeast cell cultures were drop on quadruple dropout (QD, -Leu/-Trp/-His/-Ade) agar plates to screen potential interactors of DRM2 protein. As shown in Figure 4.1, I identified some associations of type III effectors with DRM2 by yeast two-hybrid assays (Y2H). Effector HopO1-2 had strongest interaction with the DRM2 protein. HopAJ2, HopAG1 and HopAS1 showed strong interaction with the DRM2 protein. In addition, AvrE1, HopD1, and HopAA1-2 could also interact with DRM2.

Construction of overexpressing effectors-HA transgenic plants.

Next, in order to find out whether those effectors could change DNA methylation levels through their association with DRM2, recombined plasmid *35S: HopO1-2-HA*, *35S: HopAJ2-HA*, *35S: HopAG1-HA*, *35S:HopAS1-HA* were transferred into *Agrobacterium tumefaciens* and positive strains were used to make transgenic plants in WT Col-0 background individually. T1 potential positive seedlings were selected on the ½ MS agar plates with kanamycin. And total protein extracts were obtained to further detect expression of effectors-HA proteins by western blots. For each kind of overexpressing effector-HA transgenic plant, I picked up two lines that expressed effectors-HA well for further study (Figure 4.2).

Detection on methylation levels of DRM2 targets in effectors-HA transgenic plants.

A previous study showed that *AtSN1* and *MEA-ISR* are two useful transposable elements (TE) for examining DRM2-mediated RdDM pathways (Groth et al., 2016). The method Chop-qPCR was used to check changes of methylation levels on those two TEs in effectors-HA transgenic plants. To our surprise, I found that transgenic lines overexpressing HopAS1-HA and HopO1-2-HA had lower DNA methylation levels at both TE loci compared with positive control Col-0 (Figure4.3). Transgenic lines of overexpressing HopAJ2-HA and HopAG1-HA had similar DNA methylation levels at both TE loci compared with positive control Col-0 (Figure4.3).

Discussion:

Although DRM2 is mainly in charge of establishment of *de novo* methylation and maintenance of non-CG DNA methylation in plants, how post-translation modifications (PTMs) of DRM2 affect DNA methylation of its targets is still needed to be further clarified (Denis et al., 2011). And previous research showed that the *ddc* (*drm1-2, drm2-2, cmt3-11*) was resistant to plant pathogen *P.syringae,* though *drm2* mutants did not have an obvious resistant or susceptible phenotype (Yu et al. 2013). This inspired us to explore the relationship between bacterial type III effectors and plant DNA methylation. In this study, I have demonstrated that DRM2 interacted with Type III effectors, especially HopO1-2, HopAJ2, HopAG1 and HopAS1 by YH2 assays (Figure 4.1). However, only overexpressing HopO1-2-HA and HopAS1 transgenic lines downregulated methylation abundances at two DRM2 targets *AtSN1* and *MEA-ISR* compared with WT Col-0 (Figure 4.3). So, it is interesting to clarify whether methylation levels of other targets of DRM2 are also affected in those transgenic lines.

Recognition of effector HopAS1 in plant cells contributes to *Arabidopsis* nonhost resistance and triggers ETI (Sohn et al., 2021). It is possible that HopO1-2 functions as a mono-ADP-ribosyl transferase based on its protein sequence and that it contributes to bacterial virulence and suppresses plant defensive pathways (Fu et al., 2007). Whether DRM2 was a real target of HopAS1 or HopO1-2 should also be verified *in planta*. Future experiments must be conducted to determine how HopAS1 and HopO1-2 modify DRM2 proteins to trigger ETI or ETS. To further explore DRM2 functions in plant immune response will further elucidate new mechanisms of plant susceptibility or plant resistance to pathogens at epigenetic levels.

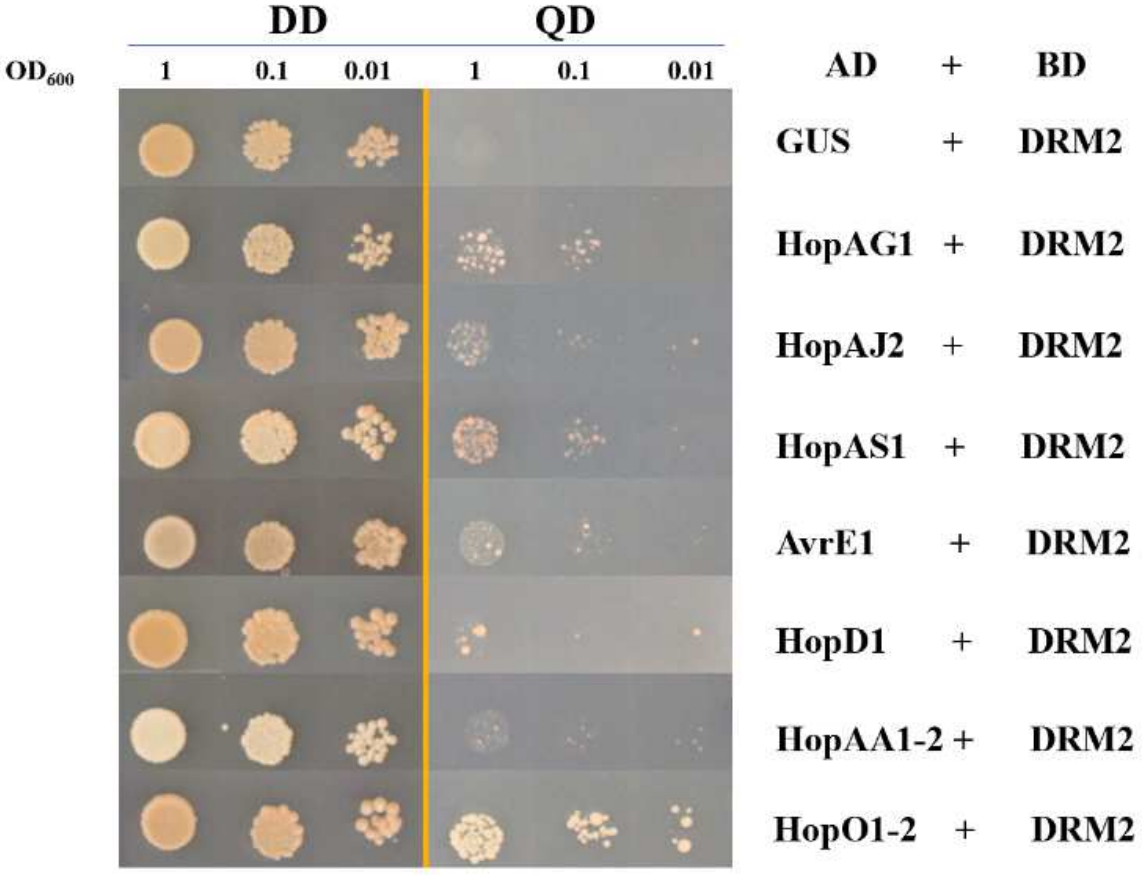

Figure 4.1 Interactions between type III effectors in *P. syringae* and DRM2 in Y2H assays.

Yeast cells containing AD-effectors were mated with yeast cells containing BD-DRM2 pair wisely. The mated culture was dropped on control plates DDO-Leu/-Trp (DDO-LW) and QDO-Leu/-Trp/-His/-Ade (QDO-LWHA) at OD_{600} =1.0,0.1 and 0.01 individually. GUS served as negative control. These experiments were performed more than four times with consistent results.

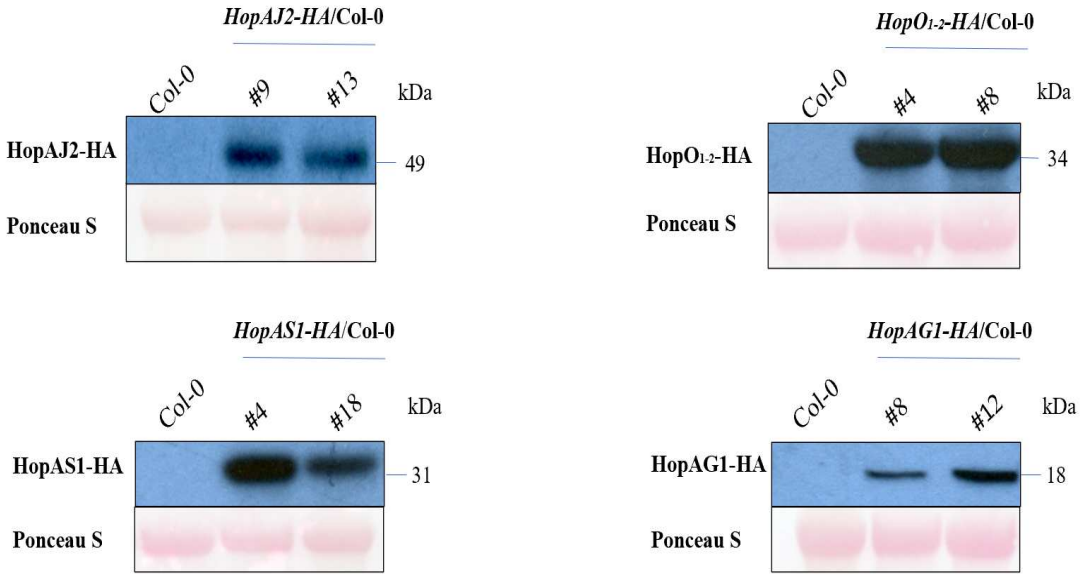

Figure 4.2 Construction of transgenic plants overexpressing HA-effectors.

HA antibody was used to detect effectors-HA (HopAJ2-HA, HopO1-2-HA, HopAS1-HA, and HopAG1-HA) proteins in WT Col-0 background. For each type of overexpressing effector-HA transgenic plants, total protein extracts of two potential positive seedlings after 50mM MG115 treatment were subjected to western blot detection. Ponceau S stained Rubisco protein was the internal reference of a total protein. KD: kinase dead. These experiments were performed more than four times with consistent results.

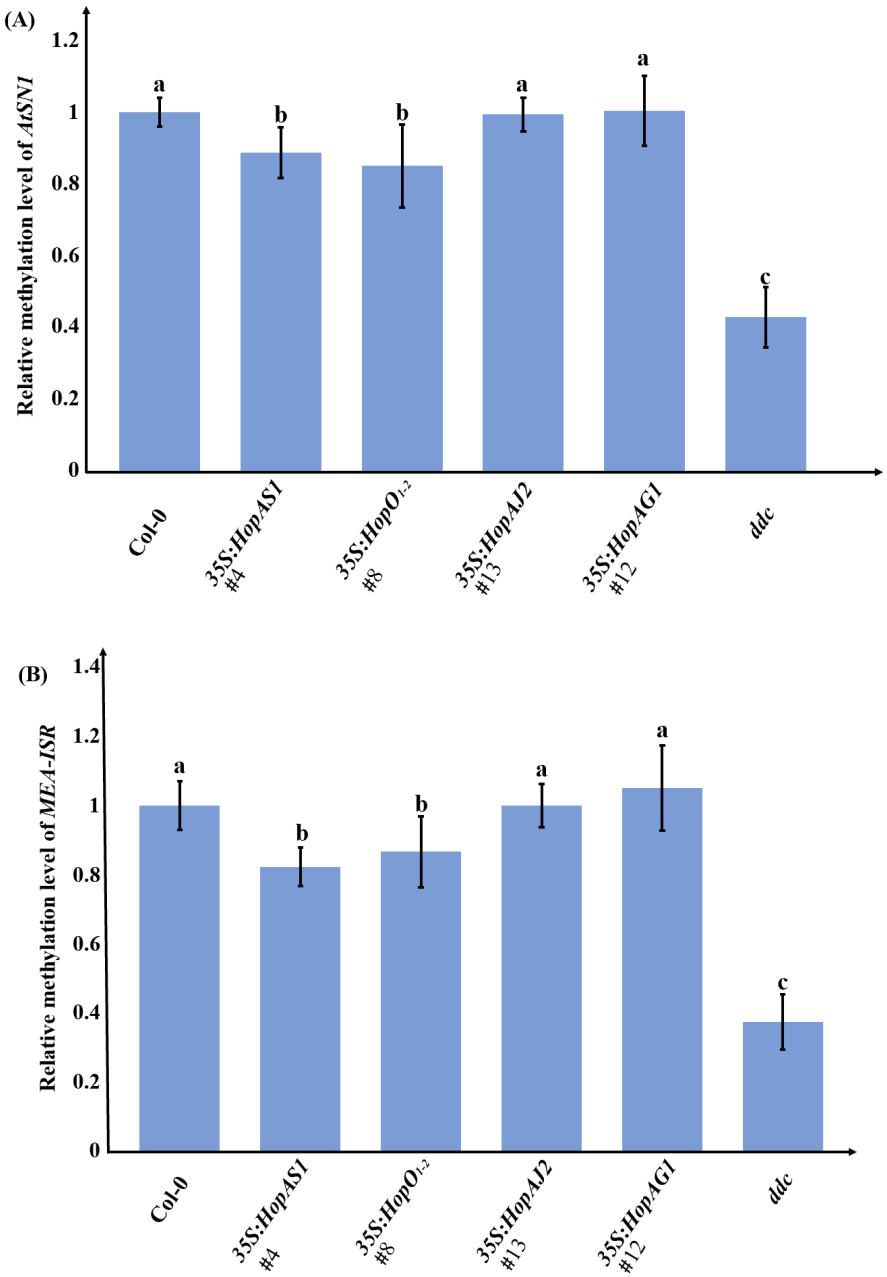

Figure 4.3 Detection of relative methylation levels of DRM2 targets *AtSN1* and *MEA-ISR* in transgenic plants expressing HA-tagged effectors by Chop-qPCR.

(A) The relative methylation level of *AtSN1* in transgenic plants overexpressing effectors-HA. All the data was calculated by normalizing to non-McrBC control. Values stand for the mean value ± s. d.(n=3). Specific lowercase letters on bars represent significant differences by Student's t-test ($p < 0.05$). (B) The relative methylation level of *MEA-ISR* in transgenic plants overexpressing effectors-HA. All the data was calculated by normalizing to non-McrBC control. Values represent the mean value ± s. d.(n=3). Specific

lowercase letters on bars represent significant differences detected by Student's t-test ($p < 0.05$). These experiments were performed more than four times with consistent results.

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