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rCBP-dependent regulation in rice innate immunity

by

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Declaration of Original and Sole Authorship

I, Nino A. Espinas, declare that this thesis entitled "rCBP-dependent regulation in rice innate immunity" and the data presented in it are original and my own work.

I confirm that:

- No part of this work has previously been submitted for a degree at this or any other university.
- References to the work of others have been clearly acknowledged. Quotations from the work of others have been clearly indicated, and attributed to them.
- In cases where others have contributed to part of this work, such contribution has been clearly acknowledged and distinguished from my own work.
- None of this work has been previously published elsewhere, with the exception of the following:

  Espinas NA*, Saze H, and Saijo Y*. 2016. Epigenetic control of defense signaling and priming in plants. *Front. Plant Sci.* 7: 1201. doi: 10.3389/fpls.2016.01201

  (Authorization has been obtained from all co-authors. * indicate corresponding authorship.)

Date: December, 2017
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Abstract

rCBP-dependent regulation in rice innate immunity

Plant innate immunity against bacterial attacks is a two-tiered inducible system capable of defense responses at local and systemic areas. These systems are the PTI and ETI. During infection, PTI has the ability to recognize microbial signatures upon bacterial contact, while ETI recognizes microbial protein secretions called effectors delivered inside the cell. The activation of PTI and ETI confers systemic tissues of infected plants a broad-spectrum immunity against later pathogen attacks termed systemic acquired resistance (SAR). Defense priming is an adaptive component of SAR that regulates the molecular storage of defense memory for a more effective defense response.

The main aim of this work is finding a novel molecular defense signaling pathway that is controlled by acetylation at the infected (local defense) and systemic tissues (priming defense).

To investigate the role of histone acetyltransferase-dependent pathway in plant immunity, I have isolated transgenic and mutant lines of *rCBP*, [rice Cyclic adenosine monophosphate response element-binding protein (CREB) Binding Protein], under Nipponbare cultivar background using RNAi silencing and gRNA/Cas9-mediated genome editing. Animal CBP was initially described as both transcriptional coactivator and histone acetyltransferase. The *rCBP*-RNAi lines with mistargeting of the other members of CBP family are characterized by massive sterility and impairment of the number of effective grains. On the other hand, the CRISPR/Cas9 mutant lines have wild-type number of effective grains.

To profile the global acetylation of histone lysine-sites *via* rCBP, I performed mass spectrometry-based proteomics in data dependent acquisition (DDA) and parallel reaction monitoring (PRM) modes. My results showed that H3 lysine sites are possibly targeted by rCBP with very high acetylation specificity on H3K9.

To implicate the role of rCBP in rice innate immunity, I conducted a pathogenesis assay with bacterial pathogen, *Pseudomonas syringiae* pv. *oryzae* (*Pso*). Pathogenesis assay showed that *rCBP*$^{-/-}$ mutants are resistant to *Pso* infection compared to segregated wild-type control.

I also performed transcriptome analysis on locally-infected tissues and on systemic tissues to investigate the genome-wide effects of *rCBP* mutation and to identify factors with roles in both local and systemic immune response. As a result, I have identified seven putative rCBP-dependent transcriptional repressors that possibly explain the resistance phenotype of *rCBP* mutant lines in locally-infected tissues. On the other hand, non-infected systemic tissues in mutant lines show diminishing number of genes with significant expression

Overall, these data preliminary indicate that *rCBP* is both a positive regulator of developmental processes and a negative regulator of rice immunity. These data also suggest that rCBP may execute this dual regulatory function either through H3K9ac and/or co-transcriptional activity on target gene loci. It is also tempting to hypothesize that *rCBP* might potentially regulate systemic defenses through an unknown mechanism at distal non-infected site in preparation for future infection episodes.

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Abbreviations

PTI PAMP-triggered immunity

PAMP Pathogen-associated molecular pattern

ETI Effector-triggered immunity

NB-LRR/NLR Nucleotide-binding site leucine-rich repeat

HR-PCD Hypersensitive response- programmed cell death

SAR Systemic acquired resistance

HAC701 Histone acetyltransferase 701

rCBP rice cyclic adenosine monophosphate response element-binding protein (CREB) binding protein

ROS Reactive oxygen species

PRR Pathogen recognition receptor

DAMP Damage-associated molecular pattern

LysM Lysin motif

EGF Epidermal growth factor

FLS2 Flagellin sensitive2

EFR EF-Tu receptor

R-gene Resistance gene

R protein Resistance protein

EIX Ethylene-inducing xylanase

CERK1 Chitin elicitor receptor kinase1

LYM LysM domain protein

CEBiP Chitin elicitor-binding protein

LYP LysM receptor-like protein

RK Receptor kinase

RD Non-arginine-aspartate kinase

RLP Receptor-like proteins

OG Oligogalacturonide

CC Coiled-coil

TIR Toll-interleukin 1 receptor

WRKY WRKYGQK amino acid motif

Avr Avirulence

T3SS type III bacterial secretion system

SA Salicylic acid

NO Nitrogen oxide

PR1 Pathogenesis-related1 protein

NPR1 Non-expressor of pathogenesis-related1

MeSA Methylsalicylic acid

JA Jasmonic acid

MAPK Mitogen-activated protein kinase

siRNA small interfering RNA

RdDM RNA-directed DNA methylation

ssRNA single-stranded RNA

dsRNA double-stranded RNA

Pol Polymerase

TE Transposable elements

TSS Transcriptional start sites

HAT Histone acetyltransferase

HDAC Histone deacetylase

PstDC3000 *Pseudomonas syringae* pv. *tomato* DC3000

Pso *Pseudomonas syringiae* pv. *oryzae*

sgRNA single-guide RNA

RNAi RNA interference

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

Cas9 CRISPR-associated protein-9 nuclease

INDELS Insertion and deletion mutations

H3K9ac/me Histone 3 Lysine 9 acetylation/ methylation

LC-MS/MS Liquid chromatography tandem mass spectrometry

DDA Data-dependent acquisition

PRM Parallel reaction monitoring

GO Gene ontology

TF Transcription factor

rTF repressor transcription factor

DEG Differentially expressed gene

To my parents and siblings

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Chapter 1

Introduction

Condensed Introduction

Plant innate immunity operates either locally or systemically. Localized immunity is generally divided into two tiers of inducible defense mechanisms namely the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), that protect the plants from its natural enemies. PTI utilizes cell surface receptors that recognize bacterial protein signatures, while ETI recognizes bacterial effectors via resistance proteins (R proteins). PTI and ETI are identifiable by specific physiological hallmarks they induce among others. PTI usually is characterized by callose deposition among others, whereas ETI often results in hypersensitivity response coupled to programmed cell death (HR-PCD). Both PTI and ETI induce the systemic acquired resistance (SAR), which is a distal form of immune defenses aimed to reduce fitness costs during subsequent pathogen attack. SAR is deemed to be fast, sustained, and intense due to the stored memory of the initial bacterial attack. This memory is termed as defense priming. Defense priming is mechanistically regulated by a number of molecular, biochemical, and physiological interactions. Epigenetic control of defense memory is an alternative mechanism to explain the regulation of defense priming. Epigenetic regulators such as methylation of the DNA strand and histones as well as histone modifications through acetylation and others are thought to be primary modulators of defense priming. Studies also showed that these epigenetic regulators maintain the stored memory via transgenerational inheritance of *bona fide* memory marks.

This work is ultimately aimed at finding a molecular defense signaling pathway that is controlled by acetylation at the local (infected-site defense) and systemic tissues (priming defense). This work utilizes transgenic and mutant rice lines of histone acetyltransferase 701 (HAC701)/rCBP since earlier experiments showed a possible role of this gene in defense responses during PTI signaling. The work presented here describes mainly the genetic and phenotypic features of the *rCBP* transgenic and mutant lines, its possible acetylation targets, its role in local defenses, and its possible role in systemic signaling.

1.1 The plant local immune defense signaling

Plants are in constant battle with its surroundings in a very distinctive manner. They have been fighting mobile opponents over evolutionary time scales mostly by way of stationary defense. This defense mechanism entails plants to be equipped with highly specific and fast evolving recognition systems. In conjunction with preformed physical barriers such as dynamic remodeling of cell walls (Bellincampi et al., 2014; Malinovsky et al., 2014; Underwood, 2012), plants have evolved early signaling defense mechanisms for instance reactive oxygen species (ROS) production and inducible innate immune systems that recognize and respond to pathogens. These inducible resistance mechanisms consist of two tiers, namely pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Chisholm et al., 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006; Pritchard and Birch, 2014). PTI represents the first tier of plant immunity and is conferred by pattern recognition receptors (PRRs) that recognize PAMPs (*e.g.* bacterial flagellin) or endogenous elicitors, termed damage-associated molecular patterns (DAMPs) (*e.g.* systemin, AtPep1), generated by pathogen assaults (Boller and Felix, 2009; Macho and Zipfel, 2014; Zipfel, 2014). On the other hand, ETI is typically mediated by nucleotide binding (NB)-leucine rich repeat (LRR) receptors (NLRs) (Cui et al., 2015; Takken and Goverse, 2012; Wu et al., 2014).

PTI in plants is the primary layer of resistance mechanism utilizing pattern recognition receptors (PRRs) (**Fig. 1.1**) (Macho and Zipfel, 2014; Zipfel, 2014). The perception of PAMPs by PRRs triggers a cascade of intracellular signaling leading to gene expression of defense-related genes. PRRs contain extracellular and intracellular domains connected by a single-pass transmembrane domain (Liu et al., 2014; Schwessinger and Ronald, 2012). The extracellular domains are ligand-binding domains that can vary from leucine-rich repeats (LRRs), LysM motifs, and EGF-like. LRRs include the *Arabidopsis* and rice FLS2 and EFR,

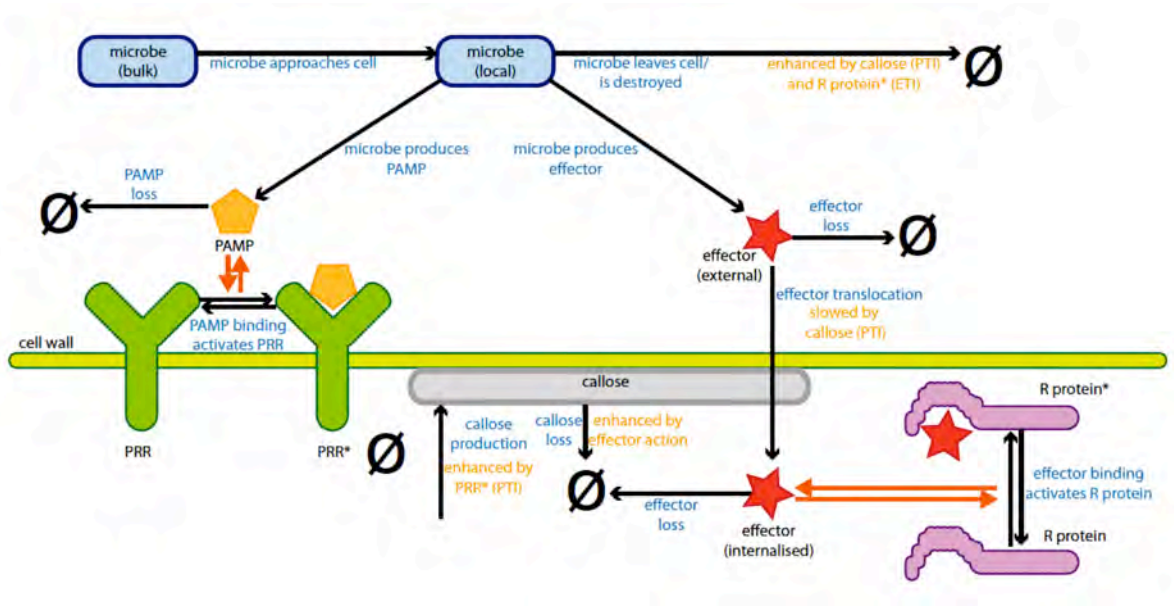


Figure 1.1. The model plant immune system. The system is divided into extracellular and intracellular compartments in reference to cell wall. In the extracellular compartment, the local microbial population derived from a remote bulk population is 'destroyed' as indicated by the arrow pointing to the empty set symbol (Φ). The rate at which the microbe is 'destroyed', as either microbial movement or death, is enhanced by pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). While local to the plant, the microbe produces two species namely pathogen-associated molecular patterns (PAMP) and effectors. These species can be lost by diffusion or destruction in the extracellular compartment. The PAMP may bind reversibly with plant pattern-recognition receptor (PRR) to produce an activated PRR* species. The effector may be translocated into the cell and may interact reversibly with plant R protein to produce an activated R protein* species. In the intracellular compartment, the presence of an activated PRR* induces the production of callose as a proxy for PTI activity. PRR* in this case is degraded within the plant cell. As a proxy for PTI, callose prevents the increase of local microbial population, and it also acts to reduce the rate of effector translocation into the cell. Activated R protein*, also prevents the increase of local microbial population, as a form of abstraction of ETI. Figure and description were adapted with modifications from Pritchard and Birch (2014).

rice XA21 and tomato EIX. *Arabidopsis* CERK1, LYM1 and LYM3 as well as rice CEBiP, LYP4 and LYP6 are LysM motif-containing binding domains. WAK1 in *Arabidopsis* is the

only EGF-like representative identified so far. These PRRs are mainly differentiated by the structure of their intracellular domain. Receptor kinases (RKs) have non-RD/RD kinase domain and a C-terminal tail, while receptor-like proteins (RLPs) simply have C-terminal tail attached to a single-pass transmembrane domain (Bohm et al., 2014; Macho and Zipfel, 2014; Schwessinger and Ronald, 2012). PRRs are capable of recognizing highly conserved ligands from different microbial pathogen sources. *Arabidopsis* FLS2 receptor binds to flagellin or the epitope flg22 (Chinchilla et al., 2006; Chinchilla et al., 2007); although rice can recognize different regions of flagellin, it showed weak perception of flg22 (Felix et al., 1999; Takai et al., 2008; Takai et al., 2007). It is also interesting to note that Ax21-derived peptides are recognized in *Arabidopsis* via the FLS2 receptor (Danna et al., 2011). EFR in *Arabidopsis* recognizes the elongation factor thermo unstable (EF-Tu) and the epitope elf18; however rice EFR can only recognize EF-Tu (Furukawa et al., 2014; Zipfel et al., 2006). The ligand for rice XA21 receptor is reported to be the *Xoo* protein, RaxX (Pruitt et al., 2015), while EIX in tomato perceives ethylene-inducing xylanase (EIX) signatures (Ron and Avni, 2004). LysM motif-containing domains in *Arabidopsis*, AtCERK1/LysM RLK1/AtLYK1 and AtLYK4-5, bind chitin (Cao et al., 2014), while AtLYM1 and AtLYM3 in conjunction with AtCERK1 sense bacterial peptidoglycans (PGNs) (Miya et al., 2007; Wan et al., 2008; Willmann et al., 2011). In rice, OsCEBiP binds chitin and forms a complex with OsCERK1, whereas both OsLYP4 and OsLYP6 bind chitin and PGNs (Hayafune et al., 2014; Kaku et al., 2006; Liu et al., 2012a; Shimizu et al., 2010). However, bacterial pathogens release proteins called effectors that suppress PRR triggered immune signaling, which bypasses PTI. In such cases, ETI is executed via species-specific R-gene- mediated resistance.

ETI acts as the secondary layer of resistance mechanism and is mainly composed of nucleotide binding-leucine rich repeat (NB-LRR) R proteins (**Fig. 1.1**) (Cui et al., 2014). These proteins are encoded by polymorphic resistance loci R-genes distributed in the cell as

intracellular receptors. Structural analysis of R proteins showed that they contain four major domains namely the N-terminal, nucleotide binding (NB), leucine-rich repeat (LRR), and the C-terminal extension domains (Takken and Goverse, 2012; Wu et al., 2014). NB and LRR domains are highly conserved domains in majority of R proteins, while the N- and C-terminal domains are highly variable and the bases for classifying R protein types. N-terminal domain is either coiled-coil (CC) or Toll-interleukin 1 receptor (TIR), whereas C-terminal is either present or absent (Panstruga et al., 2009; Wu et al., 2014). Examples of well-known CC-NB-LRR proteins are RPS2 (Axtell and Staskawicz, 2003; Leister et al., 1996; Mackey et al., 2003), RPM1 (Boyes et al., 1998; Gao et al., 2011; Leister et al., 1996), and MLA (Halterman et al., 2001; Shen et al., 2007). PRF CC-NB-LRR has an extra SD domain in its N-terminal region (Gutierrez et al., 2010). Another class of NB-LRR is the TIR-NB-LRR represented by RPS4 (Gassmann et al., 1999; Wirthmueller et al., 2007), N (Whitham et al., 1994), and L6 (Dodds et al., 2004; Ellis et al., 1999) R proteins. RRS1-R is a TIR-NB-LRR protein whose C-terminal domain contains WRKY extension (Deslandes et al., 2002). Differences in the presence of these types of NB-LRR proteins were found to be partially conserved in two major plant lineages, monocotyledonous and dicotyledonous plants, such that CC-NB-LRR were found in both lineages, whereas TIR-NB-LRR was found only in dicotyledons (Jacob et al., 2013; Yue et al., 2012). A survey analysis done by Monosi et al. (2004) showed that rice genome completely lacks TIR-like NB-LRRs, but contains unique NB-LRRs whose N-terminal domains remain poorly understood. These plant R proteins recognize their cognate microbial effectors known as avirulence (Avr) proteins typically delivered via type III bacterial secretion system (T3SS) (Cui et al., 2014; Galan et al., 2014).

PTI is characterized by transcriptional reprogramming and increased expression of pathogenesis-related genes (*e.g. PR1*) and salicylic acid (SA)-induced genes as well as physiological hallmarks including oxidative burst, deposition of callose, ethylene production,

and alkalinization (**Fig. 1.1**) (Boller and Felix, 2009; Felix et al., 1999; Gomez-Gomez and Boller, 2002). It is also known that PTI can inhibit growth if exposed at a particular concentration of flg22 peptides (Gomez-Gomez et al., 1999). PTI operates primarily by utilization of its extracellular leucine-rich repeat domains (LRR) that bind to epitope-regions of certain microbial PAMPs (Macho and Zipfel, 2014; Monaghan and Zipfel, 2012; Panstruga et al., 2009; Zipfel, 2014). This then generates further molecular reorganization (*e.g.* FLS2-BAK1 or EFR-BAK1 interaction) and transmission of signals downstream of these receptors (Panstruga et al., 2009; Zipfel, 2014). In the case of ETI, a few of the PTI-related physiological features were also observed including the ETI-associated hypersensitive response (HR) characterized by programmed cell death (PCD) of the locally infected tissues (**Fig. 1.1**) (Dangl et al., 1996). In some cases, intracellular R proteins are activated by ADP binding and its NB-LRR interacts with its cognate effectors in cases of direct physical recognition (Caplan et al., 2008; Lukasik and Takken, 2009; Takken et al., 2006; Wu et al., 2014). Indirect interaction utilizes the N-terminal domain along with a host factor. During HR-PCD, the hormone salicylic acid (SA) seemed to co-regulate with ROS and nitrogen oxide (NO) at the locally infected site eventually leading into systemic induction of defenses (Coll et al., 2011; Wu et al., 2014). The SA-mediated defense appears to be regulated by the key transcriptional coregulator and central redox switch, Non-Expressor of PR1 (NPR1) (Fu and Dong, 2013; Mukhtar et al., 2009; Spoel and Dong, 2012; Spoel et al., 2009).

Hormone-mediated signaling in plants plays a very important role in innate immunity. In rice, the phytohormones salicylic (SA) and jasmonic acid (JA) as well as ethylene (ET) regulate defenses against biotrophic, hemibiotrophic, and necrotrophic bacteria (De Vleesschauwer et al., 2013; Yang et al., 2013). In *Arabidopsis*, there is a relatively clear distinction of roles modulated by SA and JA signaling during pathogenesis. SA most likely regulates immunity against biotrophic pathogens, while JA against necrotrophic pathogens.

However, in rice there is no clear distinction of SA and JA's roles in immunity, such that both of these phytohormones tend to regulate as well as uniquely co-regulate defenses against (hemi)biotrophic and necrotrophic pathogens. Another feature that is unique in rice is that SA tends to be maintained at high levels (Chen et al., 1997; Silverman et al., 1995). Endogenous SA accumulation though was not observed during pathogen infection, but only during exogenous SA-treatment (Silverman et al., 1995). This implies that rice immunity via SA-pathway is not dependent on SA level, but rather SA signaling. JA-mediated defense, on the other hand, is dependent on rice *Coronatine Insensitive 1* (*COI1*), a major JA receptor. It is a major player in defense responses indeed that Os*COI1* was found to be indispensable in the activity of *OsNPR1* (Yang et al., 2013).

Plant immunity is characterized by such multilayered structures, which likely enable fine-tuning of defense responses. Fine control of receptor-mediated pathogen recognition and defense signaling downstream of the receptor are fundamental to avoid precocious activation of immune responses that negatively influence plant growth. How do plants mount effective immune response at a minimal fitness cost?

1.2 The plant systemic immune defense signaling and priming

Local defense response stimulated by recognition of microbial PAMPs or effectors is the first step in developing defense priming in plants. This event will trigger signals delivered systemically in plant cells resulting in a broad-spectrum immunity called systemic acquired resistance (SAR) (Conrath, 2011). SAR acquired from pathogen-challenged site primes non-challenged sites and mounts a defense state termed as defense priming. Defense primed plants are typically characterized by having a swift defense-response compared to unprimed plants and relies on mobile immune signals released from local infection to establish systemic immunity. Mobile signals are either proteins or molecules, which are lipid-derived or

hormone-like (*e.g.* methylsalicylic acid (MeSA), azelaic acid, or G3P) and chemical species (*e.g.* reactive oxygen species (ROS), or Ca) that prompts salicylic acid (SA) production effecting antimicrobial activity, transgenerational immune memory, and priming of immune-related genes (Kachroo and Robin, 2013; Spoel and Dong, 2012; Vlot et al., 2017). The lipid-derived phytohormone, jasmonic acid (JA), has also been implicated as a mobile signaling hormone required for systemic acquired resistance (SAR) in plants (Fu and Dong, 2013; Gao et al., 2015; Shah, 2009; Truman et al., 2007; Vlot et al., 2017). Although, its role in SAR is still debatable since JA-pathway compromised *Arabidopsis* mutants, *opr3* and *jin1*, and JA-insensitive mutants, *coi1* and *jar1*, present a functional SAR during pathogenesis (Attaran et al., 2009; Shah, 2009). In addition, petiole exudates from leaves of plastid desaturase mutant, *fad7*, which provides fatty acids for JA synthesis, showed that JA did not co-purify with SAR-inducing activity in this mutant (Chaturvedi et al., 2008; Shah, 2009).

Plant vascular tissues such as xylem and phloem are instrumental in inducing SAR (Notaguchi and Okamoto, 2015). Most notably, the accumulation of phytohormones in phloem vasculature is a critical step in relaying information on the state of local tissues during pathogenesis to distal tissues. Systemic gene expression is characterized by transcriptional reprogramming of SA-independent and SA-dependent genes involved in pathogen responses such as *NPR1* (non-expressor of pathogenesis-related 1), transcription factors, flavin-dependent monooxygenase (*FMO1*), disease resistance genes, genes that modify cell wall, fatty acid and secondary metabolism gene networks, and others (Bernsdorff et al., 2015; Kachroo and Robin, 2013; Schenk et al., 2003). It is also important to note that chemicals such as phytohormones are not traveling alone during long distance systemic signaling. Electrical and hydraulic signals as well as mobile RNAs are essential components of systemic signaling to enable systemic acquired resistance (Ham and Lucas, 2017; Huber and Bauerle, 2016).

Defense priming is an important component of systemic acquired resistance. Mobile immune signals traveling from the local tissues to distal parts of the plants induce the accumulation of phytohormones in distal tissues setting in motion the regulatory mechanisms of immune memory, thus 'priming'. However, it is necessary to fulfill basic experimental requirements before defense priming can be established (**Fig. 1.2**) (Martinez-Medina et al., 2016). Defense priming needs to be evaluated based on the presence of immune memory, a lower fitness costs during stresses, a more robust defense state, and better performance gaining obvious advantages during and after pathogen stress. First, immune memory is usually stored after plants are exposed to stresses or any form of priming stimulus. This plant memory or the 'primed state' could be characterized by elevated levels of defense receptors or transcription factors, attenuated DNA methylation, and regulated chromatin modification. The 'primed state', therefore, is a poised state where defense gene promoters are induced yet nearly at the basal expression. However, in the event of a second stress or a triggering stimulus, primed plants can activate a more intense, likely sustained, and faster defense responses. Second, unlike the cost of the initial basal defenses deployed at the local site, the systemic defenses of 'primed' tissues are less costly and yet more effective. This is due to the physiological alterations conferred by the poised state to primed plants as compared to unprimed. The physiological alterations are the molecular and biochemical remodeling events stored at distal tissues post local infection. Direct activation of defenses are typically costly resulting in impaired growth and reproduction (Karasov et al., 2017). An example would be the constitutive immune response of *Arabidopsis NPR1* in transgenic rice, which resulted in stunted growth in the absence or presence of a pathogen (Bailey-Serres and Ma, 2017). Defense priming mitigates the costly tradeoff of deploying full-scale defense whenever there is pathogen infection, thus enabling plants to allocate resources for growth and seed production. Third, primed plants have robust defenses during the secondary attack as

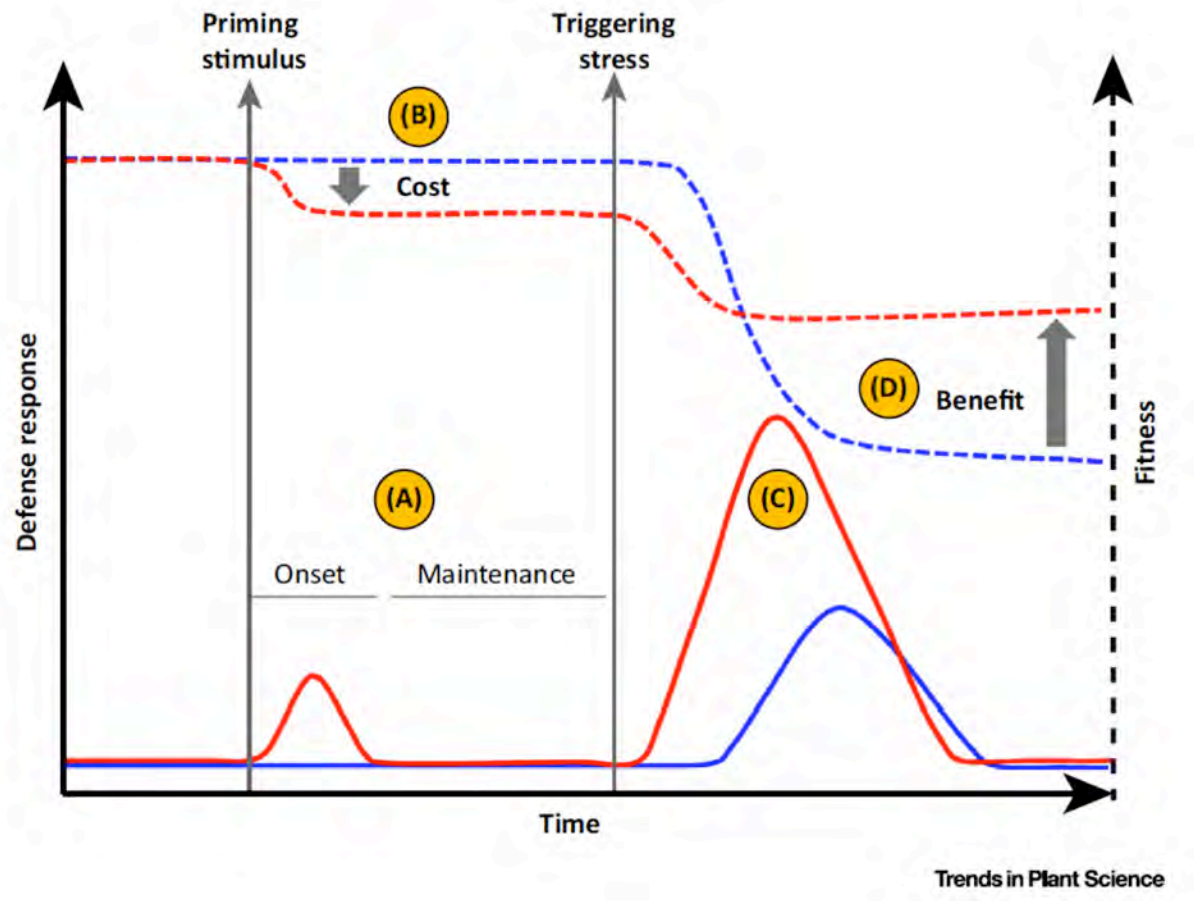


Figure 1.2. Diagram showing the relation between defense responses (solid lines) and fitness (dashed lines) in primed (red) versus unprimed (blue) plants. Defense priming is evaluated based on plant defenses and the associated cost-benefit balance. The criteria below may help in deciding whether defense priming is present or not: (A) Memory: two sequential environmental events (*e.g.* priming stimulus and triggering stress) are required for asserting memory in the absence of molecular markers. During priming and in the primed state, which is before the triggering stress, plant defenses are expected to be only transiently and generally faintly induced. (B) Low fitness costs: the maintenance of the primed state, which is before the triggering stress, has low fitness costs compared with the direct activation of defense. (C) A more robust defense response: after the triggering stress is applied, primed plants exhibit a faster, earlier, stronger, and/or more sustained cellular defenses than do unprimed plants. (D) Better performance: primed plants are expected to defend successfully against a given stressor than unprimed plants. Therefore, priming enhances plant fitness in stressful environments. Figure and description were adapted with modifications from Martinez-Medina et al. (2016).

compared to the initial infection. These responses are a combination of highly modulated activation and repression of defense response genes and gene networks, signaling compounds and biosynthetic pathways, or defense metabolites broadly targeted to sources of stresses. Lastly, better performance of primed plants indicate that the broad spectrum nature of defenses are fine-tuned such that one defense mechanism does not compromise defenses of another nature at a different period of time. Such coordinated defenses that builds on the previous infection memory is the most effective and least costly defense to plant fitness.

How then does a defense priming work? There are several proposed mechanisms of establishing defense priming in plants and this introduction will focus next on the epigenetic nature of defense priming and memory.

1.3 Epigenetic mechanisms that control defense signaling and priming in plants

1.3.1 DNA methylation: A dynamic regulator of defense genes

Cytosine methylation of the DNA bases in all sequence contexts, CG and non-CG (CHG and CHH, where H is non-G), is triggered by small interfering RNAs (siRNAs) via a *de novo* methylation pathway termed RNA-directed DNA methylation (RdDM). Canonical RdDM begins by production of RNAs by Polymerase (Pol) IV via nuclear RNA polymerase D (NRPD) subunits, and after several processing steps, the processed RNAs are loaded into argonaute 4 (AGO4) and base-paired with an RNA scaffold produced by Pol V. Recruitment of AGO4 involves its interaction with nuclear RNA polymerase E1 (NRPE1) of Pol V. Subsequent interaction with domains rearranged methyltransferase (DRM) leads to methylation of DNA target sequences. On the other hand, in the non-canonical Pol II-RDR6-dependent RdDM pathway, Pol II-transcribed single-stranded RNA (ssRNA) is converted into double-stranded RNA (dsRNA) by RNA-dependent RNA polymerase 6 (RDR6), and then processed into 21-22nt siRNA. The siRNA is loaded into AGO6 that can be directed to

the scaffold RNA transcribed by Pol V, which establishes DNA methylation. These methylation marks are maintained through mitosis and meiosis via a pathway catalyzed by methyltransferase 1 (MET1) and chromomethylase 3 (CMT3) methyltransferases, while repressor of silencing 1 (ROS1), demeter-like 2 (DML2) and DML3 are DNA glycosylases that dynamically erase DNA methylation via a base excision repair process (details of the RdDM pathway are referred to Du et al., 2015; Law and Jacobsen, 2010; Matzke et al., 2015; Matzke and Mosher, 2014). DNA methylation is a vital process that is also linked to other epigenetic pathways, such as histone methylation and acetylation (Du et al., 2015; Eden et al., 1998; Qian et al., 2012).

Recent studies have extended our understanding of epigenetic control of plant immunity (Alvarez et al., 2010; Ding and Wang, 2015; Sahu et al., 2013; Saijo and Reimer-Michalski, 2013). High-resolution DNA methylation profiling by Dowen et al. (2012) provides the first genome-wide insight into biotic stress-responsive genes in *Arabidopsis*, expression of which is modulated by DNA methylation and demethylation. *met1-3* and *ddc* (*drm1-2 drm2-2 cmt3-11*) plants that are globally defective in maintaining CG and non-CG methylation, respectively, show enhanced defense responses when exposed to *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000). The same results were obtained in mutants partially defective in CG and non-CG methylation. Moreover, in rice, application of 5-azadeoxycytidine, a DNA demethylating agent, enhances bacterial resistance to *Xanthomonas* (Akimoto et al., 2007). These results are consistent with findings that enhanced RdDM in *ros1-4* plants leads to lowered resistance to *Pst* DC3000 (Yu et al., 2013). In addition, flg22 treatment results in inhibition of transcriptional gene silencing (TGS) as it de-represses RdDM targets. Yu et al. (2013) also confirmed increased bacterial resistance in *ddc* and *met1 nrpd2* plants. *met1 nrpd2* plants also exhibit hypersensitivity response (HR)-like cell death and high *PR1* expression, pointing to de-repression of ETI-like defenses. Furthermore, *ros1 dml2 dml3*

(*rdd*) plants, simultaneously disrupted for the three DNA demethylases, show lowered fungal resistance (Le et al., 2014).

Pol V, but not Pol IV, has been implicated in plant immunity (Lopez et al., 2011; Matzke and Mosher, 2014). However, Le et al. (2014) showed an overlap of down-regulated genes between *rdd* and the RdDM mutants, *nrpe1* and *nrpd1*, suggesting that Pol V and Pol IV both regulate defense responsive genes. In addition, fungal infection is enhanced in *nrpe1* and *ago4* plants, while it is slightly reduced in *nrpd1* plants. These results clearly suggest that genome-wide disruption of DNA methylation leads to defense activation, in a way reminiscent of ETI, and that DNA methylation down-regulates immune responses. However, this is not the case for all defense-related genes, as evident in the blast resistance gene, *Pib*, in rice (Li et al., 2011b) and in the genome-wide methylation analysis of tobacco plants infected with *Tobacco mosaic virus* (TMV) (Kathiria et al., 2010). Future investigation will be required to determine whether RdDM pathways play a distinctive role in different plant species, between different target genes, against different pathogens or combinations thereof. It is of particular importance to elucidate the regulatory components, the mode of control, and specific target sites in the genome for canonical and non-canonical RdDM pathways in plant immunity, not only in *Arabidopsis* but also in other plant models.

These *Arabidopsis* studies also offer insight into methylation states in plant genomes and how changes influence immune responses. In response to *Pst* challenge or flg22 application, DNA methylation levels are globally reduced in all sequence contexts, while the decrease following SA application is restricted to CG and CHG contexts (**Fig. 1.3**). Intergenic transposable elements (TEs) seem to be among the main targets for both canonical and non-canonical RdDM pathways during pathogen challenge. Stress-associated differential methylation in the CG context occurs predominantly ~1 kb upstream of transcriptional start sites (TSS) for protein-coding genes, whereas such methylation in the CHH context occurs

high in intergenic regions. Differential methylation in both contexts is over-represented at both ends of protein coding genes. *At3g50480*, a locus encoding a homolog of RPW8 disease-resistance (R) protein, undergoes differential methylation changes during pathogen infection. Another R gene, *RMG1* (*At4g11170*), is highly induced in response to flg22 and in *met1 nrpd2* plants, while it is compromised in *ros1* plants in which TSS-flanking regions are highly methylated. In *rdd* plants, TEs inserted adjacent to or within 200 bp of promoters and gene bodies, represent major targets of methylation. It is important to note that not only TEs, but also sequences surrounding them are methylated. This is particularly true for those inserted in promoter regions, as shown for *CC-NBS-LRR (At1g58602)* and *jacalin lectin (At5g38550)*. Work on cytosine DNA methylation (mC) in rice and *Arabidopsis* also indicates that proximal regions of TEs, when they are within or in proximity to stress-inducible genes, play a critical role in responsiveness to environmental stress cues (Secco et al., 2015). These findings suggest that regulatory processes modulating methylation at or near gene boundaries, particularly in *R* gene loci, help to fine-tune defense responses, at least in these plant models. Future studies will be required to determine the precise function of these DNA sequences and the molecular mechanisms underlying their recognition and modification.

1.3.2 Transposable elements (TEs) in plant immunity

A major class of R proteins are the NLR immune receptors that mediate ETI to various pathogens. *NLR* genes often form gene clusters in the genome that contain repetitive sequences and TEs (Meyers et al., 2003). The repetitive nature of *NLR*-gene clusters is thought to facilitate rapid expansion and sequence diversification of these genes, possibly by promoting unequal recombination (Friedman and Baker, 2007). It is well documented that TEs inserted in the promoter region often regulate neighboring genes in both animals and

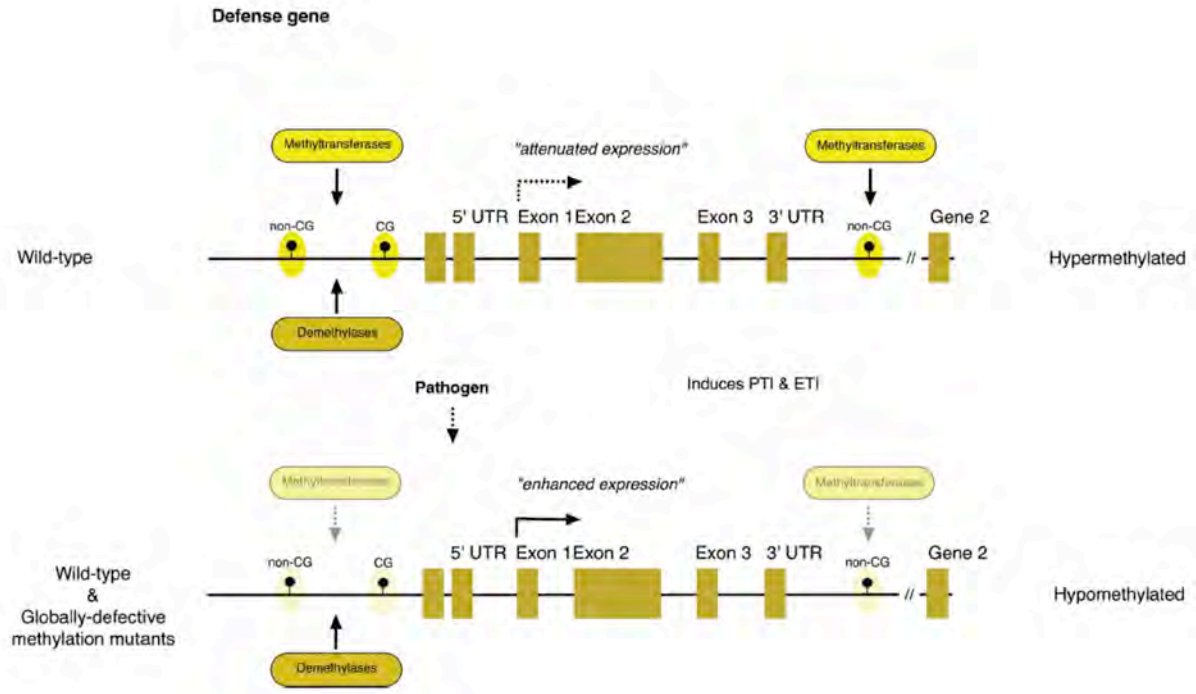


Figure 1.3. A general model of epigenetic regulation of defense-related genes. Hypomethylation of regions flanking both ends of defense-related genes enhances their expression during pathogen challenges. Filled lollipops indicate transposable elements (TEs) or repetitive elements that may be methylated or de-methylated. Figure and description were adapted from Espinas et al. (2016).

plants by changing their epigenetic states (Slotkin and Martienssen, 2007). A recent report shows that TEs in intronic regions can regulate *NLR* expression in *Arabidopsis* (Tsuchiya and Eulgem, 2013). *Arabidopsis RPP7* encodes a CC-NBS-LRR class of NLR that confers resistance to downy mildew, *Hyaloperonospora arabidopsidis* (*Hpa*) (Eulgem et al., 2007). Proper transcription and splicing of *RPP7* requires a protein named ENHANCED DOWNY MILDEW2 (EDM2), which encompasses PHD domains that recognize H3K9 methylation and a putative RNA methyltransferase domain at the C-terminus (Lei et al., 2014; Tsuchiya and Eulgem, 2014). In the *edm2* mutant, transcription of *RPP7* is attenuated due to premature

termination of the transcripts at the TE within the 1st intron, termed *ECL* (exon 1-containing LTR-terminated transcript). Interestingly, intronic TEs, including COPIA-type retrotransposon in the 1st intron, are targeted by repressive epigenetic marks, such as DNA methylation and H3K9 methylation, as are their intergenic copies, even though they are embedded within the actively transcribed gene unit (Saze et al., 2013; Tsuchiya and Eulgem, 2013). Maintenance of repressive epigenetic marks in intronic TEs seems to be important for proper expression of *RPP7*, since RPP7-mediated ETI to *Hpa* is impaired in plants deficient for H3K9 methylation, recapitulating the immuno-compromised phenotype of *edm2* plants. Similarly, reduced DNA methylation in *DECREASE IN DNA METHYLATION1* (*DDM1*) mutants or *CMT3* results in a transcription defect of *RPP7* (Le et al., 2015). Interestingly, even though *RPP7* shows sequence polymorphism among different *Arabidopsis* accessions due to TE insertions within intronic regions (Tsuchiya and Eulgem, 2013), most of these natural accessions harbor the COPIA element in the 1st exon. This implies that TE insertion has selective advantages, possibly by providing a fine-tuning mechanism for *RPP7* expression (McDowell and Meyers, 2013). As reported, epigenetic states of TEs are dynamically altered in response to biotic stress (Dowen et al., 2012). Epigenetic control of intragenic TEs may thus act as a regulatory mechanism for *NLR* gene expression in plant-pathogen interactions.

1.3.3 Histone modification and its role in systemic acquired resistance

Defense activation at recognition sites to PAMPs or effectors generates and delivers systemic signals throughout the plant, which result in enhanced immunity to a broad spectrum of pathogens, called systemic acquired resistance (SAR) (Conrath, 2011; Conrath et al., 2015; Fu and Dong, 2013; Kachroo and Robin, 2013). During and after SAR, defense-related genes become sensitized to subsequent pathogen attack at distal, non-challenged sites, known as

defense priming. Defense-primed plants are enabled to mount a swift defense response, which involves "kick starting" of up- and down-regulation for priming target genes.

Among potential mechanisms underlying defense priming, histone modifications are of particular interest since they affect the landscape of transcription of defense-related genes through evolutionarily highly conserved functions (Waterborg, 2011). Recent studies in plants have implicated H3K4me3, H3K4me2, H3K9ac, H4K5ac, H4K8ac, and H4K12ac in defense priming. In particular, H3K4me3 is considered as a primary chromatin marker of stress memory (Conrath et al., 2015). Recent studies on heat stress acclimation in *Arabidopsis* present a model in which transient binding of the heat-inducible transcription factor HSFA2 leads to sustained H3K4 methylation and thus the maintenance of heat stress memory, *i.e.* acquired thermotolerance (Lamke et al., 2016). Notably, HSFA2 function is dispensable for the acquisition of thermotolerance *per se*, but indispensable for its maintenance (Charng et al., 2007). On the other hand, Mozgová et al. (2015) have shown that the histone chaperone, CAF-1, mediates a repressive chromatin state of defense genes, by retaining nucleosome occupancy and suppressing H3K4me3 marking. However, loss of CAF-1 alone is insufficient to activate SA-related defense genes. These findings suggest that CAF-1-conditioned chromatin modification prevents inappropriate defense activation. Further investigation will be required into the mechanisms by which defense signaling triggered upon pathogen recognition overcomes this barrier and leads to a priming state, partly through increasing H3K4me3 deposition, at both challenged and non-challenged sites.

Histone acetyltransferases (HATs) and deacetylases (HDACs) also participate in control of defense priming. *hac1-1* (histone acetyltransferase 1) plants are compromised in bacterial resistance and defense priming following PTI (Singh et al., 2014). This is the first evidence that an HAC1-dependent pathway is responsible for defense priming after exposure to

recurring abiotic stress cues. HAC1 does not seem to direct resistance to *Pst per se*, suggesting that HAC1 links recurring stress response activation to defense priming. It remains to be shown how HAC1 establishes the epigenetically primed states at open chromatin target sites. Consistent with a positive role for histone acetylation in defense activation, loss of HDAC19 results in de-repression of SA-based defenses (Choi et al., 2012) and depletion of the HDAC HDT701 enhances H4 acetylation and resistance to both fungal and bacterial infection (Ding et al., 2012).

It has been reported that defense priming and these histone marks are transgenerationally inherited (Crisp et al., 2016; Heard and Martienssen, 2014; Iwasaki and Paszkowski, 2014; Kinoshita and Seki, 2014; Lamke and Baurle, 2017). A study in yeast has proven for the first time that H3K9 methylation is heritable over several generations (Audergon et al., 2015). In addition, a very recent work of Jiang and Berger (2017) detailed a DNA-replication dependent inheritance of H3K27me3 in *Arabidopsis* possibly via a combination of self-propagation and *cis*-recruitment mechanisms (De and Kassis, 2017). Given the evolutionary conservation for functions of these histone marks, it is conceivable that histone modifications provide a basis for heritable immune response memory. At least for Polycomb-mediated silencing in flowering locus C (FLC) of *Arabidopsis*, physical presence of memory at local chromatin states has been established already (Dean, 2017).

A subset of, if not all, defense genes activated in SAR, seems to be primed as a consequence of interplay between different histone modifications, via mechanisms that are still poorly understood (Conrath, 2011; Conrath et al., 2015; Ding and Wang, 2015; Gutzat and Mittelsten Scheid, 2012; Saijo and Reimer-Michalski, 2013; Spoel and Dong, 2012). Priming of defense-related genes has a fitness advantage compared to their substantial activation (van Hulten et al., 2006). It is tempting to speculate that this has contributed to the

evolution of genomic regions that undergo histone modifications to establish such a priming state at target genes, which enables effective transcriptional reprogramming toward enhanced resistance in response to second challenge. In animals, enhancer and promoter sites are often marked with H3K4me1/H3K27ac and H3K4me3/H3K27me3, respectively (Azuara et al., 2006; Bernstein et al., 2006; Calo and Wysocka, 2013; Voigt et al., 2013; Zhou et al., 2011) (**Fig. 1.4**). These combinatorial histone marks can occur in a gene-autonomous manner, and seem to exert complex regulatory effects, as is the case of H3K4me3/H3K27me3 in the promoter region (called a bivalent promoter)(Bernstein et al., 2006). It should be noted, however, that bivalency is not restricted to narrow genomic regions, as enhancers can influence target genes as much as a million bases distant (Pennacchio et al., 2013). Thus, cautions need to be taken when considering bivalency, which can occur at the same nucleosome unit harboring two antagonizing marks in different histone molecules or in one histone molecule (*e.g.* H3K4me3/H3K27me3 in promoters; **Fig. 1.4A**), or in separate nucleosome units (*e.g.* H3K27me3/H3K27ac in promoters and enhancers, respectively; **Fig. 1.4B**). In acclimation to abiotic stress, an increase of transcription-permissive H3K4me3 occurs when plants are exposed to recurring stress cues without removing transcription-repressive H3K27me3 (Avramova, 2015; Saleh et al., 2007). Given that not only pathogen recognition, but also adverse abiotic conditions can induce defense priming in plants (Singh et al., 2014; Vivancos et al., 2015), it is of high interest to test whether bivalent histone modification also plays a role in defense priming. Future studies will be required to clarify the functional significance of bivalent modification, which may be distinct from that of either transcription-permissive or -repressive modification alone.

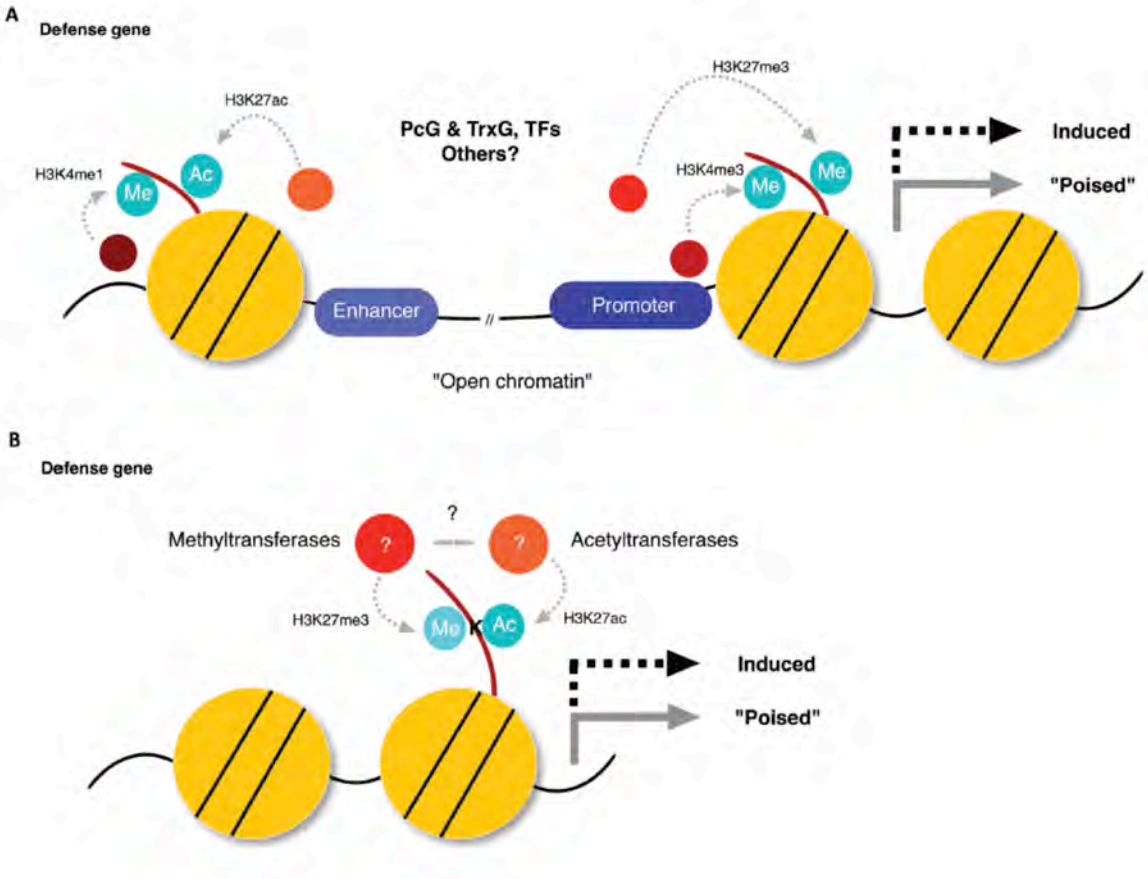


Figure 1.4. Bivalent phenomena hypothesis in plant defense priming. (A) An interplay of opposing histone modification marks in enhancer and promoter regions modulate the expression status of defense-related genes. Polycomb-group (PcG) and Trithorax (TrxG) proteins may assemble with interacting proteins, such as transcription factors (TFs). (B) Opposing histone marks on the same lysine-site (*K*-site) act as a switch to modulate the expression status of defense-related genes. Figure and description were adapted from Espinas et al. (2016).

1.4 Histone acetylation and transcription

Acetylation is a posttranslational modification on protein substrates via the introduction of an acetyl group through covalent bonding. The acetylation reaction is highly reversible and is catalyzed by members of lysine acetyltransferases families, KATs, (*i.e.* also known as histone acetyltransferases, HATs, for histone-specific acetylation events) localized in cytoplasm and

in nucleus (as reviewed in Sadoul et al., 2011; Wang et al., 2011; Yang, 2004). About 50 years ago, acetyl groups were first detected in a very rich (F1) to slightly (F2a) lysine-rich histone fractions of calf thymus (Phillips, 1963). Afterwards, the role of histone acetylation on gene expression was first demonstrated by the binding of purified histone proteins with DNA sequence *in vitro* and by showing that this binding manifests inhibitory effects on transcription (Allfrey et al., 1964). Thus, it was clear from these experiments and the succeeding findings that transcriptional regulation of the gene in eukaryotic cells is regulated by protein acetylation modification on histones (Gershey et al., 1968; Pogo et al., 1966). However, it took another 30 years to provide a causal relationship linking histone acetylation and transcription by isolating the first nuclear protein, histone acetyltransferase A (HAT A), a *Tetrahymena*-derived protein homologous to yeast Gcn5p with acetyltransferase activity able to modify histones and thus affecting DNA transcription (Brownell et al., 1996). Prior to this discovery, functional significance for site-specific acetylation especially on histone tails was investigated in histone H4 by producing antisera that recognize various acetylated histone sites (Turner, 1993; Turner et al., 1992). It turned out that acetylation on H4 sites 5, 8, 12, and 16 are distributed uniquely, such that H4K5ac and H4K8ac are dispersed on four chromosomes, H4K12ac is distributed on heterochromatin region, and H4K16ac is found mainly in the X-chromosome of *Drosophila* genome. Kuo et al. (1996) also showed that Gcn5p enzyme has preferred acetylation sites on H3 and H4 histones albeit exhibiting a certain degree of functional specificity. After the discovery of HAT A, the yeast transcriptional coactivator, Gcn5p, was also found to exhibit histone acetyltransferase activity targeted at gene promoters necessary for transcriptional activation (Kuo et al., 1998). In addition, multiple substitution mutations on Gcn5 abolished its ability to enhance gene expression in Ada and SAGA complexes (Wang et al., 1998). Studies on yeast and chicken β-globin have also shown that acetylation is an important component for regulating gene

transcription on certain histone domains, as well as for maintenance of the open conformation of chromatin allowing action by the transcription initiation complex (Braunstein et al., 1993; Durrin et al., 1991; Hebbes et al., 1994; Hebbes et al., 1988). Overall, these findings suggest that *in vitro* preliminary studies on the ability of histone acetylation to regulate transcription is strictly correlated with transcriptional activity *in vivo*. For histone acetylation to be dynamic in nature, evidence was needed for the subsequent removal of acetylation marks. Around the time of the discovery of the first histone acetyltransferase, the first deacetylation enzyme in yeast, Rpd3p, was discovered (Taunton et al., 1996). These series of events established the mechanistic role of a reversible histone acetylation reaction in remodeling chromatin structure that in turn affects gene transcription (Jenuwein and Allis, 2001). The modulation of the chromatin structure through histone acetylation as one of the many posttranslational modifications correlates with gene regulation in the cell (Lee and Workman, 2007; Li et al., 2007).

1.5 Histone acetylation in plants

Transcriptional eukaryotic regulatory mechanisms in plants are conserved and resemble that of yeasts and animals (Yilmaz and Grotewold, 2010). An important component of this similarity is evidenced by sequence conservation of the core histones in both plants and animals. Sequence comparison of histone H3 and H4 in these organisms revealed a highly conserved sequence in which they differed only at two H4 residues, I60 and R77 in plants *versus* V60 and K77 in animals, respectively. Histone H3 also showed comparatively conserved sequences with a few residue substitutions (**Fig. 1.5**) (Waterborg, 2011). In addition, plants and animals show identical acetylation pattern of H4 isoforms, where transcriptionally active gene regions contain H4 with extensive acetylation at sites H4K5, H4K8, and H4K12, while heterochromatic regions are associated with underacetylated H4

(Belyaev et al., 1998). Histone acetylation modifications were earlier postulated to play a role in phaseolin (phas) gene transcriptional regulation, one of the best characterized model of transcriptional activation using French bean seeds (Li et al., 2001). Within plants, genome-wide investigation revealed a highly conserved posttranslational modification sites associated with transcriptional activation (H3K9ac and H3K27ac) and transcriptional competence (H3K56) (Charron et al., 2009; Wang et al., 2009; Zhou et al., 2010).

Historically, the use of Edman degradation protein sequencing on purified histones of peas (*Pisum sativum*) marked the start of studies on plant histone acetylation (Bonner et al., 1968; Fambrough et al., 1968). In plants, histone H3 was found to be highly acetylated (Waterborg, 1990). Previous work identified lysine-sites 4, 9, 14, 18, 23, and 27 of histone H3 as targets for acetylation, while H4 has five acetylated isoforms on sites K5, K8, K12, K16, and K20 (H4K20 is typically methylated in yeasts and animals) (Earley et al., 2007; Matthews and Waterborg, 1985; Waterborg, 1992; Zhang et al., 2007).

In plant model *Arabidopsis*, histone acetyltransferases (HATs) and deacetylases (HDACs) are grouped into four and three families, respectively. HATs consist of GNAT, MYST, CBP/p300, and TAF1/TAFII250, while HDACs are RPD3/HDA1, HD2-like, and SIR2. A previous review of HATs and HDACs in plants listed about 12 putative HAT and 18 HDAC proteins exhibiting acetylation/deacetylation activities with differential site specificities (Pandey et al., 2002). However, recent analysis showed reduction in number of these proteins to five HATs and nine HDACs only (Berr et al., 2011). *Arabidopsis* GCN5 is the most characterized member of the GNAT-family of plant acetyltransferases (Berr et al., 2011). As a HAT component of Ada and SAGA transcriptional adaptor complexes, it acetylates a number of lysine sites in histone H3 (Grant et al., 1997; Lee and Workman, 2007). At*gcn5* is characterized by pleiotropic effects with phenotypes ranging from dwarfism, loss of apical dominance, aberrant meristem function, root and leaf development phenotypes, short petals

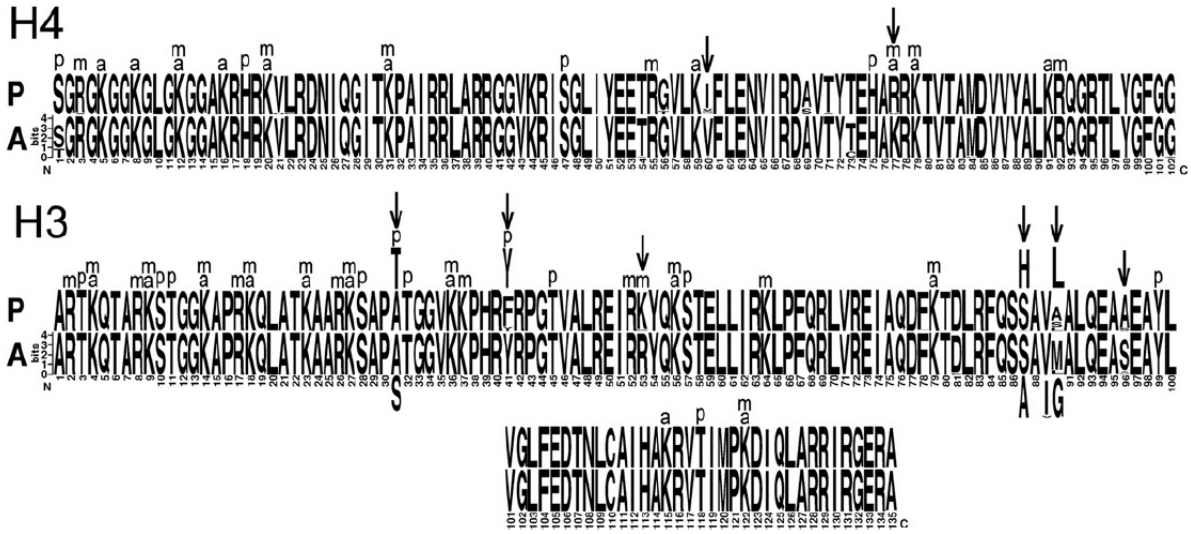


Figure 1.5. Sequence conservation of core histones H4 and H3 among plants and animals, illustrated by WebLogo. In the absence of any variability in the amino acid found at a sequence position, the LOGO algorithm displays the amino acid in the single letter code at full size. Any sequence variability reduces the size of the primary amino acid code(s). (H4 panel) 24 plant (P) and 26 animal (A) species, broadly representing viridiplantae and metazoan, were used to create the upper and lower scaled representations, respectively. Vertical arrows mark the distinctive sequence differences between plant and animal H4 histones. Sites of posttranslational modifications, observed within eukaryotic H4 proteins, are marked by 'a' for acetylation, by 'm' for methylation and by 'p' for phosphorylation. (H3 panel) 322 animal and 179 plant species were used. The amino acid sequence of the replication-coupled variants at positions 31, 41, 87, 89, and 90 is shown in line with the residues that are the same for the two H3 variant types. The amino acids in the replication-independent variants of plant and animal H3s are shown above and below the replication-coupled ones, respectively. Figure and description were adapted with modifications from Waterborg (2011).

and stamens, floral organ identity, and reduced expression of light and cold-inducible genes (as reviewed in Servet et al., 2010). Two *Arabidopsis* MYST-family members, HAM1 and HAM2, were shown to acetylate H4K5 *in vitro* (Earley et al., 2007) and mutation of MYST generally affected gametogenesis (Latrasse et al., 2008). CREB-binding protein (CBP)/p300-

family member, AtHAC1/PCAT2 *in vitro* acetylates lysine sites of the four core histone proteins (Bordoli et al., 2001). Functional mutagenesis of CBP/p300 affected *Arabidopsis* flowering time by modulation of flower repressor *Flowering Locus C*, *FLC,* gene (Deng et al., 2007; Han et al., 2007). HAF2, a TAF1/TAFII250-family member, was reported to acetylate H3 and/or H4 sites where *Arabidopsis* mutants exhibited reduction in chlorophyll levels, thus affecting light regulation and greening (Benhamed et al., 2006; Bertrand et al., 2005). Histone deacetylase RPD3/HDA1-superfamily member, AtHD1/HDA19/RPD3A, affects significantly the *in vitro* acetylation of histones H3 and H4 wherein *Arabidopsis* mutants exhibit accumulation of acetylation coupled with various developmental defects (*e.g.* early senescence, male and female sterility, *etc*.) (Tian and Chen, 2001; Tian et al., 2003). AtHDA19, an RPD3 homolog, affects acetylation levels in overexpression and RNAi plants and may regulate expression of genes of jasmonic acid and ethylene signaling pathways in response to pathogen infection (Zhou et al., 2005). Another deacetylase, AtHDA6/RPD3B, causes loss of promoter cytosine methylation and trimethylation of H3K4, acetylation of H3K9 and H3K14, as well as tetra-methylation of H4 sites (Earley et al., 2006; Probst et al., 2004). The study also implicated this HDAC in rRNA gene silencing characterized by decondensation of the nucleolus organizer region (NOR). It was also found to modulate transposable elements as it interacts with MET1 (Liu et al., 2012c), although other reports revealed that it minimally contributes to developmental processes evidenced by mutants' wild-type phenotype. SIR2-family of deacetylases is unique in the sense that it depends on NAD^+ for its catalytic activities (Imai et al., 2000; Xing and Poirier, 2012). Original work in yeast showed that SIR2 deacetylates K9 and K14 of histone H3 and K16 of H4 with silencing activity on transcription (Imai et al., 2000). *Arabidopsis* SRT2 negatively regulates basal defense mechanism as demonstrated by increase in resistance against PstDC3000 and expression of PR1 gene upon knocking-out At*SRT2* (Wang et al., 2010). A plant-specific

HDAC family, HD2, first reported in maize (Lusser et al., 1997) represses transcription. Similarly, *Arabidopsis* HD2A, HD2B, and HD2C were proposed to function in repression as well having shown to have identical biological function as RPD3-like HDACs (as reviewed in Berr et al., 2011).

Earlier work on acetylation in other model plants such as maize (*Zea mays*) identified also quite a number of HATs and HDACs protein activities (Loidl, 1994; Lusser et al., 2001). Work on rice (*Oryza sativa*), however, has just begun with a preliminary investigation of eight histone acetyltransferases (Fang et al., 2014; Liu et al., 2012b) and deacetylases (Ma et al., 2013). It is clear from these preliminary researches that novel mechanistic models of acetylation and deacetylation will become apparent in certain epigenetic areas of study where maize and rice provides an excellent model system.

1.6 Histone acetylation via rice-CREB binding protein (rCBP): A role in rice biotic defense responses

Several studies have shown that histone acetylation and deacetylation control defense signaling in plants in response to phytohormone or pathogen application (refer to Ding and Wang, 2015; Song and Walley, 2016; Zhu et al., 2016). More specifically, histone acetyltransferases (HATs) and deacetylases (HDACs) in rice model were shown to be responsive to abiotic stresses and can be modulated by phytohormones, thus implicating a role in biotic stresses as well (Fu et al., 2007; Liu et al., 2012b). HATs in rice can be classified into four families including the CBP family (*HAC701*/*rCBP*, *HAC703*, and *HAC704*), the TAFII250 family (*HAF701*), GNATs (*HAG702*, *HAG703*, and *HAG704*), and lastly, *HAM701* of the MYST family (Liu et al., 2012b). On other hand, HDACs are represented in two families, RPD3/HDA1-like and SIR2-like with no known rice member belonging to HD2 family (Ma et al., 2013).

As HATs have shown to be modulated by biotic stress-inducible hormonal pathways, I hypothesized that it could be a potential system for studying rice pathogenesis. Indeed, our initial results showed that *HAC701* gene is significantly upregulated upon flg22 treatment indicating a possible role in defense responses (**Fig. 2.1**). This finding was partially confirmed by a report that *Arabidopsis* HAC1 is involved in priming PTI (Singh et al., 2014).

OsHAC701 was reported earlier as a putative rice CBP-related acetyltransferase, herein after referred to as *rCBP*, of the p300/CBP acetyltransferase (PCAT) family of proteins (Yuan and Giordano, 2002). On the other hand, the *Arabidopsis* genome contains five CBP genes (*AtHAC1*, *AtHAC4*, *AtHAC5*, *AtHAC12*, and *AtHAC2*) having broad acetyltransferase specificity on histones (**Fig. 1.6**) (Liu et al., 2012b).

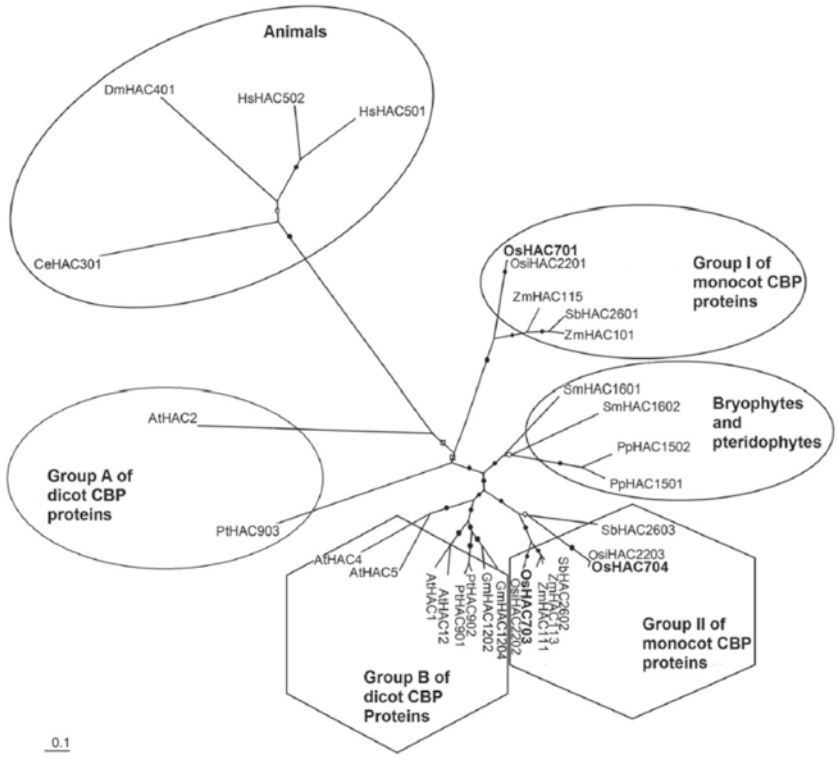


Figure 1.6. Phylogenetic tree showing the members of CREB-binding (CBP) proteins in plants and animals. This unrooted tree was constructed using neighbor-joining distance method of the Phylip package. Figure and description were adapted with modifications from Liu et al. (2012b).

Currently, there is no clear consensus on the similarity of biological functions of rice and *Arabidopsis* homologs of CBP family proteins. In animals, p300 and CBP are paralogs and originally described as transcriptional coactivator that exhibit histone acetyltransferase activity on all four core histones specifically at H4 N-terminal tail sites K5, K8, K12, and K16 (**Fig. 1.7**) (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). Other sites include H3 sites K14, 23 (Henry et al., 2013), K18, K27 (Jin et al., 2011; Tie et al., 2009), and K56 (Das et al., 2009); H2B sites are K12 and K15 (Schiltz et al., 1999). H3K9 is mainly acetylated by GCN5/PCAF (Jin et al., 2011), however acetylation by p300/CBP was also reported (Henry et al., 2013; Modak et al., 2013; Sakamoto et al., 2012). Its highly conserved function is mainly found in multicellular organisms as it probably participates in complex physiological processes acting as a limiting factor in various pathways due to its high cellular demand (Yuan and Giordano, 2002). PCAT proteins are also considered integrators or adaptors as they were shown to interact with DNA-binding activators and the basal transcriptional machinery (Goodrich and Tjian, 1994; Janknecht and Hunter, 1996).

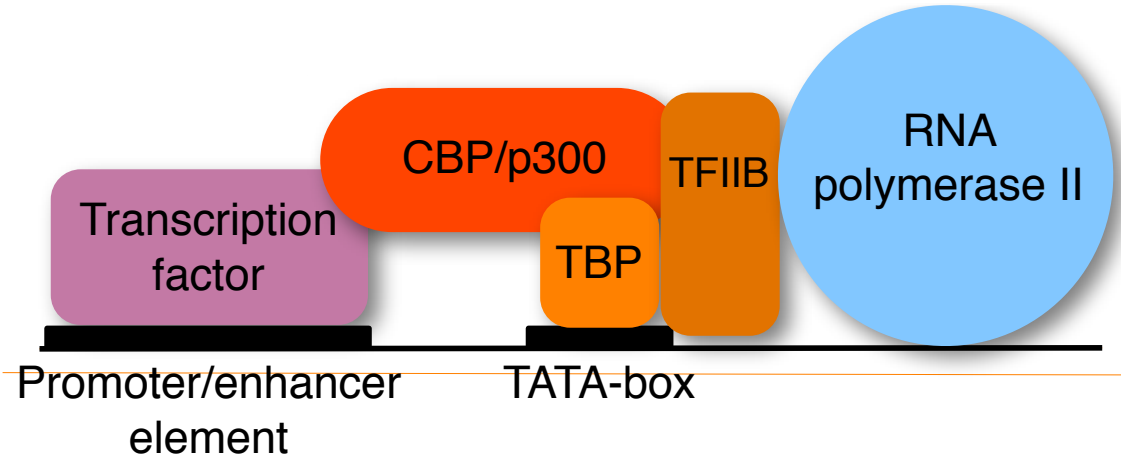


Figure 1.7. Model of p300/CBP bridging between a sequence-specific transcription factor and components of the basal transcription machinery. TBP, TATA-box-binding protein; TFIIB, transcription factor IIB. Figure and description were adapted with modifications from Janknecht and Hunter (1996).

Although, histone acetyltransferase-dependent pathway (HAC1-dependent) has been shown to regulate priming of repetitively stressed *Arabidopsis* plants, it is not known whether these functions extend to rice cereal crop model, where increased biotic stress tolerance accompanies domestication selection pressures [Meyer & Purugganan (2013) *Nat. Rev. Genet.* **14**: 840-852]. Also, it remains to be elucidated how HAC1 and/or its homologs establish the epigenetically primed states at open chromatin target sites. Moreover, rCBP, maybe involved in a switching mechanism over antagonistically functioning chromatin site/s with a possible role in pathogenesis resistance (Holmqvist and Mannervik, 2013).

The work presented here is primarily aimed at deciphering the role of *rCBP* in:

- developmental and non-developmental aspects of rice;
- the acetylation of histone lysine target sites;
- local defense responses; and
- systemic defense responses

Chapter 2

rCBP is involved in non-developmental and developmental processes in rice

Summary:

Rationale:

The purpose of this chapter is to investigate the roles of *rCBP* in PAMP-triggered immunity using flg22 as an elicitor. This chapter also investigates the developmental phenotypes through silencing and mutagenesis of *rCBP* gene. These purposes are addressed mainly by performing flg22-induced gene expression analysis on wild type *Oryza sativa* ssp. *japonica* cv. Nipponbare. This chapter also describes the isolation of single transgenic and mutant lines of *rCBP* gene.

Results:

This chapter presents two major results:

1. The rice CREB-binding protein gene (*rCBP*) is significantly induced under flg22 [QRLSTGSRINSAKDDAAGLQIA (30-51 aa of Q83WT8), Flic, *P. aeruginosa*)] treatment, suggesting its possible role in rice defense against bacterial pathogen.
2. RNA interference and mutagenesis of *rCBP* gene induced embryonic lethality in rice.

RESULTS

To implicate the role of rCBP-dependent pathway in non-developmental processes in rice, I asked whether *rCBP* is inducible using flg22 peptide, a known defense-related gene elicitor in rice (Takai et al., 2008). Although, it was reported that perception of flg22 in cultured cells was weak, the perception of flagellin showed some conservation in rice. To answer this question, I did an expression analysis by RT-qPCR on a representative pathogenesis-related gene (PR-gene), *PR10a* (Agrawal et al., 2001; Choi et al., 2015; Huang et al., 2016; McGee et al., 2001), and on eight HAT genes found in rice (Liu et al., 2012b). The results showed a 3 to 5 fold-induction of pathogen-responsive gene, *PR10a*, after flg22 treatment for 24h (**Fig. 2.1A; Fig. 2.1B**). Among the eight rice HATs, *rCBP* was the sole acetyltransferase gene induced with at least 2 fold-changes upon flg22 treatment at varying concentrations (**Fig. 2.1A; Fig. 2.1C**). It is important to note that the increase of flg22 peptide concentration at 1.0 and 1.5 μM did not further induce the gene expression of *PR10a* and *rCBP*. It might be that the rice OsFLS2 receptors have reached the saturation point resulting to the absence of further induction by flg22 ligand. These results suggest a possible role of *rCBP* in non-developmental biological processes such as bacterial pathogen response and sensing.

To functionally analyze whether *rCBP* is involved in plant immunity, I isolated RNAi transgenic lines by targeting the endogenous *rCBP* gene at the 5'- and 3'-regions (**Fig. 2.2A**). Analysis of the rCBP protein domains suggests that majority of the functional domains are found at the C-terminal region containing the PHD, KAT, ZZ, and TAZ domains (**Fig. 2.2B; Fig. 2.3**). I generated nine 5' and eight 3' RNAi lines (**Fig. 2.2F**) and randomly genotyped three representative first generation (T0) lines from each by detecting the expression of *gus linker* as an indirect measure of the trigger RNA against *rCBP* (**Fig. 2.2C**) (Miki and Shimamoto, 2004). The steady-state transcripts of 5' lines (*5'-1*, *5'-2*, and *5'-14A*) show a weak knockdown of the target gene; however, the other three 3' lines (*3'-1*, *3'-2*, and *3'-3*) are

characterized by >80% knockdown of *rCBP* gene (**Fig. 2.2D**). These data indicate the isolation of weak and strong transgene expression in these RNAi *rCBP* lines at T0 generation. Next, I investigated whether the knockdown of *rCBP* controls developmental processes in rice by examining the embryonic lethality rate of all the generated transgenic lines. Our results show that 3' lines have lethality at about 63% of the total transgenic lines isolated, while the weaker line of *rCBP* in 5' and the GFP control lines are mostly non-lethal (**Fig. 2.2F**). I then compared the effective grain production of the few 3' fertile lines with 5' and wild type lines and found that the number of effective grains is highly reduced in 3' lines significantly (**Fig. 2.2G; Fig. 2.2E**). Together these data suggest that reduction of *rCBP* transcripts in RNAi lines most notably in 3' lines compromise the production of effective or viable grains in rice. Furthermore, the knockdown of the target gene region, which in this case corresponds to the *rCBP* histone acetyltransferase coding region in 3' lines suggest the involvement of this particular gene region in the observed impairment of grain lethality.

In addition to RNAi transgenic lines, I also isolated null mutant lines by independently targeting two sites of *rCBP* gene (**Fig. 2.4A; Fig. 2.5A**) using CRISPR/Cas9 editing technology (Xie and Yang, 2013). I initially targeted the first exon of the *rCBP* gene using a CRISPR/Cas9 vector construct containing one single-guide RNA (sgRNA) (**Fig. 2.4A; Supp Table 2**). The isolation of the first generation (T0) *CRISPR/Cas9-rCBP-S2* lines yielded 90 positive independent lines (**Fig. 2.4E**), and among these, randomly chosen representative lines were further genotyped to characterize the identified DNA mutation. PCR and RFLP assays resulted in the isolation of monoallelic lines characterized mostly by deletions and a few insertions on or surrounding the targeted site of sgRNA (S2) (**Fig. 2.4B; Fig. 2.4C; Supp Table 2**). The T1 *rCBP-S2* generation as observed from seeds showed conservation of mutation directly from parental lines (**Fig. 2.4B**). The *rCBP* lines isolated by targeting the first exon likewise feature an embryonic lethality at about 38% among the independently

isolated positive lines and is mainly characterized by frequent occurrence of empty grains in panicles (**Fig. 2.4D; Fig. 2.4E**). I then isolated mutant lines using the same technology targeting the fifth exon of the *rCBP* gene using a construct that also contains a single-guide RNA (S5) (**Fig. 2.5A; Supp Table 2**). The *CRISPR-Cas9-rCBP-S5* (T0) lines generated 11 positive independent lines of which three were genotyped for verification of mutations (**Fig. 2.5F; Fig. 2.5B**). Second generation (T1) lines also showed conservation of mutations (**Fig. 2.5B; Fig. 2.5E**). Similar to *rCBP-S2* lines, genotyping showed insertions and deletions (INDELS) in proximity to or on the target site. The phenotype is similarly characterized by the presence of abnormal outgrowths of reproductive structures and empty grains in panicles causing embryonic lethality of up to 36% the total of all *rCBP-S5* independent lines isolated (**Fig. 2.5C; Fig. 2.5D; Fig. 2.5F**). The observed weak embryonic lethality in these mutant lines could be attributed to the loss of function of *rCBP* especially at T0 generation. It is important to assess whether the embryonic lethality phenotype in the succeeding generations are maintained or lost due to genetic or environmental variations. These findings indicate the isolation of monoallelic and biallelic *rCBP* lines at T0 and T1 generation using CRISPR/Cas9 genome editing technology in rice. Phenotypic analysis shows impairment to produce effective grains in both *rCBP-S2* and *S5* lines as a consequence of targeting the first and fifth exons of the *rCBP* gene. Overall, the findings in these two CRISPR/Cas9 lines validate the phenotype observed in RNAi transgenic lines with regard to grain production.

Lastly, I investigated the seemingly stronger phenotypic effect of RNAi as compared to CRISPR/Cas9 editing in these *rCBP* lines examined. To answer this question, I did multiple sequence alignment analysis of *rCBP* and two genes that also belong to the same family as *rCBP* (Liu et al., 2012b). The analysis showed that the histone acetyltransferase domain of rCBP, HAC703, and HAC704 are highly conserved (**Fig. 2.6B**), while the upstream amino acid sequences of these three proteins are not (**Fig. 2.6A**). I confirmed the mistargeting of

RNAi on the expression of *HAC703* and *HAC704* in RNAi *rCBP* transgenic lines (**Fig. 2.6C**). These data suggest that the severity of phenotype in RNAi *rCBP* lines is putatively a collective effect of knocking down all members of rice CBP family namely: rCBP, HAC703, and HAC704. It also indicates that the CRISPR/Cas9 targeting is mostly specific to the gene of interest (GOI), which is *rCBP*. Although, there is a need to further examine the expression of HAC703 and HAC704 in these RNAi lines as well as to isolate knockdown and knockout lines targeting these three rice CBP members to fully clarify their roles in embryonic lethality.

DISCUSSION

The molecular mechanism of CBP/p300 in different model organisms have been studied extensively and revealed an important function in gene regulation as it acted as global transcriptional coactivator and acetyltransferase (Bannister and Kouzarides, 1996; Janknecht and Hunter, 1996; Ogryzko et al., 1996; Yuan and Marmorstein, 2013). Here, I demonstrate that rice CBP is possibly involved in pathogen response as wild type *rCBP* gene expression showed a significant up-regulation specific to flagellin (flg22) induction. The possible involvement of *rCBP* in regulation of rice innate immunity is consistent with the recent work on *Arabidopsis* HAC1 (Singh et al., 2014). Although in this work, HAC1 seemed not to directly regulate *Arabidopsis* innate immunity, but to bacterial resistance induced after recurring exposure to abiotic stresses such as heat, cold, and salt. To this effect, HAC1 is proposed to regulate defense priming through mediation of open chromatin reconfiguration. On the other hand, my results show that *rCBP* expression is significantly up-regulated upon application of PAMP-elicitor, flg22, without the need for repetitive abiotic stress treatment (**Fig. 2.1C**). To discard the effect of abiotic stress such as wounding in this assay, floated leaf disc was utilized to test the *rCBP* response upon PAMP treatment. Previous studies have showed that leaf discs floated on water overnight do not exhibit wounding symptoms (Heese et al., 2007; Heese et al., 2005; Nozue et al., 2011). This result suggests that rCBP-dependent regulation of innate immunity is extended in rice cereal crop model and biotic stress tolerance may have been optimized as a result of domestication selection pressures (Meyer and Purugganan, 2013). However, it is not known yet whether rCBP functions similarly as HAC1 in *Arabidopsis* considering their conserved evolutionary function. It is also valuable to test the expression of *rCBP* gene using various PAMP treatments (*e.g.* chitin, *etc*) to address whether *rCBP* gene induction is specific to bacterial PAMP, flg22, or not.

My results also show that *rCBP* participates in the regulation of embryonic development during reproductive stages of rice. The percentage of embryonic lethality in transgenic and mutant *rCBP* lines and the number of effective grains produced indicate that *rCBP* has a role during embryonic developmental processes (**Fig. 2.2E-G; Fig. 2.4D-E; & Fig. 2.5C, F**). This phenotype is not surprising as the early mutagenesis work on CBP in mouse yielded embryonic lethal lines characterized by defects in cell proliferation, nervous, cardiac, and skeletal maldevelopment, and Rubinstein-Taybi syndrome (RTS) (Tanaka et al., 2000; Vo and Goodman, 2001; Yao et al., 1998). Using the *rCBP* locus, LOC_Os01g14370, as input to PlantGOSlim ontologies of the Rice Genome Annotation Project database, it showed that rCBP protein is implicated to activities related to reproduction, post-embryonic development, and flower development. These data clearly implicates *rCBP* activity to regulation processes in the development of rice embryo.

FIGURES & TABLES

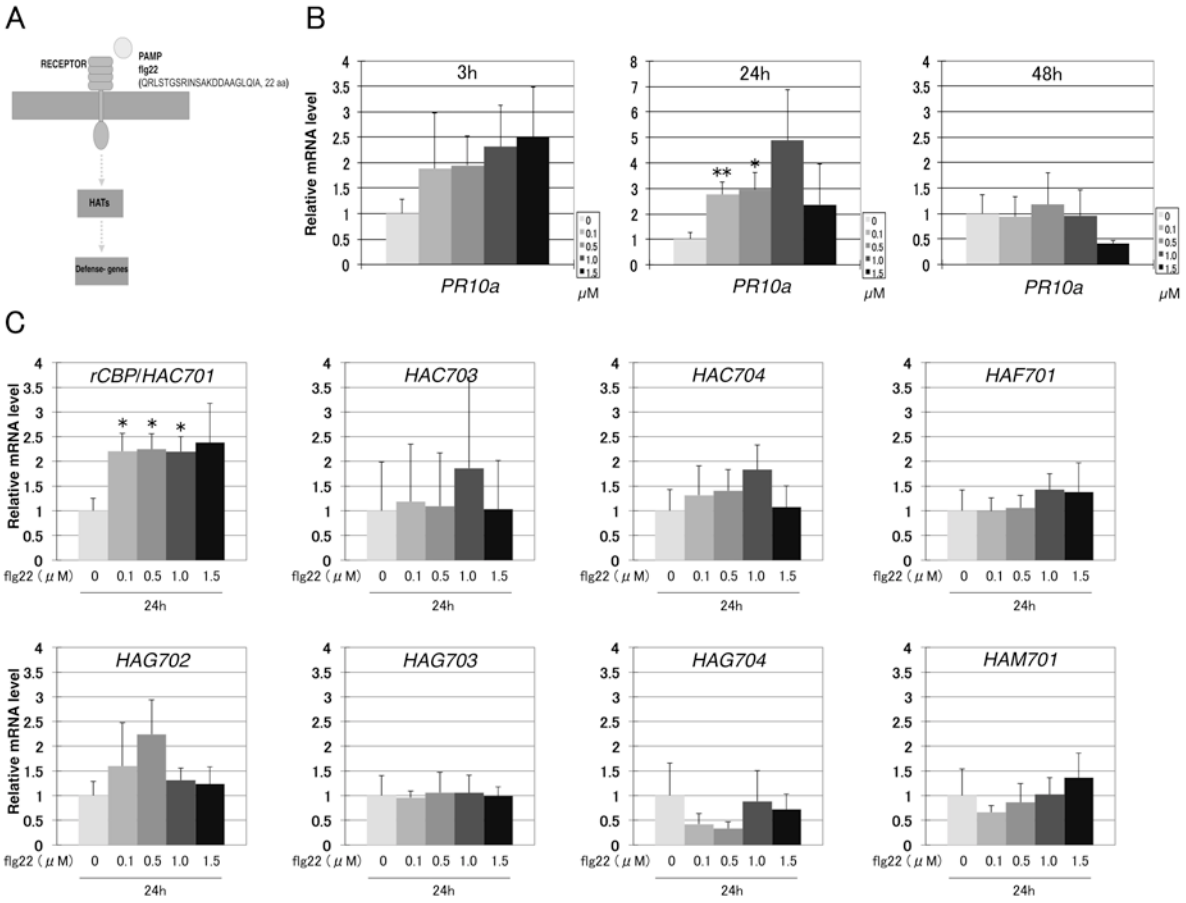


Figure 2.1. flg22 induced the expression of pathogenesis-related and histone acetyltransferase (HAT) genes

(A) Schematic diagram of a putative plant defense response signal transduction pathway under flagellin (flg22) induction. (B) Transcriptional levels of pathogenesis-related gene, *PR10a*, upon flg22 induction at concentrations in μM units. (C) Transcriptional regulation of eight HAT genes in response to flg22 treatment. Data shown are means ± SE; n= 3. The significant difference in transcription is computed using two-tailed Student's *t*-test where asterisks: ***$P \leq 0.01$, **$P \leq 0.03$, *$P \leq 0.05$.

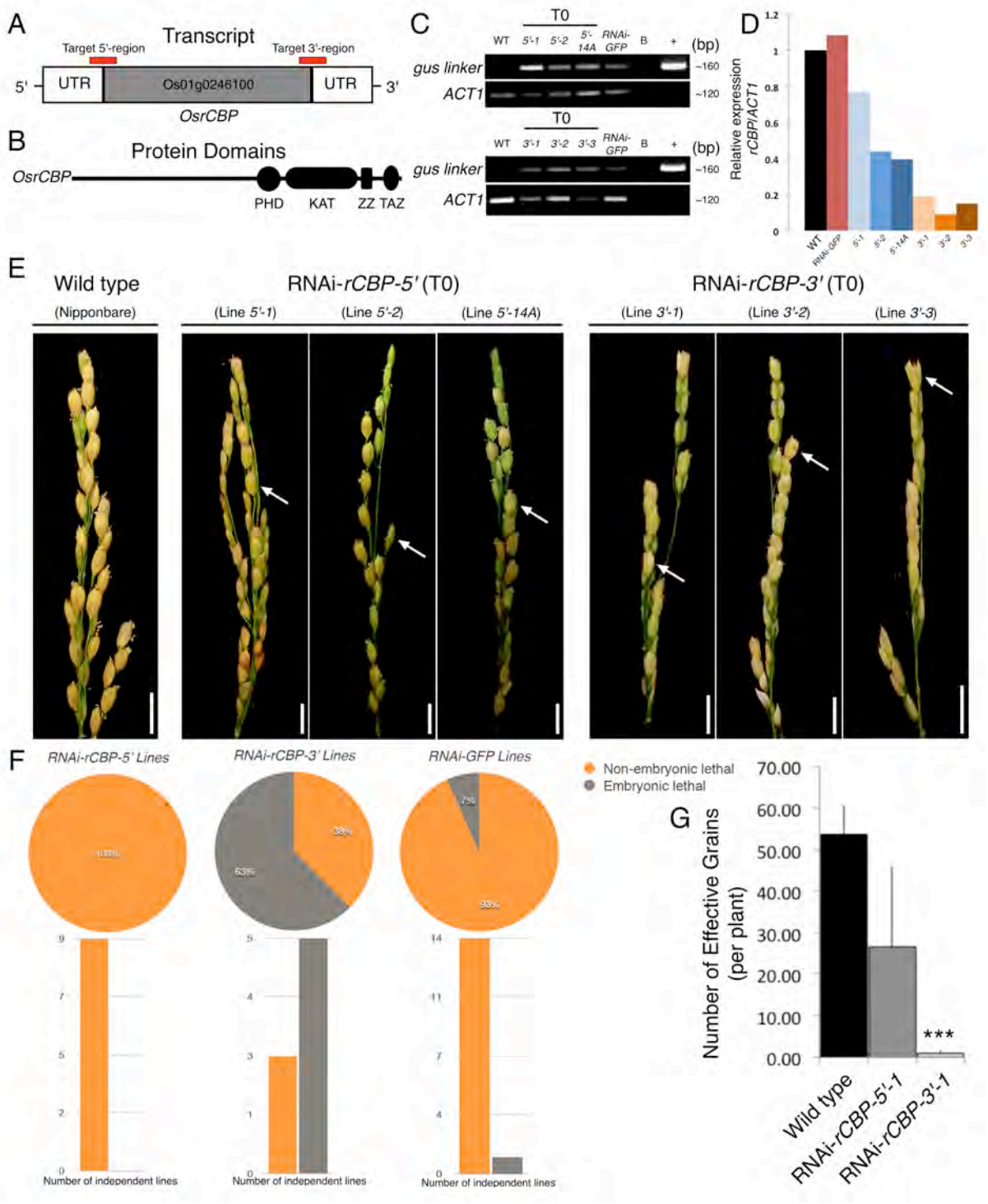


Figure 2.2. Characterization of RNAi *rCBP* knockdown transgenic rice plants

(A) Schematic illustrating the target-regions of RNA silencing in *rCBP* gene. (B) Schematic illustrating the location of protein domains in rCBP protein namely the plant homeodomain (PHD), lysine acetyltransferase (KAT), ZZ-type zinc finger, and Zf-TAZ superfamily. (C) RT-PCR and PCR genotyping of six first generation (T0) RNAi rice plants showing the expression of *gus linker* and *ACT1* mRNA. *RNAi-GFP*, B (blank), and + represent the positive, negative, and vector positive controls, respectively.

(D) RT-qPCR of six representative RNAi rice plants showing varying degrees of down regulation in *rCBP* transcript. (E) Images of six representative *RNAi-rCBP* transgenic lines showing frequent occurrence of empty grains (arrows). (F) Total non-embryonic and embryonic lethality of independent plants isolated for each RNAi positive transgenic lines. (G) Number of mature grains per plant in RNAi lines targeting the 5' and 3'-regions of *rCBP* gene. Data shown are means ± SE; n = +3 (G). The significant difference in number of effective grains is computed using *F*-test (equal variance) and two-tailed Student's *t*-test where asterisks: ***$P \leq 0.01$, **$P \leq 0.03$, *$P \leq 0.05$. Scale bars: 1 cm (E).

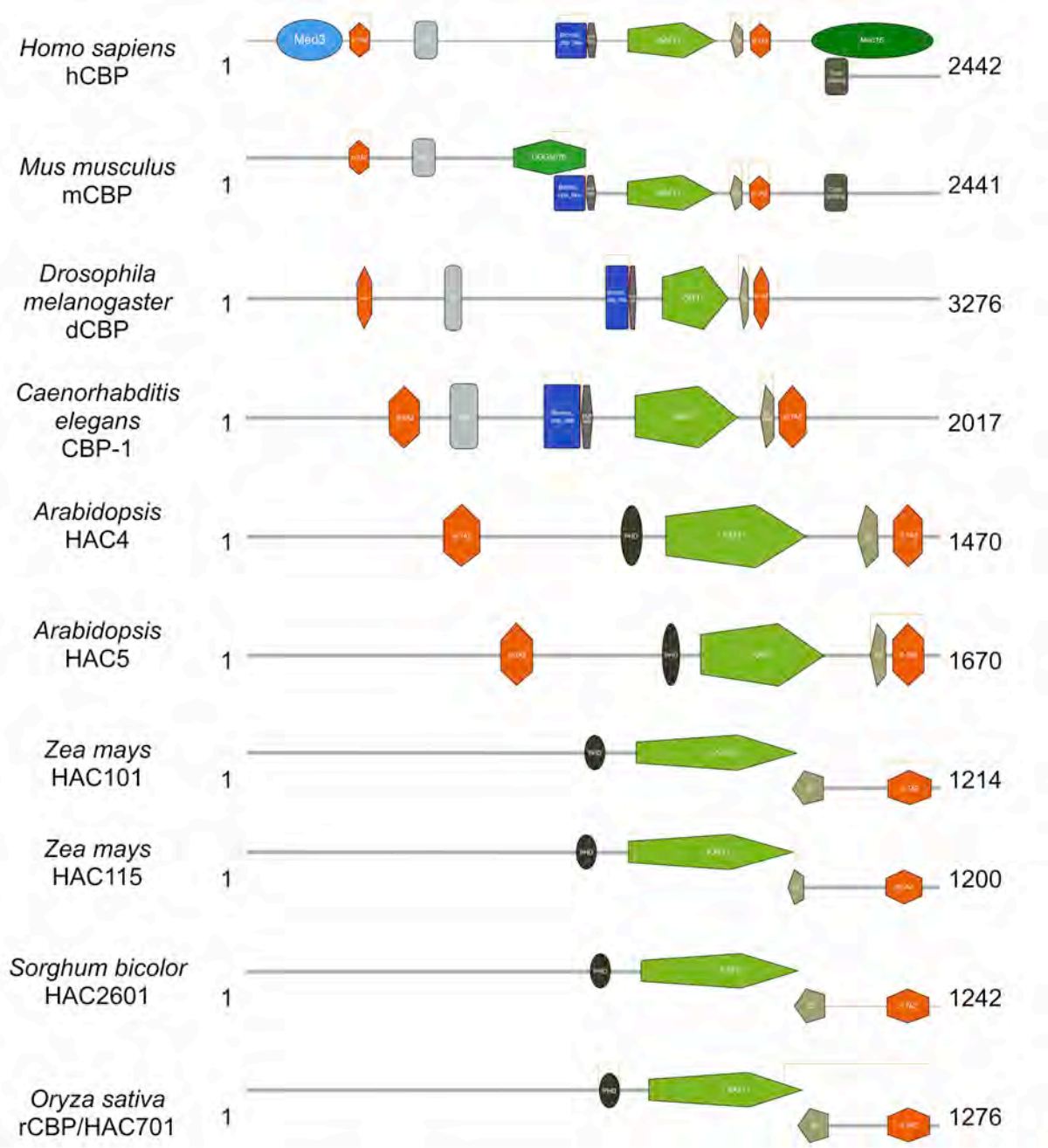


Figure 2.3. CBP domain architecture from different representative taxa

The protein domains represent the highly conserved regions of p300/CBP-family of acetyltransferases. Plant domain structures represent group I of the monocotyledon taxon and group B of the dicotyledon taxon as classified in Liu et al. (2012b). Protein regions are Med3, zf-TAZ, KIX, Bromo_cbp_like, DUF902, KAT11, ZZ_CBP/ZZ, Med15, COG5076, PHD, and Creb_binding. The grey lines illustrating the connections of some protein domains are omitted due to space limitations.

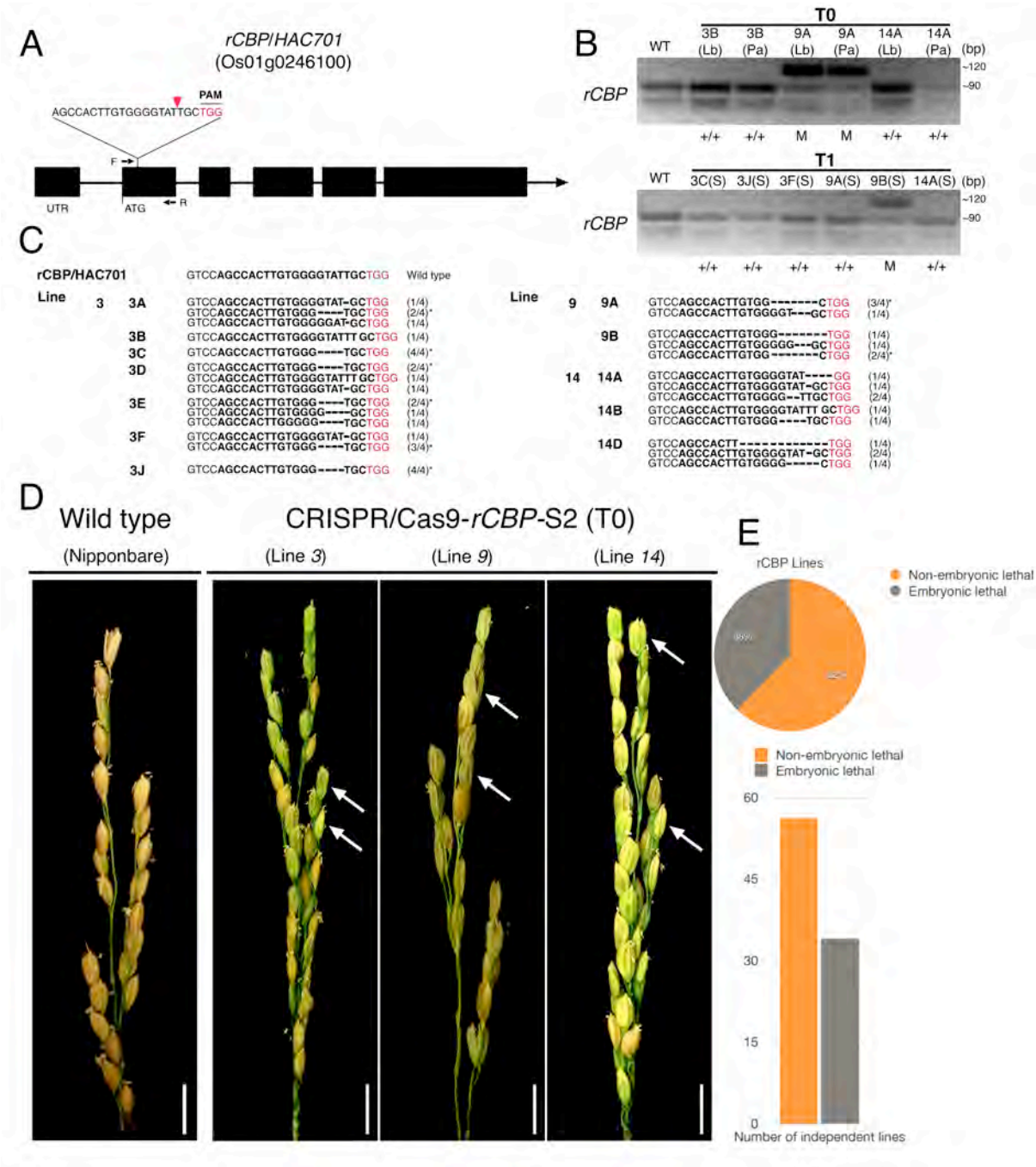


Figure 2.4. Characterization of mutations generated by CRISPR/Cas9 editing on the first exon of rice acetyltransferase gene, *rCBP*

(A) Schematic showing an sgRNA targeted to the first exon of *rCBP* gene. (B) PCR and RFLP assays of representative T0 and T1 generation lines from leaf blade (Lb), panicle (Pa), and seed (S) DNA samples. +/+ and M represent the zygosity of the line, where +/+ refers to wild type and M refers to monoallelic. (C) Alleles from 12 T0 generation lines identified by cloning and sequencing the PCR products from *rCBP* target regions using the forward (F) and reverse (R) primers as found in **Supp Table 2.2**. Similar line number indicates that lines came from the same callus. For each line, four DNA amplicons were

cloned and sequenced and the fraction indicates the number of times the type of mutations were found in each line. In case not indicated, it means wild type. The asterisk (*) indicates the most common mutation found within and across different lines. (D) Images of *rCBP-S2* engineered lines with arrows showing empty grains. (E) Total non-embryonic and embryonic lethality of independent plants isolated among positive *rCBP-S2* lines. Scale bars: 1 cm (D).

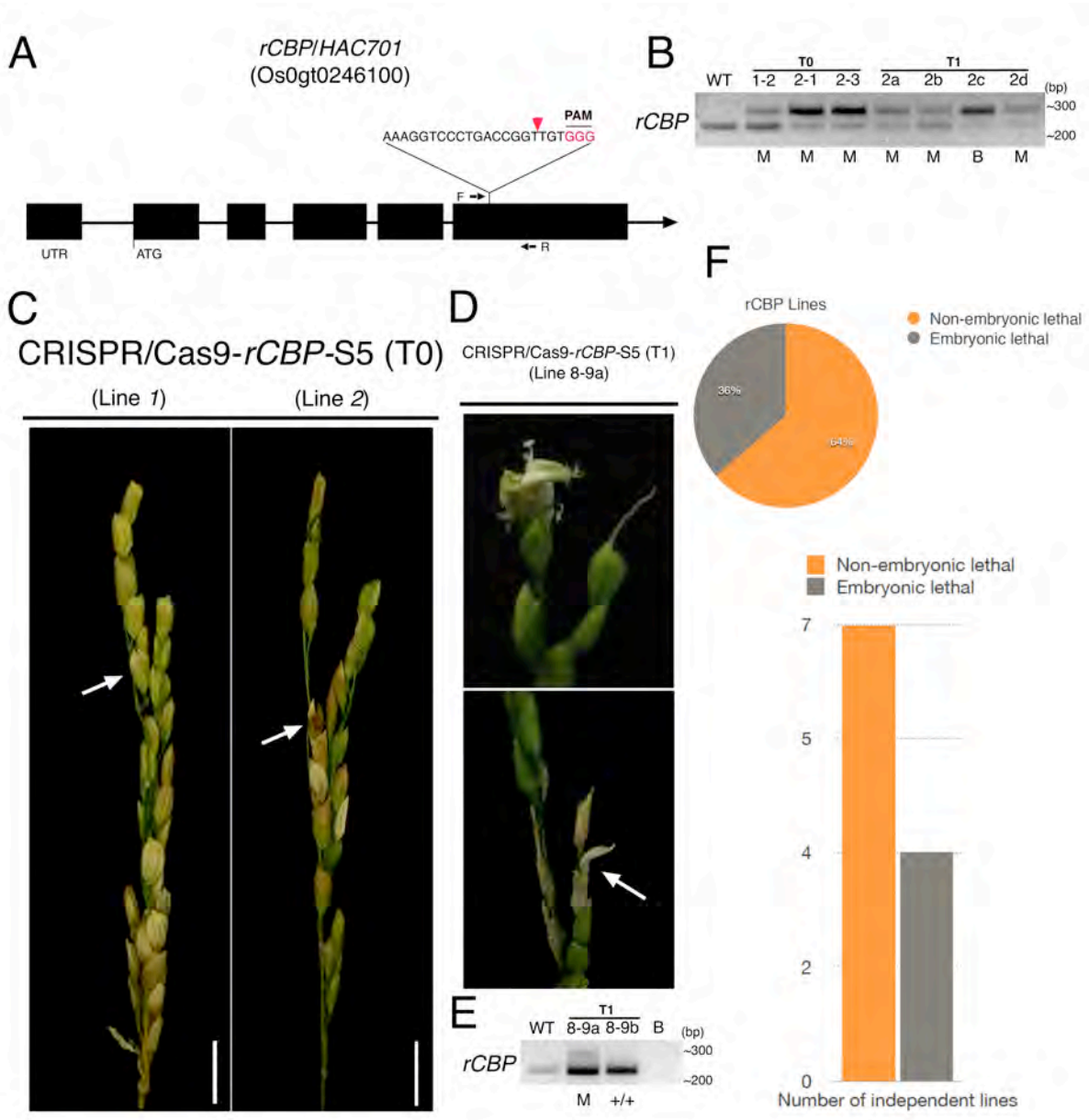


Figure 2.5. Characterization of mutations generated by CRISPR/Cas9 editing on the fifth exon of rice acetyltransferase gene, *rCBP*

(A) Schematic showing an sgRNA targeted to the fifth exon of *rCBP* gene (B) PCR and RFLP assays of representative T0 and T1 generation lines from leaf blade DNA samples. +/+, M, and B represent the zygosity of the line, where +/+ refers to wild type, M refers to monoallelic, and B refers to biallelic. T1 lines came from parental 2-3 line. (C) Images of *rCBP-S5* (T0) engineered lines with arrows showing empty grains. (D) Images of *rCBP-S5* (T1) engineered lines with arrows showing undeveloped grains. (E) PCR and RFLP assays of representative, 8-9a and 8-9b, T1 generation lines. Blank (B) acts as a negative control. (F) Total non-embryonic and embryonic lethality of independent plants isolated among positive *rCBP-S5* lines. Scale bars: 1 cm (C).

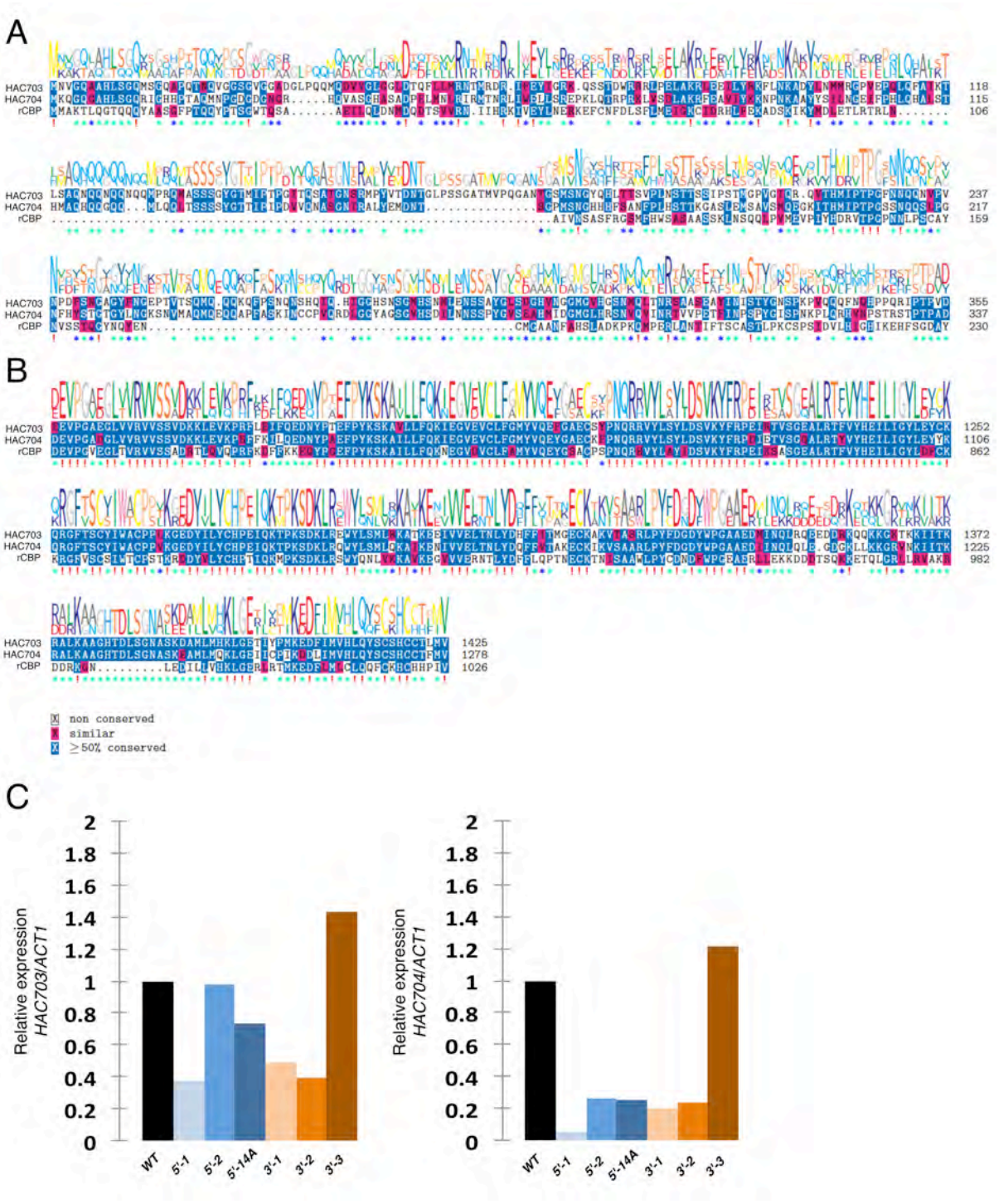


Figure 2.6. Mistargeting of two rCBP-related proteins in RNAi *rCBP* knockdown transgenic lines

(A) Multiple sequence alignment of rCBP, HAC703, and HAC704 upstream amino acid sequences. (B) Multiple sequence alignment of rCBP, HAC703, and HAC704 histone acetyltransferase domain. The domain location coordinates uses the predicted histone acetyltransferase domain of rCBP protein. (C) RT-qPCR of six representative RNAi lines showing the expression of non-targeted genes, *HAC703* and *HAC704*.

Table 2.1. Oligonucleotides used for PCRs, RT-PCRs, RT-qPCRs, vector constructs, and genotyping. PCR (1), RT-PCR (2), RT-qPCR (3)

Primer Name	Forward (5'-3')	Reverse (5'-3')	Purpose
ACT1	TCCATCTTGGCATCTCTCAG	TGGCTTAGCATTCTTGGGTC	3
PR10a	AAGTCATGTCCTAAAGTCGGATG	ATAGTAGCCATCCACGATGTCCT	3
HAC701/rCBP	TGGCGGTGCTTGGTTTGCCT	ACGGGCACGGGTATGACATCGT	3
HAC703	TGTTGAAGAGGTGAAACGTGGG	GCTTCAACCGTTTAAAAAGCCGA	3
HAC704	CAGTGACGAACCAGAGGAAGGGTG	AGGCATGCGCAAACCACGTT	3
HAF701	ACCAGTGCCGCAGATGACGA	TCCGCCAGTGCAAAAAGGTGCT	3
HAG702	TTGCTCGGCAGCTTCCTAACATGC	CAGCATCTCGGGCATGTTGCTTCA	3
HAG703	TGCTGCAAATGAGGGCTGGGA	CGGCCACATTTTCGCAATCGCA	3
HAG704	AAGCGGCTCGTCCAAATGCC	TTGCCGCGTGAGGTGACGTT	3
HAM701	TCCAGTACCGGAAAGGTCAG	AGGGTGTCCAGATCAGCTTG	3
gus linker	TGCTGTCGGCTTTAACCTCT	TTTTTGTCACGCGCTATCAG	1, 2

Table 2.2. sgRNA oligonucleotides used for CRISPR/Cas9 mutagenesis and primers for genotyping. Bold sequences in the guide RNAs are the PAM motifs.

Gene	Guide RNA sequences	Primers used for genotyping	
		Forward (5'-3')	Reverse (5'-3')
rCBP (S2)	AGCCACTTGTGGGGTATTGC**TGG**	GGCGAGATAGAAGCATGATGGC	CGGTGAATAATATTGCGAAC
rCBP (S5)	AAAGGTCCCTGACCGGTTGT**GGG**	CCGATGGATTCTTTCTCAACAG	CCTGGAGAAACCTTCATGATCAG

Chapter 3

Acetylation through rCBP-dependent regulation

Summary:

Rationale:

The purpose of this chapter is to quantify the global lysine site acetylation level catalyzed by rCBP acetyltransferase. It also aims to identify specific histone sites putatively targeted by rCBP activity. These aims were addressed by measuring the acetylation levels in second generation (T1) RNAi lines through biochemical and computational approaches and through mass spectrometry-based proteomics.

Results:

This chapter presents three major results:

1. Silencing of *rCBP* gene in *RNAi-3′* lines depleted the global levels of acetylation to about 80% compared to wild type.
2. Histone H3 is a possible target of rCBP acetylation.
3. rCBP putatively acetylates H3K9 target site.

RESULTS

To identify the acetylation targets of rCBP acetyltransferase, I first characterized the acid extracted proteome (*i.e.* histones and other acid soluble proteins) of second generation (T1) RNAi lines that originally express weak (*RNAi-5′*) and strong (*RNAi-3′*) transgene expression against wild type background. There were 2, 373 unique proteins identified from the pool of wild type, *RNAi-5′*, and *RNAi-3′* proteomes. Acid extracted proteins were chemically derivatized via propionylation of N-termini and empty lysine (K) sites before and after trypsin/Lys-C digestion (Garcia et al., 2007; Maile et al., 2015; Meert et al., 2015; Shechter et al., 2007). Propionylation reaction on histone proteins and peptides confers two benefits, namely, relatively longer peptides are produced after enzymatic digestion and it neutralizes the peptides making them hydrophobic. These changes in chemical properties of histone peptides enhance discoverability during mass spectrometry detection. The peptides were then resuspended in Milli-Q water and were analyzed by a 70 min liquid chromatography tandem mass spectrometry (LC-MS/MS) gradients on a hybrid Quadrupole-Orbitrap Q-Exactive Plus mass spectrometer. All the spectra were collected at high resolution using higher energy collisional dissociation (HCD) technology and the peptide sequences were identified by a combination of SEQUEST and Mascot search algorithms with preference to peptides having variable modifications including propionyl (N-term), propionyl (K), acetyl (K), acetyl (protein-N-term), and methylation. A total of 31, 954 unique peptide spectrum matches were collected at a false discovery rate (FDR) <0.01 (**Fig. 3.1**). Of these peptides, 98% were propionylated at N-termini and 99% of the total intensity belongs to those peptides with N-terminal propionylation (**Fig 3.1**). These data suggest that the efficiency of chemical derivatization via propionylation at the N-terminal branch of the peptides is >98% efficient. These also suggest that majority of the peptides have been efficiently converted into N-termini propionylated state.

To further verify whether the proteome datasets collected in triplicates from wild type, *RNAi-5′*, and *RNAi-3′* samples were not skewed due to sampling errors and or due to unequal chemical derivatization, I analyzed the distribution properties of relative protein abundance of wild type and RNAi lines by sum of peptide intensities. Using box whisker plots, protein abundance from 3 technical replicates of wild type and RNAi lines upon log2 normalization show that the variances were homogenous indicating that the distribution of the data does not contain atypical observations (**Fig. 3.2; Fig. 3.7A**). Although equal amounts of proteins were loaded, peptide intensities of RNAi lines are significantly less than the wild type suggesting that these lines might have overpropionylated peptides that further prevents identification (**Fig. 3.2**). To find out whether the reduction of relative protein abundance in RNAi lines is due to reduction of acetylation of histones possibly targeted by rCBP acetyltransferase, I characterized the histone peptide subset containing 10, 048 histone peptides from the acid extracted proteome (**Fig. 3.3C**). These peptides constitute about 31% of the total acid extracted proteome, and from these peptides I utilized only the N-terminally propionylated histones for downstream analysis. My search resulted to the identification of 28 canonical and non-canonical histone proteins with histone H3.2 and H3.3 having the most abundantly identified peptide spectra match (**Fig. 3.3A; Fig. 3.3B;**). I then compared the acetylation level of these 28 histone proteins in wild type and RNAi lines and found that histone H3, H3.2 (Q2RAD9) and H3.3 (Q0JCT1), are highly targeted for acetylation with observed reduction in RNAi lines especially in *RNAi-3′* sample (**Fig. 3.4A**). These results only showed the sum of the raw peptide intensities of histone proteins without normalization. To confirm the reduction of H3 acetylation in RNAi lines, I performed enzyme-linked immunosorbent assay (ELISA) (Colorimetric; Epigentek) and western blot analysis on wild type and RNAi protein samples. The results showed that global acetylation, H3K27ac, and H4K12ac were reduced in RNAi lines especially in *RNAi-3′* at the same amount of total H3 (**Fig. 3.4B**). I

then validated the reduction in acetylation in *RNAi-3′* using a gradient-based western blot analysis. The validation experiment shown as a ratio of signals from global acetyl-lysine (K) antibody to histone H3 antibody validated the reduction of global lysine-site acetylation (**Fig. 3.4C**). The differences in molecular weights of the bands are due to the differences of percent gel concentration utilized. Nonetheless, the gradient-based western blotting profile shows band sizes consistent with histone proteins. Overall, these data suggest that H3 can be one of the targets, either directly or indirectly, of rCBP and that silencing of *rCBP* gene reduced the global acetylation of RNAi lines specifically at lysine acetylation sites. Whether the reduction of acetylation in RNAi lines is caused by pleiotropic phenotype needs to be verified.

To find the specific histone H3 target site of rCBP, I first verified the possible lysine-target sites available in canonical histone H3 using UniProt's amino acid manual assertion database that was inferred from the similarity of sequences from other species (**Fig. 3.5A**). Of these lysine sites, H3K4 and H3K27 are almost always methylated and never acetylated; H3K9, H3K18, and H3K23 are bivalent sites that can be regulated by both acetylation and methylation; while H3K14 can only be possibly acetylated. From this manual assertion, I asked whether I can confirm these predicted modifications using DDA results. The results showed that H3K4 site is always dimethylated and H3K9 site is a true bivalent site that can be acetylated and dimethylated (**Fig. 3.5B**). Additionally, H3K14, H3K18, and H3K23 are only acetylated with no methylation observed. These data demonstrate that UniProt's manual assertion on histone modifications in canonical histone H3 lysine sites is verifiable using DDA-mass spectrometry search.

Bivalent lysine sites are important sites for regulatory control of biological processes associated with these sites. These sites are usually regulated by opposing molecular mechanisms that aid in fine-tuning the control of these biological processes (Espinas et al., 2016). In line with this, I further investigated the confirmed bivalent site, H3K9, whether it

can be one of the possible candidate target sites of rCBP acetyltransferase. To answer this question, I performed an *in silico* enrichment-based approach (ASEB) to find the statistically possible acetylation target sites of histone acetyltransferases and deacetylases on rice canonical histone H3 amino acid sequence. ASEB predicts the substrate sites on known substrate proteins using amino acid sequences. The algorithm to find acetylation sites in this method has been validated on known human acetylation sites using a predefined peptide set. Using ASEB, I searched for highly possible histone acetyltransferase that can acetylate rice H3K9 site and found that of three possible acetyltransferases, CBP/p300 highly likely acetylates H3K9 having the lowest p-value (**Fig. 3.6A**). On the other hand, among deacetylases, SIRT1 will most likely deacetylate H3K9 site (**Fig. 3.6B**). To confirm this observation, I performed parallel reaction monitoring (PRM)-mass spectrometry to specifically quantify H3K9 site and to investigate its relative abundance and proportion in RNAi lines normalized to wild type (**Table 3.1**). PRM-mass spectrometry results suggest that H3K9 acetylation (H3K9ac) is reduced in RNAi lines when normalized to wild type; although its proportion to other observed conditions are minute (**Fig. 3.7A**). Further analysis using PRM-mass spectrometry to quantify other sites are valuable. Next, I confirmed the reduction of H3K9ac using ELISA and the results showed that acetylation level was reduced in *RNAi-3′* to about 80% relative to wild type (**Fig. 3.7B**). These data indicate that H3K9 site is one of the major target acetylation sites of rCBP acetyltransferase in rice cells. The small proportion of H3K9ac possibly indicates a tight regulatory control of this site along with the biological processes it modulates.

DISCUSSION

Acetylation at protein and histone lysine sites is a reversible enzymatic reaction that regulates myriads of cellular and metabolic processes in eukaryotic cells (Choudhary et al., 2009; Choudhary et al., 2014; Sabari et al., 2017). Among these enzymes that catalyze acetylation is CBP/p300 acetyltransferase. A protein that is central to several regulatory signaling pathways and modulation of target gene expression in cells (Dancy and Cole, 2015). Here, I show that RNAi silencing of *rCBP* gene in RNAi lines reduced the global lysine site acetylation level of chemically derivatized bulk histones (**Fig. 3.4**). It is a well-known fact that CREB-binding protein (CBP) and its homologue, p300, act as a transcriptional regulator and also as an acetyltransferase of proteins especially of histones (Bannister and Kouzarides, 1996; Janknecht and Hunter, 1996; Ogryzko et al., 1996). Among the canonical histones, my data show that histone H3 is the main source of global depletion of lysine site acetylation in RNAi lines (**Fig. 3.4**). The level of lysine site acetylation depletion is concentrated on canonical histone H3.2 and variant histone H3.3 indicating that these histones are highly targeted for acetylation reaction possibly through rCBP (**Fig. 3.3**). This is not surprising as diverse functionality have been attributed to histone H3.3 and other replacement histone variants in developmental and non-developmental processes in plants and in animals (Buschbeck and Hake, 2017; Stroud et al., 2012; Szenker et al., 2011; Talbert and Henikoff, 2017).

My results also demonstrate that H3K9 site is a probable acetylation target of rCBP (**Fig. 3.5**; **Fig. 3.6**; **Fig. 3.7**). An 80% reduction in H3K9ac level in RNAi lines indicates a collective silencing of rCBP and other members of CBP family in rice as mistargeting of HAC703 and HAC704 is evident (**Fig. 2.6**). It is interesting to note that *Phytophthora sojae* effector, PsAvh23, destabilizes ADA2/GCN5-mediated acetylation of H3K9 in soybean; thus enhancing susceptibility to infection (Kong et al., 2017). On the other hand, my results show

that majority of H3K9ac is probably catalyzed by CBP family members in rice and the remaining 20% by other acetyltransferase complexes (**Fig. 3.7B**). A deep genomic insight into the targets of rCBP in this model system is a prerequisite to mechanistic elucidation of rCBP-dependent rice immunity.

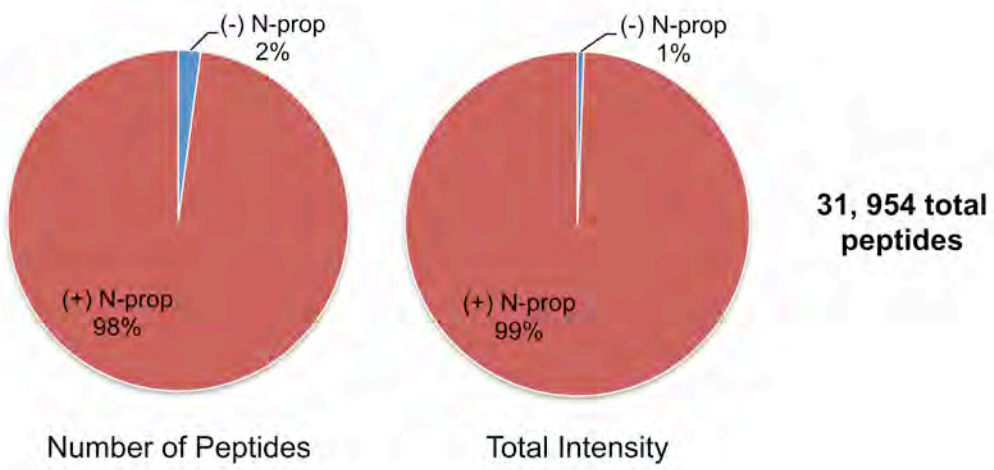


Figure 3.1. Efficiency of double propionylation chemical labeling method of peptides
Comparison of the amount of propionylation at the N-terminus of peptides. The pie chart shows N-terminal propionylation in terms of number of peptides (left) and intensity (right). Peptides were generated from doubly propionylated species of wild type, *RNAi-5′*, and *RNAi-3′* samples.

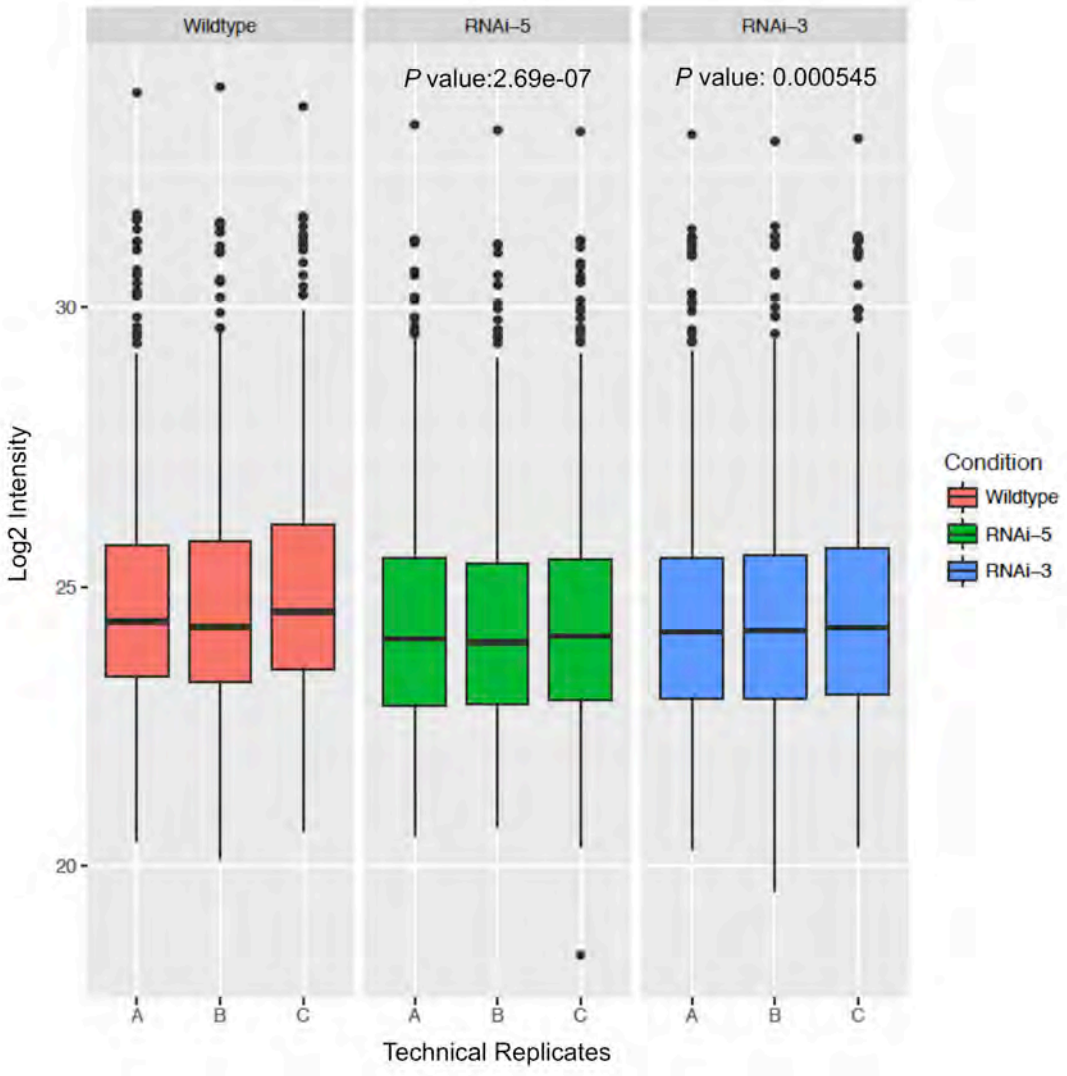
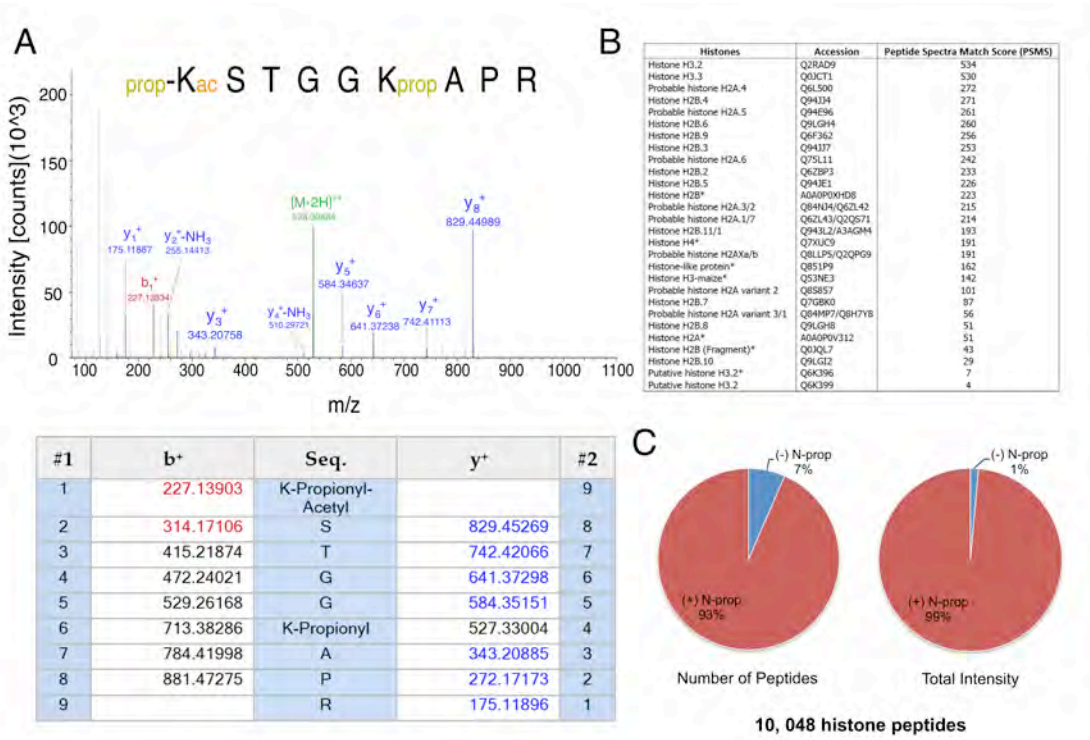


Figure 3.2. Box whisker plots showing the normalized sum of peptide intensities of corresponding proteins from wild type, *RNAi-5'*, and *RNAi-3'* samples

The protein abundance amounts are derived from data-dependent acquisition (DDA) mass spectrometry in triplicates. The data are log2-transformed showing homogeneity of variances computed using *F*-test ($P>0.05$; $F_{comp}<F_{tab}$) and Bartlett-test ($P>0.05$) across all replicates. Statistical significance of protein abundances were computed using one-way *ANOVA* with *P*-values as shown above. The black line in the center of each box is the median value, the upper and lower edges of each box are the upper and lower quartiles (75th and 25th quantiles), and the whiskers are the highest and lowest observations of the distribution (95% confidence level). Equal amounts of 5 µg acid extracted proteins of wild type and RNAi lines were utilized for mass spectrometric analysis.



Histones	Accession	Peptide Spectra Match Score (PSMS)
Histone H3.2	Q2RAD9	534
Histone H3.3	Q0JCT1	530
Probable histone H2A.4	Q6L500	272
Histone H2B.4	Q94JJ4	271
Probable histone H2A.5	Q94E96	261
Histone H2B.6	Q9LGH4	260
Histone H2B.9	Q6F362	256
Histone H2B.3	Q94JJ7	253
Probable histone H2A.6	Q75L11	242
Histone H2B.2	Q6ZBP3	233
Histone H2B.5	Q94JE1	226
Histone H2B*	A0A0P0XHD8	223
Probable histone H2A.3/2	Q84NJ4/Q6ZL42	215
Probable histone H2A.1/7	Q6ZL43/Q2QS71	214
Histone H2B.11/1	Q943L2/A3AGM4	193
Histone H4*	Q7XUC9	191
Probable histone H2AXa/b	Q8LLP5/Q2QPG9	191
Histone-like protein*	Q851P9	162
Histone H3-maize*	Q53NE3	142
Probable histone H2A variant 2	Q8S857	101
Histone H2B.7	Q7GBK0	97
Probable histone H2A variant 3/1	Q84MP7/Q8H7Y8	56
Histone H2B.8	Q9LGH8	51
Histone H2A*	A0A0P0V312	51
Histone H2B (Fragment)*	Q0JQL7	43
Histone H2B.10	Q9LGI2	29
Putative histone H3.2*	Q6K396	7
Putative histone H3.2	Q6K399	4

#1	b+	Seq.	y+	#2
1	227.13903	K-Propionyl-Acetyl		9
2	314.17106	S	829.45269	8
3	415.21874	T	742.42066	7
4	472.24021	G	641.37298	6
5	529.26168	G	584.35151	5
6	713.38286	K-Propionyl	527.33004	4
7	784.41998	A	343.20885	3
8	881.47275	P	272.17173	2
9		R	175.11896	1

Figure 3.3. Characterization of chemically derivatized histone peptides using data-dependent acquisition (DDA) mass spectrometry

(A) A sample of an MS/MS spectrum of $[M+2H]^{2+}$ precursor ion from H3 peptide digested with trypsin and doubly propionylated. The lower panel shows the generated b^+ and y^+ ions corresponding to the amino acid sequence. (B) Identified histone proteins in DDA mode with their accession codes and peptide spectra match scores (PSMS). (C) Efficiency of double propionylation in all histone peptides shown in terms of number (left) and intensity (right).

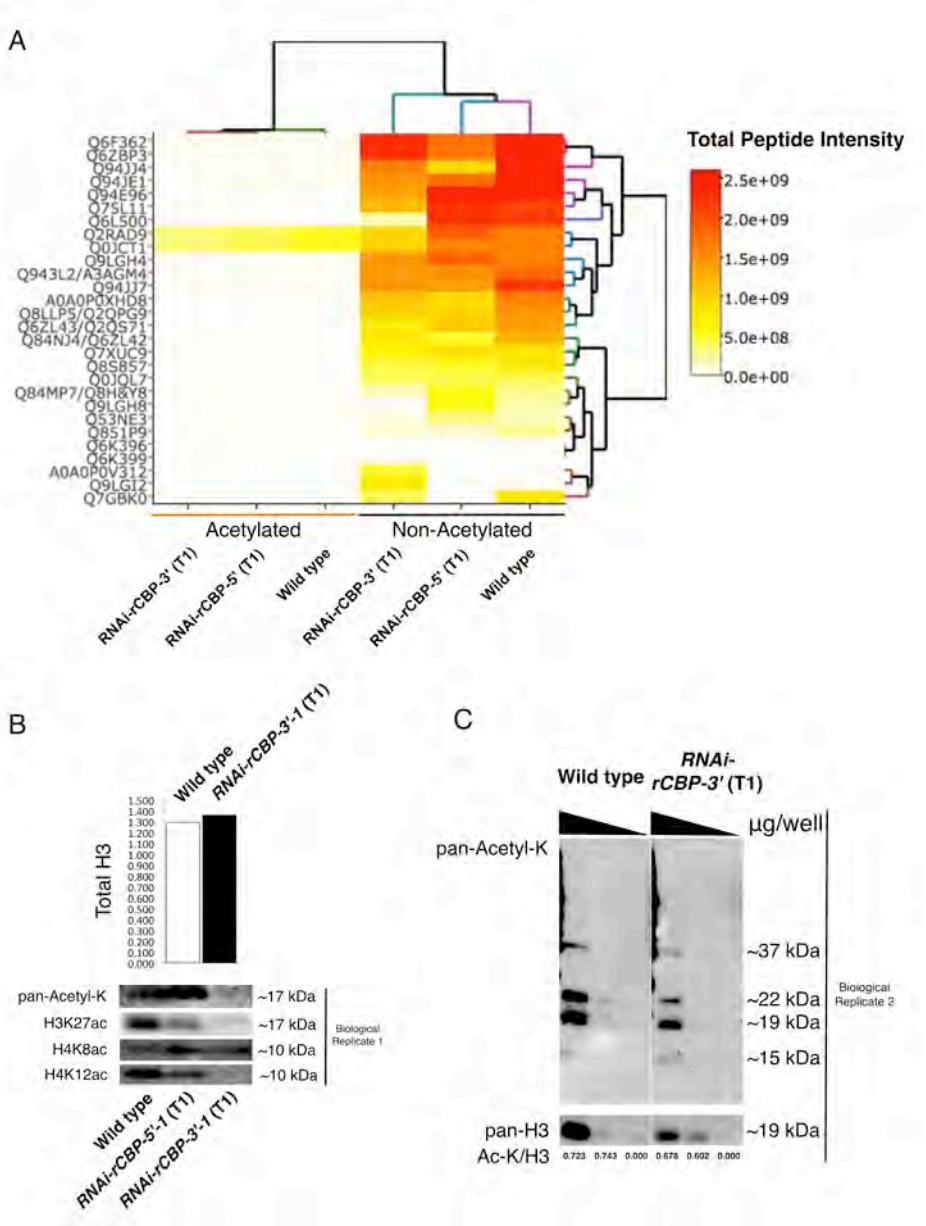


Figure 3.4. Acetylation of bulk histone and histone H3 in wild type and RNAi samples

(A) Heatmap of non-acetylated and acetylated histone proteins showing their raw peptide intensities. (B, C) Confirmation and validation of acetylation in RNAi transgenic lines using Enzyme-linked Immunosorbent Assay (ELISA) for total H3 (in OD units) and western blot analysis for pan-Acetyl-K, H3K27ac, H4K8ac, and H4K12ac antibodies (B). Gradient western blot analysis utilizing pan-Acetyl-K and pan-H3 antibodies (C). pan-H3 was used for normalization of the signals in a gradient western blot analysis. Ratios of pan-Acetyl-K and pan-H3 signals (Ac-K/H3) can be found at the bottom. Protein loading amount is 100 ng/well (ELISA), 500 ng/well (Western blot analysis), 8 µg/2 µg/ 0.5 µg per well (Gradient western blot analysis).

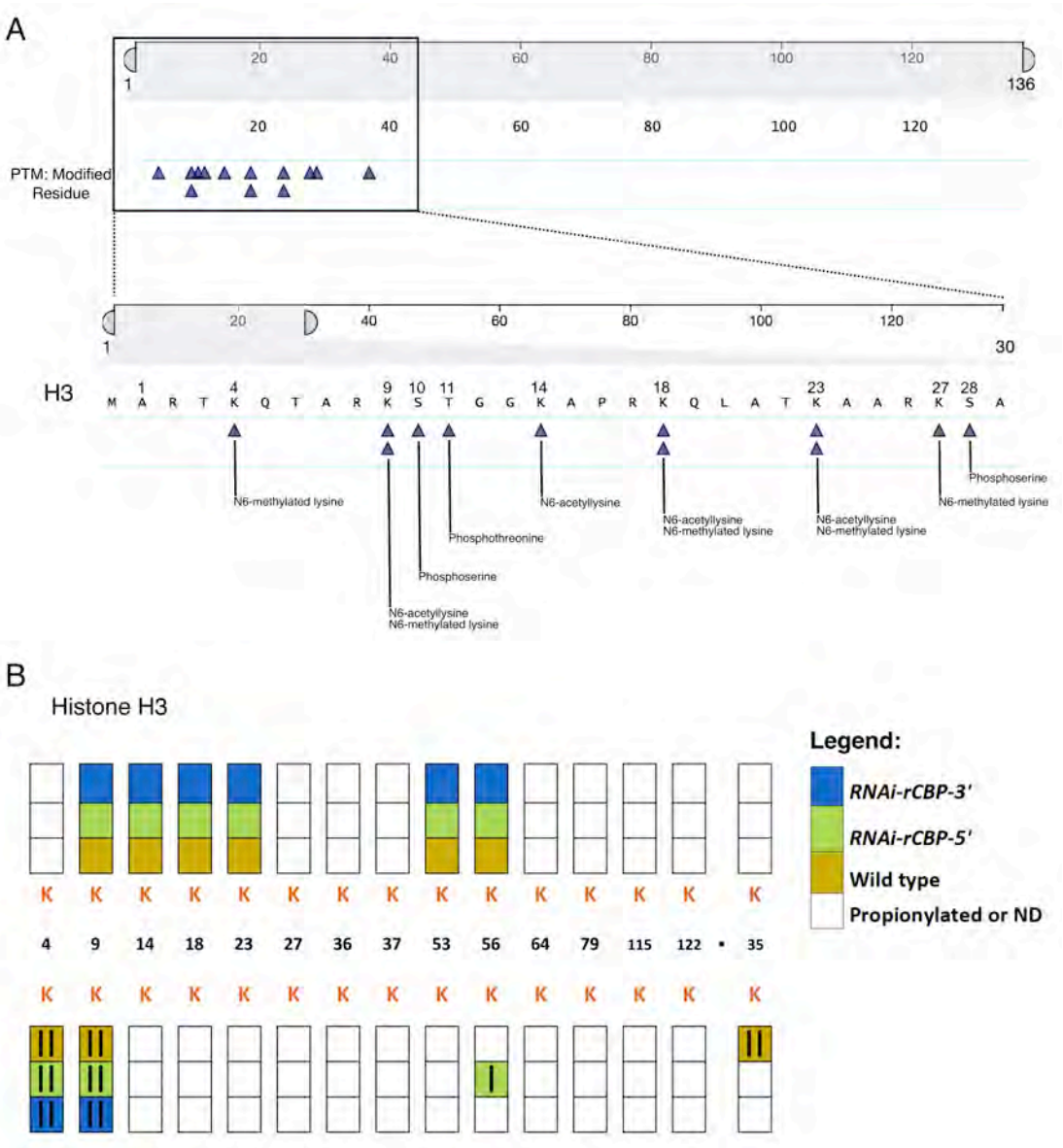


Figure 3.5. Posttranslational modifications on canonical histone H3 of rice

(A) UniProt's amino acid modification manual assertion inferred from sequence similarity on canonical H3 (Q2RAD9). (B) Confirmation of UniProt's H3 amino acid modification assertion using data-dependent acquisition mass spectrometry (DDA-MS). The upper and lower panels refer to acetylation and methylation, respectively. Methylation is marked by monomethylation (I), dimethylation (II), and trimethylation (III).

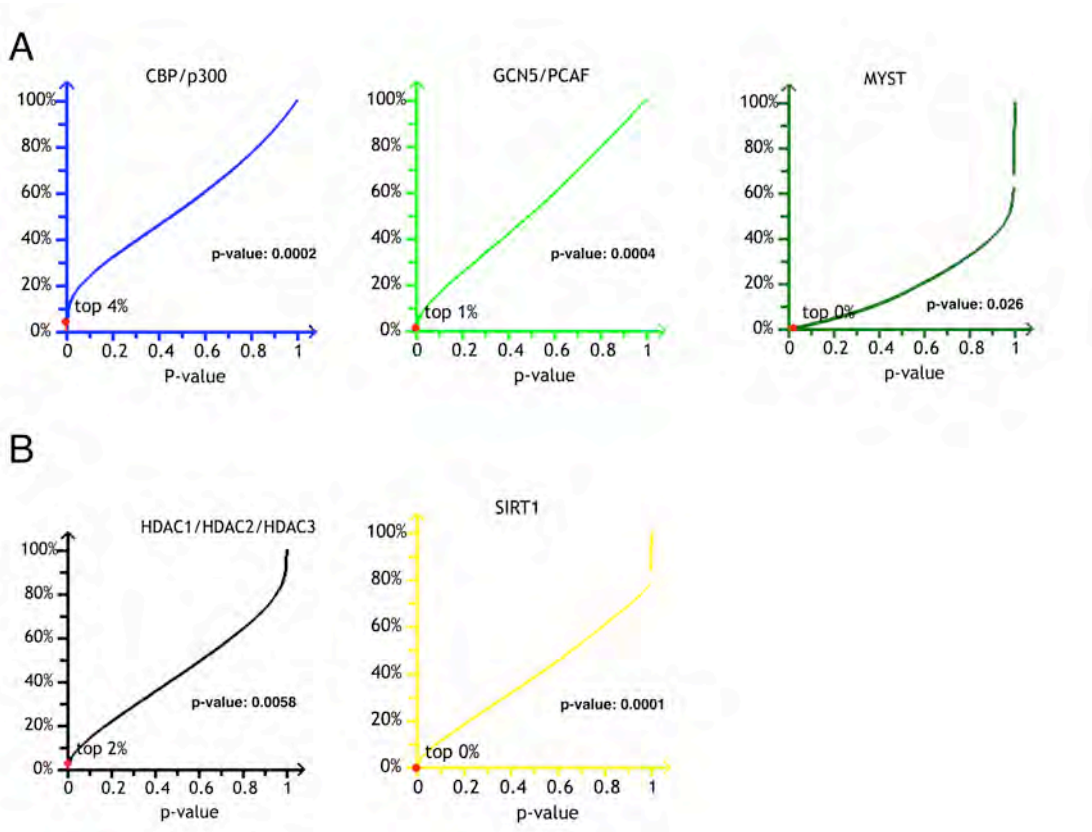


Figure 3.6. Acetylation Set Enrichment-Based (ASEB) method for lysine acetyltransferase (KAT)-specific acetylation site prediction

Probability of acetylation of a specific lysine acetyltransferase family, acetyltransferase (A) and deacetylase (B), on rice canonical H3K9 site. The percentage represents the *P*-value rank of a particular lysine acetyltransferase on H3K9 site against the background. The *P*-values were derived from all human lysine sites ranked in an increasing order and represent the probability of acetylation of the specific lysine acetyltransferase.

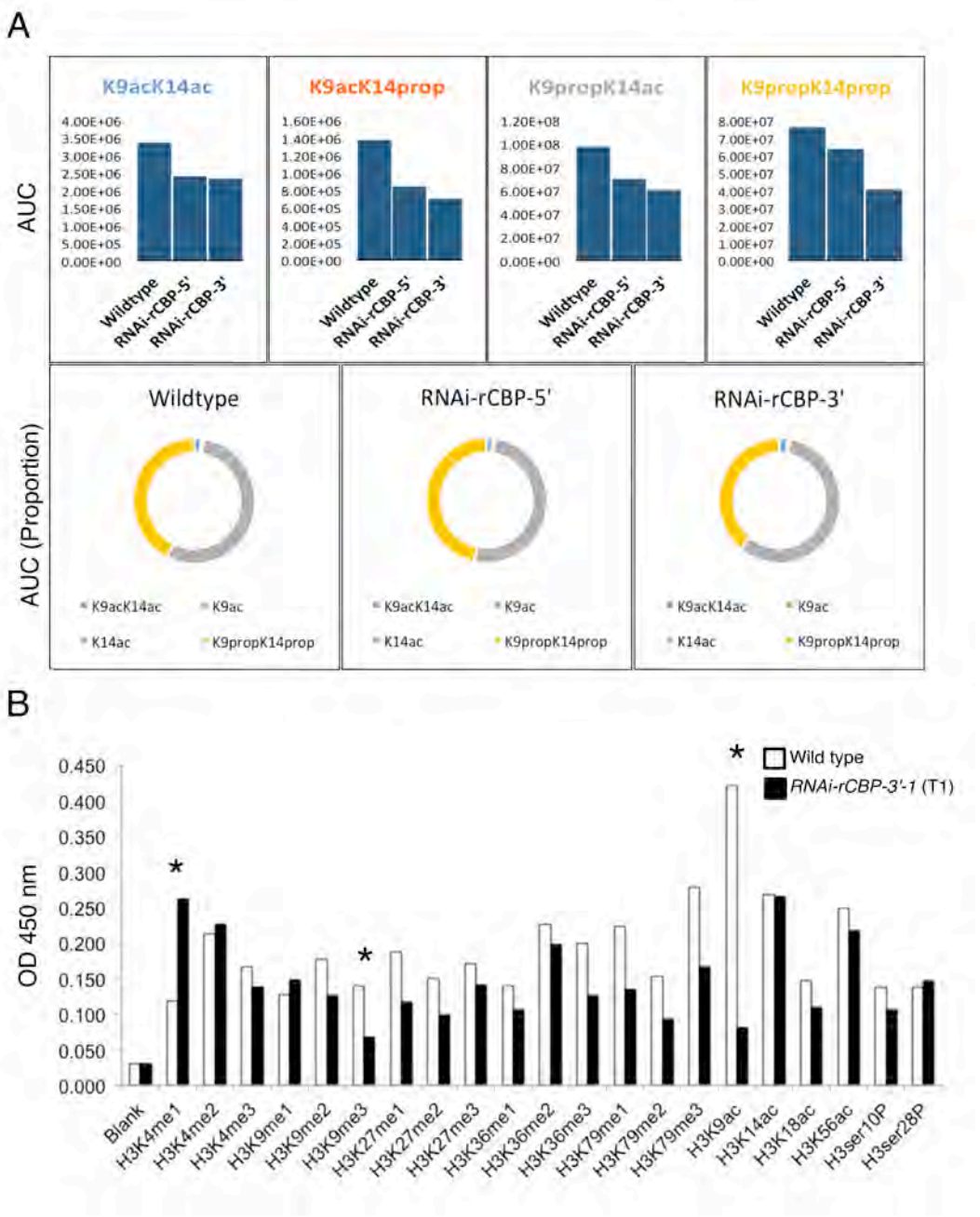


Figure 3.7. Mutagenesis of rCBP protein impairs the acetylation of H3K9 site in rice

(A) Parallel Reaction Monitoring (PRM) relative quantification for H3K9 and H3K14 acetylation using mass spectrometric analysis. AUC refers to area-under-the-curve used to quantify the intensity of each modification on a specific precursor ion and retention time (RT). The AUC proportion represents the amount measured for each modification in wild type, *RNAi-5'*, and *RNAi-3'* samples. (B) Confirmation of PRM results using Enzyme-Linked Immunosorbent Assay (ELISA). Values of H3 modifications were normalized against total H3 of each samples. Asterisks (*) indicate +/- 2x fold-change over wild type. Protein loading amount is 5 ug (PRM), 100 ng/well (ELISA).

Table 3.1. Histone peptide masses for H3K9 and H3K14 sites[1,2, 3]

Sequence	M+H	(M-H)+Prop	(M+H)+Prop [z=1]	[(M-H)Prop +2H]/2 [z=2]	[(M-H)Prop +3H]/3 [z=3]	Retention time (min)
K-S-T-G-G-K-A-P-R	901.52140	1068.59210	1069.60000	535.30335	357.20467	18
K(Ac)-S-T-G-G-K-A-P-R	943.53200	1054.57650	1055.58440	528.29555	352.53280	16
K-S-T-G-G-K(Ac)-A-P-R	943.53200	1054.57650	1055.58440	528.29555	352.53280	16
K(Ac)-S-T-G-G-K(Ac)-A-P-R	985.54250	1040.56080	1041.56870	521.28770	347.86090	15

[1]K sites are available for propionylation in case unmodified or monomethylated.

[2]Shaded masses are utilized as inclusion list for identification and quantification of specific *in vivo* histone lysine site modifications.

[3]For acetylation at K9 only or K14 only, unique transitions were utilized to identify specificity.

Table 3.2. Primary antibodies used for western blot analysis

Antibody	Type	Host	Supplier
pan-Acetyl-K (9441)	Polyclonal	Rabbit	Cell Signaling Technology
pan-H3 (ab1791)	Polyclonal	Rabbit	Abcam
H3K27ac (ab4729)	Polyclonal	Rabbit	Abcam
H4K8ac (61104)	Polyclonal	Rabbit	Active Motif
H4K12ac (39166)	Polyclonal	Rabbit	Active Motif

Table 3.3. Repository information of deposited mass spectrometry files

File name	Type	Reference No.	Database
Wildtype_1-1_DDA	.raw file	TBD	TBD
Wildtype_1-2_DDA	.raw file	TBD	TBD
Wildtype_1-3_DDA	.raw file	TBD	TBD
Wildtype_1-4_PRM	.raw file	TBD	TBD
Wildtype_1-5_PRM	.raw file	TBD	TBD
Wildtype_1-6_PRM	.raw file	TBD	TBD
5p_1-1_DDA	.raw file	TBD	TBD
5p_1-2_DDA	.raw file	TBD	TBD
5p_1-3_DDA	.raw file	TBD	TBD
5p_1-4_PRM	.raw file	TBD	TBD
5p_1-5_PRM	.raw file	TBD	TBD
5p_1-6_PRM	.raw file	TBD	TBD
3p_1-1_DDA	.raw file	TBD	TBD
3p_1-2_DDA	.raw file	TBD	TBD
3p_1-3_DDA	.raw file	TBD	TBD
3p_1-4_PRM	.raw file	TBD	TBD
3p_1-5_PRM	.raw file	TBD	TBD
3p_1-6_PRM	.raw file	TBD	TBD

*To be deposited (TBD): Files will be deposited to OIST Institutional Repository & Research Data Archive after the final version of the thesis is completed.

Chapter 4

rCBP is a component of rice innate immune system

Summary:

Rationale:

The purpose of this chapter is to investigate the involvement of *rCBP* in the regulation of rice innate immunity against *Pseudomonas syringiae* pv. *oryzae* (*Pso*) pathogen. This aim was addressed by performing pathogenesis assay on segregated wild type and CRISPR/Cas9-*rCBP-S5* mutant lines. Total RNA-sequencing on locally infected tissues was done to analyze the genomewide effects of *rCBP* mutation during *Pso* pathogenesis.

Results:

This chapter presents three major results:

1. *rCBP* is involved in the regulation of rice defenses against *Pso* infection.
2. rice-*Pso* pathosystem is characterized by up-regulation of tryptophan and oxylipin biosynthetic processes involved in defense by serotonin production and defense by JA-biosynthesis, respectively.
3. Seven candidate repressor transcription factors were found to negatively regulate both growth and innate immunity in rice upon *Pso* treatment.

RESULTS

To compare the histone modifications on histone H3 sites of the wild type and *rCBP 9-12b*$^{-/-}$ protein samples, I performed ELISA (Colorimetric; Epigentek) and found that consistent with the observations in *rCBP* RNAi lines (**Fig. 3.7B**), histone H3 lysine site 9 (H3K9) acetylation is reduced (**Fig. 4.1E**). I then investigated the involvement of rCBP in basal defense in rice, I inoculated *rCBP* segregated wild type and *rCBP 9-12*$^{-/-}$ *and 9-5*$^{-/-}$ biallelic homozygous mutant lines with mock (10 mM $MgCl_2$) and *Pseudomonas syringiae* pv. *oryzae* (OD = 0.2) resuspended in 10 mM $MgCl_2$ for 72 h (**Fig. 4.1A**; **Fig. 4.1B; Fig. 4.1C**). *Pso* is a causative agent of halo blight in rice characterized by brown lesions and yellow halo-like blotches on leaves (Kuwata, 1985). Infection of *Pso* in rice elicits hypersensitive response with observable programmed cell death. The pathogenesis assay showed that *rCBP*$^{-/-}$ mutants are resistant to *Pso*-treatment as compared to wild type with similar results in six independent biological replicates (**Fig. 4.1C; Fig. 4.3A**). The grounded tissue suspensions were serially diluted six times to be able to count with accuracy the *Pso* colonies on Luria-Bertani (LB) agar medium (**Fig. 4.1C**, left panel). *Pso* colonies were counted from 4th until 6th serial dilution in two independent and genotypically dissimilar *rCBP*$^{-/-}$ mutant lines (**Fig. 4.1C**, right panel; **Fig. 4.1A**). In addition, *rCBP 9-12b*$^{-/-}$ line was grown from the seeds of the third generation (T2), while *rCBP 9-5*$^{-/-}$ line was embryonically rescued about 15 days post flowering from the second generation (T1) parental plants. Rice embryos were grown into plantlets in Murashige and Skoog (MS) agar medium before being transferred to soil for further growth. The phenotype in terms of effective grain number and tiller number of mostly all biallelic homozygous mutants did not show impairment as compared to wild type (**Fig. 4.1D**). This is in complete opposite with the observed phenotype of *rCBP* RNAi lines and T0 CRISPR/Cas9 lines characterized by embryonic lethality (**Fig. 2.2; Figure 2.4; Figure 2.5**). The discrepancy in CRISPR/Cas9 lines could be explained by the differences in the

generation analyzed and needs further validation especially in the T2 and succeeding generations. These data suggest that *rCBP* is involved in the regulation of rice innate immunity acting either in its capacity as an acetyltransferase or as transcriptional coactivator. Additionally, morphological phenotypes of *rCBP*$^{-/-}$ mutants are not impaired suggesting that resistance phenotype does not interfere with the developmental growth of the non-infected plants.

To account for differences in genotype of *rCBP*$^{-/-}$ mutants generated using CRISPR/Cas9 editing, I checked the effect of insertion and deletion mutations (INDEL) in the translation of rCBP amino acid sequences (**Fig. 4.2**). Insertional mutation in *rCBP 9-12a*$^{-/-}$ showed a potentially much stronger mistranslational effect on rCBP protein compared to *rCBP 9-5*$^{-/-}$ and *9-12b*$^{-/-}$ mutant lines (**Fig. 4.2B**; **Fig. 4.2C**; **Fig. 4.2D**). To verify if this insertional mutation genotype still confers resistance to *rCBP 9-12a*$^{-/-}$ mutant lines, I did a pathogenesis assay on five independent replicates and found that resistance phenotype was conserved in this mutant (**Fig. 4.3B**), further indicating that all types of *rCBP* mutation genotypes contribute to heightened resistance against *Pso* bacterial infection.

rCBP gene expression among the eight rice histone acetyltransferase genes is the only significantly up-regulated acetyltransferase gene upon application of flg22 at different concentrations (**Fig. 2.1**). To further link the role of *rCBP* activity in regulating innate immune responses in rice, I induced the *rCBP* gene similarly by application of flg22 at the same concentration gradient in *rCBP*$^{-/-}$ mutant. My results showed that *rCBP* gene in the mutant background is not induced to a level similar to wild type (**Fig. 4.4A**). It could be that mutations in *rCBP* gene fail to recognize upstream signals induced by PAMP-treatment. By checking the gene expression of other *rCBP* gene regions within the histone acetyltransferase domain, my results revealed that there is even a slight reduction in *rCBP* gene expression induced by flg22 treatment (**Fig. 4.4B**). Additional data is needed to fully verify the reduction

of *rCBP* gene expression upon PAMP-treatment in *rCBP* mutant lines. Overall, these indicate that *rCBP* regulates innate immunity in rice.

To profile genomewide the effect of *rCBP* in rice innate immunity, I performed RNA-sequencing on mock- and *Pso*-treated wild type and *rCBP*$^{-/-}$ mutant leaf tissues (**Fig. 4.1C**; **Fig. 4.5A**; **Fig. 4.5B; Fig. 4.7D; Fig. 4.7E; Table 4.3**). Analysis of the highly variable genes in mock- and *Pso*-treated wild type plants shows gene clusters that are dependent on *Pso* induction alone (**Fig. 4.6A**; **Fig. 4.6 B**). The top 10 highly variable genes across the wild type samples are characterized to be involved in tolerance and/or resistance such as disease resistance and stress tolerance mostly by catalysis of primary and secondary metabolism and jasmonic-acid (JA) mediated signaling pathway (*e.g.* Os07g0218200, Os12g0240900, and Os04g0659300). Gene ontology (GO) analysis of up-regulated genes in wild type reveals enrichment of genes involved in response to stimulus, biotic stimulus, and secondary metabolic process (**Fig. 4.7A**). Down-regulated genes showed enrichment of general metabolic processes associated with bacterial infection (**Fig. 4.7B**). GO analysis also identified pathways associated with the up-regulated gene networks and with consistency showed that metabolic processes involved in plant defense such as tryptophan biosynthetic process, diterpene phytoalexin biosynthetic process, and aromatic amino acid family catabolic process are activated (**Fig. 4.7C**). It is also interesting to note that oxylipin biosynthetic process, the pathway involved in jasmonic acid (JA) biosynthesis, is also activated during *Pso* infection in wild type (**Fig. 4.7C**). These results reveal that the rice-*Pso* pathosystem is characterized by up-regulation of genes involved in primary and secondary metabolic pathways and that JA biosynthesis pathway is primarily involved in defense signaling upon *Pso* infection in segregated wild type rice utilized in this work.

Jasmonic acid (JA) and salicylic acid (SA) is known to exert mutual antagonism with each other to possibly fine-tune defense regulation during pathogen attacks (Thaler et al., 2012).

To test whether SA enhances or suppresses the observed *Pso*-resistance phenotype of *rCBP*$^{-/-}$ mutants, I primed the seeds of *rCBP 9-12b*$^{-/-}$ and performed pathogenesis assay. The results indicate that SA-seed primed *rCBP 12b*$^{-/-}$ lost the resistance against *Pso* (**Fig. 4.8A**). To verify this result, I primed the mature *rCBP 12b*$^{-/-}$ with sodium salicylate by spraying it twice to entire plant. Pathogenesis assay was performed 24 h after priming and the results confirmed the loss of resistance against *Pso* infection (**Fig. 4.8B**). These results suggest that JA-mediated defense signaling confers resistance to *rCBP*$^{-/-}$ mutant lines and that priming with SA antagonizes the conferred resistance.

As *rCBP*$^{-/-}$ mutants developed plant resistance, I hypothesized that *rCBP* possibly modulates negative regulators of JA-induced genes and that the loss of *rCBP* function derepresses JA-mediated resistance gene network against *Pso*. To test this hypothesis, I searched for transcription factors (TFs) that possibly negatively regulate JA-mediated defense genes and also possible targets of rCBP. Using Venn diagram, I overlapped up-regulated genes in *Pso*-treated wild type plants normalized to mock treatment, down-regulated genes in *Pso*-treated *rCBP*$^{-/-}$ mutant plants normalized to wild type, and known transcription factors differentially expressed in wild type upon *Pso*-treatment (**Fig. 4.9A**). The overlapping of 3 gene sets yielded seven putative rCBP targeted repressor transcription factors (**Fig. 4.9A; Fig. 4.9B**). These transcription factors are up-regulated in the presence of rCBP and down-regulated upon loss of rCBP activity during *Pso* infection (**Fig, 4.9B; Table 4.4**). Characterization of these TFs showed that they are all involved in plant immunity (Tsuda and Somssich, 2015). These results suggest that loss of rCBP diminishes enhancement of target repressor transcription factors in JA-mediated defense signaling during *Pso* infection. The decrease in enhancement of repressor TFs by loss of rCBP possibly resulted in derepression of target immunity genes causing enhanced resistance against *Pso*.

DISCUSSION

The role of acetylation of proteins including histones in plant-pathogen interaction has been recently explored and is in need of detailed investigation (Ding and Wang, 2015; Espinas et al., 2016; Song and Walley, 2016; Zhu et al., 2016). Several examples of histone acetyltransferases and deacetylases have been implicated in the regulatory control of transcriptional complexes involved in plant immunity and defense priming (*Note:* Refer to Chapter 1 for references). Quite recently, an *Arabidopsis* histone acetyltransferase, *HAC1*, is involved in plant responses to pathogens (Singh et al., 2014). Specifically, *HAC1* mutants are deficient in priming of the PAMP-triggered immunity (PTI) (Ding and Wang, 2015; Espinas et al., 2016; Singh et al., 2014). Here, I demonstrate that the rice CREB-binding protein, rCBP, an acetyltransferase and transcriptional coactivator is involved in local defense regulation of rice innate immunity. Using pathogenesis assay, I have shown that mutation of *rCBP* through CRISPR/Cas9 editing enhanced the resistance of $rCBP^{-/-}$ mutants against *Pseudomonas syringiae* pv. *oryzae* (*Pso*) infection (**Fig. 4.1C**; **Fig 4.3**). Contrastingly, HAC1 does not seem to directly regulate defenses in *Arabidopsis*; however, my data suggest that rCBP participates in local defense responses to *Pso* infection. Whether the immunity related phenotype observed and the acetyltransferase or co-transcriptional activator activities might be independent of each other or not is still unknown and will be further addressed in future experiments.

Morphological phenotypes of CRISPR/Cas9-*rCBP-S5* lines utilized in this section are in contrast with RNAi *rCBP* lines (**Fig. 2.2**; **Fig 2.5**; **Fig. 4.1A**; **Fig. 4.1B**). Unlike RNAi *rCBP* transgenic lines, CRISPR/Cas9-*rCBP-S5* edited lines have effective grains and tiller number similar to wild type (**Fig 4.1D**). Additionally, the level of H3K9ac is reduced in these lines similar to RNAi lines (**Fig. 3.7B**; **Fig. 4.1E**). I attribute these differences to the mistargeting of other rice CBP family members in RNAi lines and to the high accuracy of CRISPR/Cas9

editing (**Fig. 2.6**; **Fig. 4.1A**; **Fig. 4.1B**). Three off-target sites were predicted to occur in CRISPR/Cas9-*rCBP-S5* mutants, but were targeted to non-coding regions of the genome including intergenic and intronic regions. I have checked nonetheless the expression of intron and unstranslated region and found that these regions are not up- nor down-regulated in mutant lines. Whether rCBP acetylation at H3K9 site or transcriptional coactivation activity is responsible for the observed enhancement of resistance against *Pso* is not clear with the current data.

I also described here for the first time the rice-*Pseudomonas syringiae* pv. *oryzae* (rice-*Pso*) pathosystem (**Fig. 4.7**). *Pso* is a compatible pathogen of rice and the interaction can be described as a homologous interaction. The rice-*Pso* pathosystem is characterized by enrichment of genes involved in aromatic amino acid catabolic processes especially the tryptophan biosynthetic process that is known to participate in plant innate immune defenses against pathogen infections (**Fig. 4.6; Figure 4.7**) (Ishihara et al., 2008; Tzin and Galili, 2010). For example, tryptophan decarboxylase (Os08g0140300) is an important enzyme in linking inducible primary and secondary metabolism in plants (Ishihara et al., 2008). Genes such as kaurene synthase (Os12g0491800) and terpene synthase (Os07g0218200) are involved in terpenoid biosynthesis that also participates in a number of defense responses (Chen et al., 2011). Lignin and phytoalexin encoding genes for instance laccases (*e.g.* Os12g0258700, Os11g0641500) and cytochrome P450s (*e.g.* Os07g0218700, Os08g0508000) are metabolic products known to be involved in plant defenses as well (Swaminathan et al., 2009; Vanholme et al., 2010; Wang et al., 2012b). Another interesting feature of this pathosystem is the induction of oxylipin biosynthetic process that is involved in jasmonic acid (JA) production (**Fig. 4.6; Figure 4.7**) (Andreou et al., 2009; Brodhun and Feussner, 2011; Creelman and Mulpuri, 2002). Terpene synthase 3 (Os07g0218200), naringenin 7-O-methyltranferase (Os12g0240900), and root meander curling

(Os04g0659300) genes are responsive to jasmonic-mediated defense signaling (Jiang et al., 2007; Lee et al., 2015; Shimizu et al., 2012). Thus, the rice-*Pso* pathosystem is a functional system that can be utilized to analyze the defensive roles of aromatic amino acid and jasmonate-mediated pathway in rice infected with *Pseudomonas syringiae* pv. *oryzae* pathogen.

As *rCBP*$^{-/-}$ mutants gained resistance, it could be that rCBP protein targets repressor transcription factors (TFs) that play roles in JA-mediated defense signaling (**Fig. 4.9**). I have identified seven rCBP-targeted transcription factors that are documented to have negative regulatory activity to its targets. OsRAV2 (AP2/EREBP129) (OS01G0141000) is a DNA binding TF known as negative regulator of flowering by repressing florigenic molecules (Matias-Hernandez et al., 2014; Rashid et al., 2012); although, tomato RAV genes have been found to positively regulate disease resistance as it is constitutively expressed in *AtCBF1* overexpressing tomato plants (Li et al., 2011a). OsbHLH035 (Os01g0159800) is a basic/helix-loop-helix TF that possibly acts as negative regulator, although there have been few studies on this gene found so far (Li et al., 2006). ONAC085 (Os05g0194500) and ONAC103 (Os07g0683200) are candidate negative regulators found in my analysis with currently known function in viral infection and abiotic stress response in rice (Fang et al., 2008; Nuruzzaman et al., 2015). OsWRKY28 (Os06g0649000) is documented to transcriptionally repress PAMP-response and negatively regulates innate immune responses in blast-infected rice (Chujo et al., 2013; Xie et al., 2005). Interestingly, expression of OsWRKY28 is highly regulated by JA suggesting that OsWRKY28 is a DNA binding TF with negative regulatory function in JA-mediated defense responses (Chujo et al., 2013; Miyamoto et al., 2012). OsbZIP65 (Os08g0357300) is another negative regulator found to be down-regulated upon abiotic stress and also in panicle and seed development (Nijhawan et al., 2008). Lastly, OsJAmyb (Os11g0684000) is found in my analysis as putative negative

regulator of defense as it interacts with rCBP. This is in contrast with the known positive regulatory action of OsJAmyb in response to JA during fungal infection (Lee et al., 2001). This contrasting finding may indicate a reverse regulatory role of OsJAmyb in rice-*Pso* pathosystem and or during rCBP interaction and thus needed a more detailed investigation. Overall, these putative rCBP-dependent repressive transcription factors regulate rice innate immunity (Tsuda and Somssich, 2015), but may be acting on independent defense pathways at certain temporal scales during DNA regulatory binding.

It is also clear from my results that growth and developmental aspect of *rCBP*$^{-/-}$ mutants is not limited by resistance phenotype (**Fig. 4.1C**; **Fig. 4.1D**). It has been shown in several works that plant resistance imposes tradeoff to growth especially in constitutively expressed defense gene and gene networks (Gurr and Rushton, 2005; Huot et al., 2014; Karasov et al., 2017). Recently, it was shown that transgenic rice with constitutive expression of *AtNPR1* gene had stunted growth in the absence or presence of pathogen indicating that SA-mediated NPR1 regulation is characterized by high resource partitioning in favor of defenses at the expense of growth (Bailey-Serres and Ma, 2017; Xu et al., 2017a; Xu et al., 2017b). In this study, resistance phenotype in *rCBP*$^{-/-}$ mutants in the rice-*Pso* pathosystem is driven most probably by JA-mediated defense pathways instead of SA-pathway (**Fig 4.6**; **Fig 4.7**; **Fig. 4.8**; **Fig 4.9**). If this is the case, it is evident from the results that a JA-mediated pathway does not impose growth tradeoffs in *rCBP*$^{-/-}$ mutants in the absence or presence of *Pso* infection. Also, a number of candidate repressor transcription factors regulated by rCBP are negative regulators of flowering and development. This suggests that upon the loss of rCBP, these repressors are attenuated allowing growth and development. Whether these are regulated or not by JA pathway is not known. Additionally, up-regulated genes in *rCBP*$^{-/-}$ mutant during *Pso* infection showed enrichment of photosynthesis-related pathways suggesting limited restriction on growth during defense activation (**Table 4.2**).

In general, these results indicate that a repressor transcription factor (rTF) is possibly regulated by rCBP either by binding to its *cis* element, CRE, through CREB transcription factors or by interacting with transcription factors involved in basic transcriptional machinery especially during *Pso* infection (**Fig. 4.10**). The loss of rCBP down-regulates these rTFs involved not only in defense, but also in flowering and developmental processes. These in turn permit growth and development along with the conservation of resistance phenotype during pathogenesis. Genomewide analysis suggests that this proposed mechanism is regulated under JA-mediation as evidenced by gene ontology analysis and the finding of JA-regulated rTFs involved in both defense and growth. However, an alternative explanation might also give light to the results presented herein. As this model explains the result from a host perspective, it is also imperative that a pathogen perspective explanation should be considered.

FIGURES & TABLES

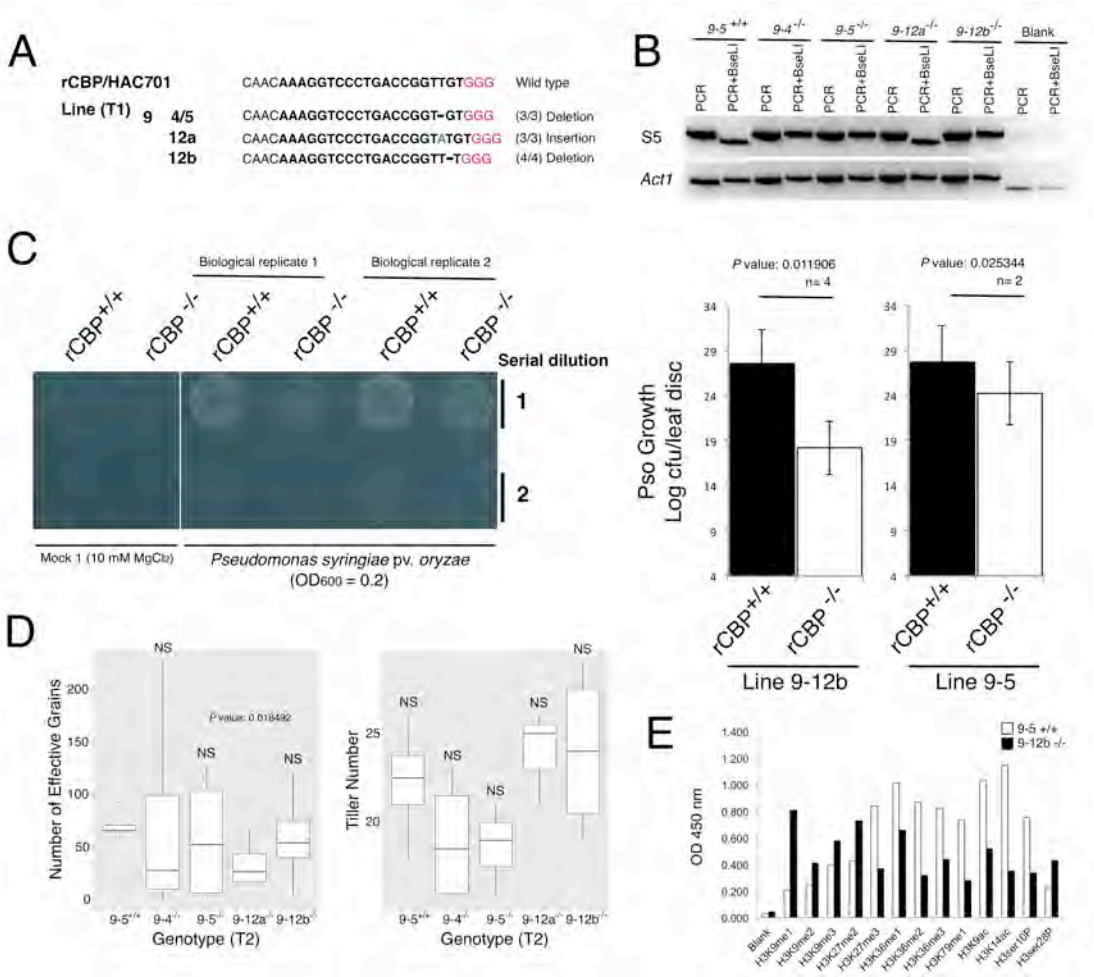


Figure 4.1. Characterization of biallelic homozygous CRISPR/Cas9 lines targeting the fifth exon of rice acetyltransferase gene, *rCBP*

(A) Alleles from four lines of the second generation (T1) plants were identified by cloning and sequencing the PCR products from *rCBP* target region using the forward (F) and reverse (R) primers found in **Supp Table 2.2**. For each line, 3-4 DNA amplicons were cloned and sequenced and the fraction indicates the number of times the types of mutations were found in each line. (B) PCR and RFLP assays of representative T1 generation lines. (C) Pathogenesis assay of *rCBP 9-5*$^{-/-}$ and *9-12b*$^{-/-}$ lines (T2) using *9-5*$^{+/+}$ segregated wild type line as control. The left panel is a representative photo of pathogenesis assay using *rCBP 9-12b*$^{-/-}$ (T2) in two biological replicates. The right panel is quantification of the pathogenesis assays using values from 4th- 6th serial dilutions. Line *9-12b*$^{-/-}$ was grown from seeds, while *9-5*$^{-/-}$ was embryonically rescued. All mock measurements yielded zero bacterial

growth for both wild type and mutant lines (C). (D) Phenotype of T2 lines showing effective grains and tiller number. (E) Histone H3 modification assay showing major modification patterns in *rCBP 9-12b*$^{-/-}$ (T2). Absorbance (OD) measurement was in duplicate and was normalized over H3 values. Bars represent the 95% confidence interval (CI) and compared from the wild type using two-tailed Student's *t*-test at $P < 0.05$ in *9-12b*$^{-/-}$; one-tailed Student's *t*-test at $P < 0.05$ in *9-5*$^{-/-}$ (C). Bars are standard error of the mean using two-tailed Student's *t*-test at $P < 0.05$ compared to the wild type (D).

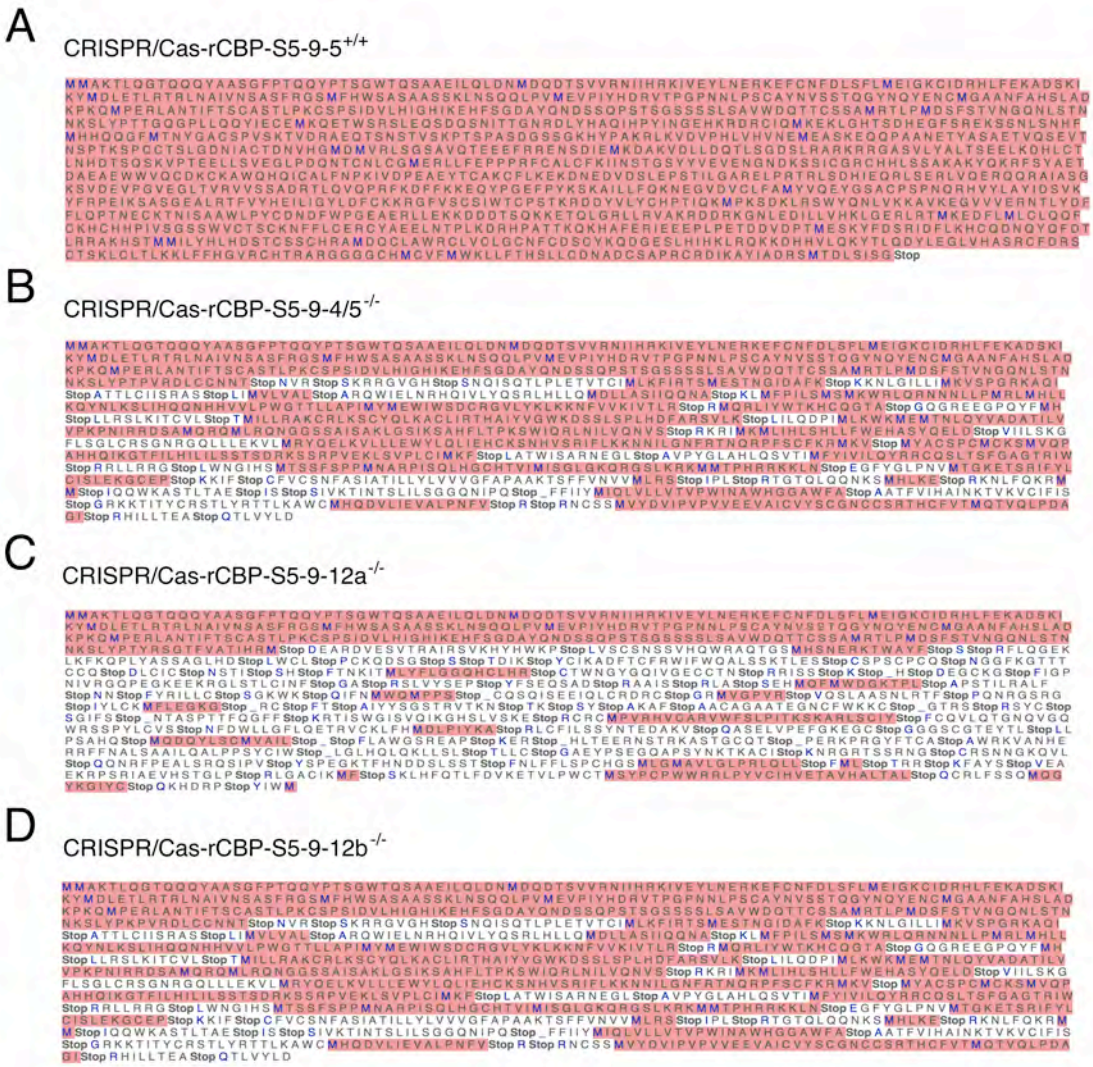


Figure 4.2. Expected open reading frameshift in rCBP protein from the insertion/deletion (INDEL) mutations catalyzed by CRISPR/Cas9 S5 sgRNA

Protein sequences of wild type rCBP (A), rCBP 9-4/5$^{-/-}$ (B), rCBP 9-12a$^{-/-}$ (C), and rCBP 9-12b$^{-/-}$ (D). Coding sequences were taken from the Rice Annotation Project Database (RAP-DB). Translation was performed using ExPASy Translation web tool.

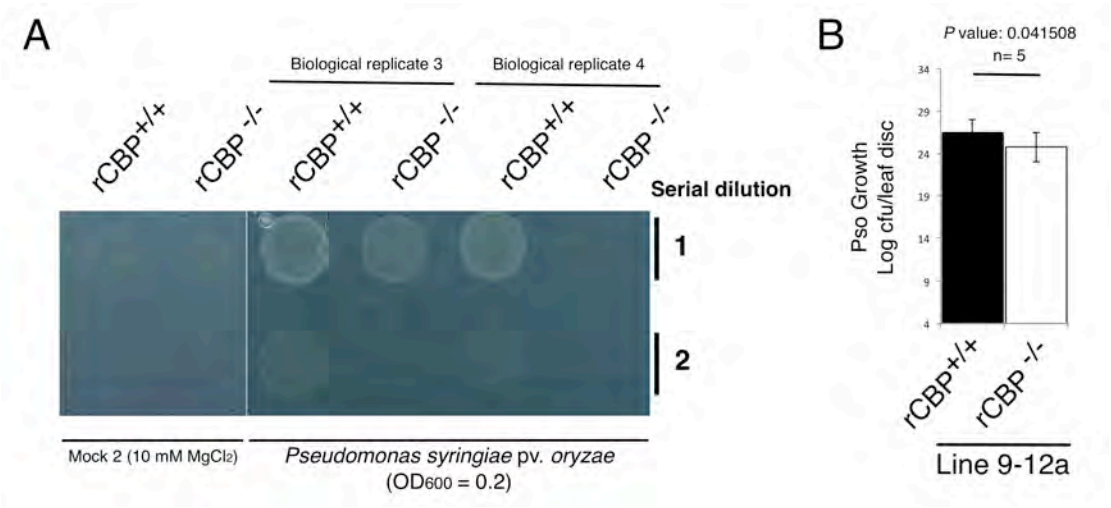


Figure 4.3. Pathogenesis assay in T2 *rCBP*$^{-/-}$ mutant lines

(A) Additional biological replicates of EDS assay in *rCBP 9-12b*$^{-/-}$. (B) Quantification of the EDS assays using values from 4th-6th serial dilutions in *rCBP 9-12a*$^{-/-}$ insertion mutation line. *9-5*$^{+/+}$ segregated wild type line was used as control. Error bars represent the 95% confidence interval (CI) and compared to wild type using one-tailed Student's *t*-test at P <0.05.

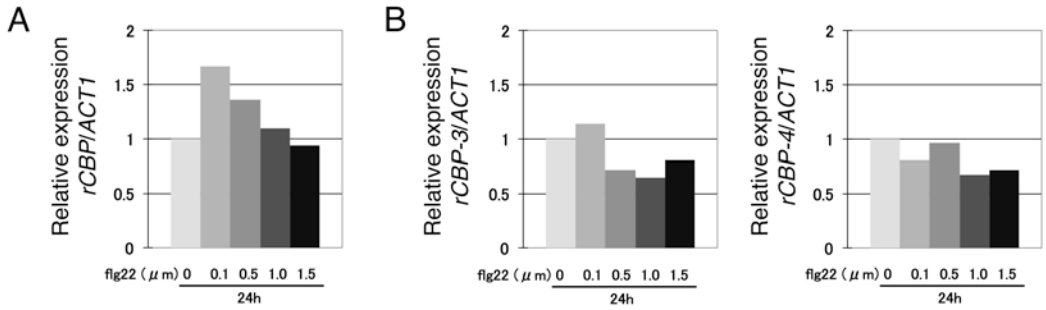


Figure 4.4. flg22 did not induce the gene expression of *HAC701/rCBP* in *rCBP 9-12b*$^{-/-}$ background mutant line

(A) Transcriptional levels of *rCBP* gene under flg22 treatment at different concentration levels in μM units under 24 h treatment. (B) Transcriptional levels of *rCBP* gene are measured at three different sites of the gene under different concentration gradients of flg22 peptide. Relative expression were normalized over the expression of 0 μM treated (Mock) mutant sample.

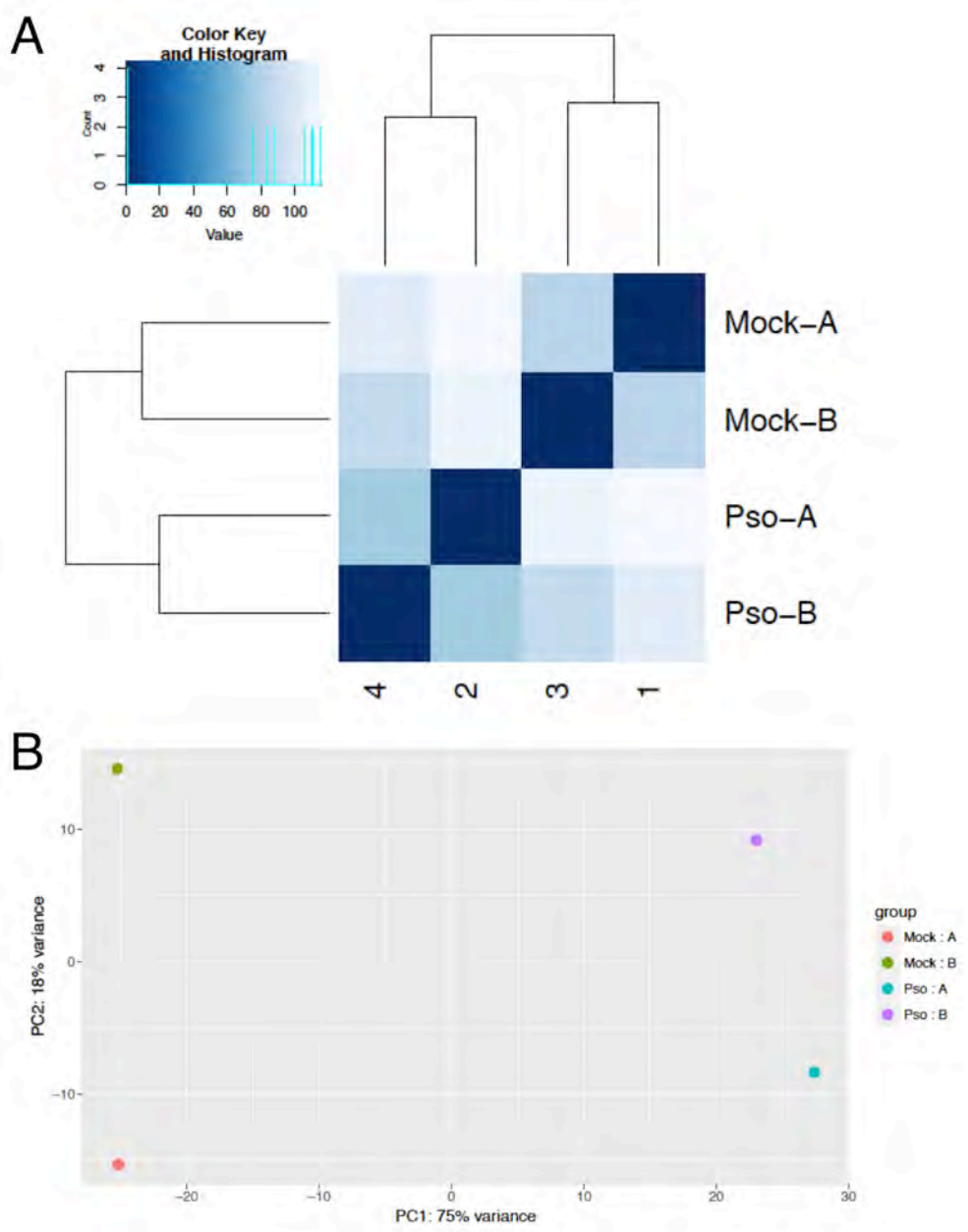
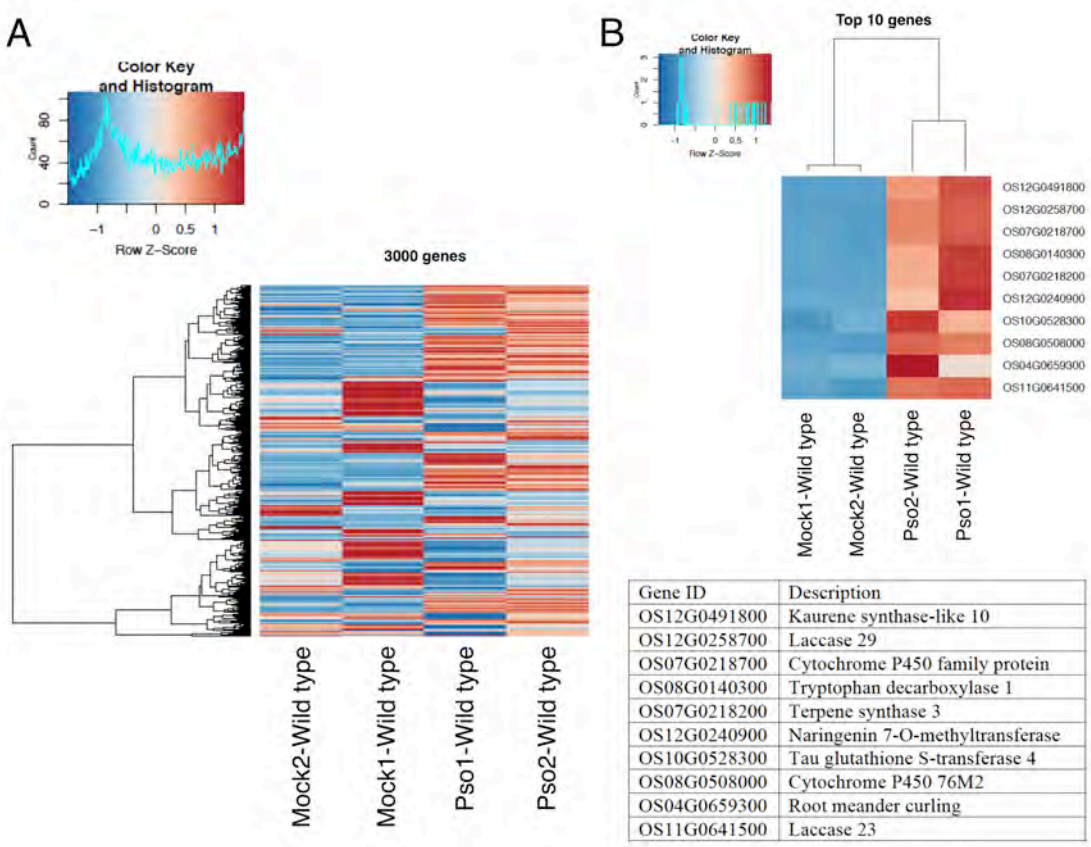


Figure 4.5. Sample distances of RNA-sequencing data after regularized-logarithm transformation (rlog)

(A) Heatmap showing the Euclidean sample distance matrix of mock- and *Pseudomonas syringiae* pv. *oryzae* (*Pso*)-treated samples in two biological replicates each. (B) Principal component analysis (PCA) of the mock- and *Pso*-treated RNA-sequencing samples. Principal component 1 (PC1) compares the variance between mock- versus *Pso*-treated samples. Principal component 2 (PC2) compares the variance between biological replicates within treatments.



Gene ID	Description
OS12G0491800	Kaurene synthase-like 10
OS12G0258700	Laccase 29
OS07G0218700	Cytochrome P450 family protein
OS08G0140300	Tryptophan decarboxylase 1
OS07G0218200	Terpene synthase 3
OS12G0240900	Naringenin 7-O-methyltransferase
OS10G0528300	Tau glutathione S-transferase 4
OS08G0508000	Cytochrome P450 76M2
OS04G0659300	Root meander curling
OS11G0641500	Laccase 23

Figure 4.6. Gene clustering of mock- and *Pseudomonas syringiae* pv. *oryzae* (*Pso*)-treated RNA-sequencing samples

(A) Heatmap of 3000 genes and (B) top 10 genes that are most highly variable in mock- and *Pso*-treated RNA-sequencing samples. Gene descriptions were derived from Oryzabase: Integrated Rice Science Database (NBRP) and Rice Genome Annotation Project (NSF). Two independent RNA-sequencing replicates are presented.

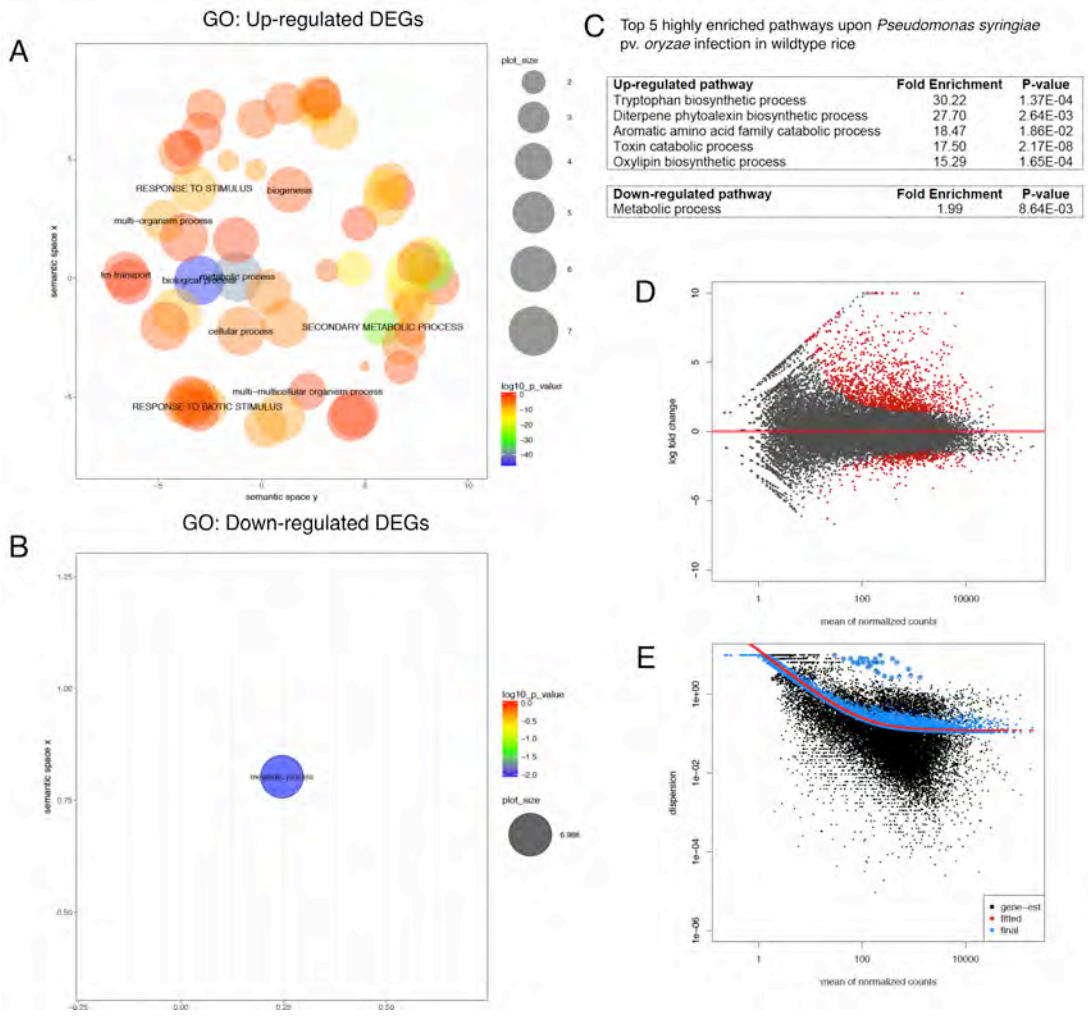


Figure 4.7. The rice-*Pseudomonas syringiae* pv. *oryzae* (*Pso*) pathosystem

(A) Gene ontology (GO) analysis of 798 up-regulated differentially expressed genes (DEGs) and (B) 72 down-regulated DEGs in wild type samples upon 72 h post-infection of *Pso.* (C) Enriched pathways in the rice-*Pso* pathosystem. (D) MA-plot of the rice-*Pso* pathosystem showing log fold changes upon 72 h post *Pso* infection. *P* adjusted value is set at < 0.1. (E) Plot of dispersion estimates of genes in the rice-*Pso* pathosystem. The red line indicates the trend showing the dispersions' dependence on the mean. The blue points are the final estimate of each gene in reference to the red line. The blue circles are genes with high gene-wise dispersion estimates and are considered outliers. Two independent RNA-sequencing data in mock and *Pso* treatments are presented where DEGs were selected at *p* adjusted value <0.01. A and B are scatter plot view based on dispensability criterion (Up-regulated genes = 0.01; Down-regulated genes = 0.15).

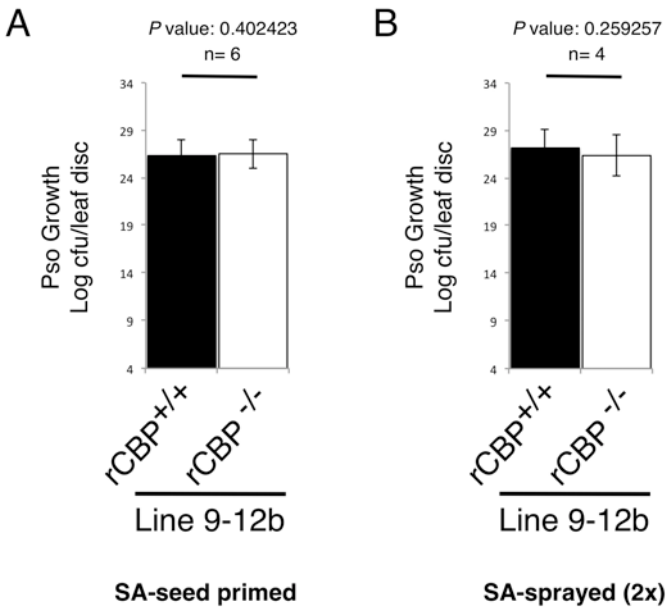


Figure 4.8. Pathogenesis assay of salicylic acid (SA)-primed plants

(A) *Pso* growth in wild type and *rCBP 9-12b*$^{-/-}$ (T2) plants seed primed with 500 µM SA for 3 days in the dark. (B) *Pso* growth in wild type and *rCBP 9-12b*$^{-/-}$ (T2) plants sprayed twice with 1 mM sodium salicylate before pathogen infection. Error bars represent the 95% confidence interval (CI) and compared to wild type using one-tailed Student's *t*-test at $P < 0.05$. Mock controls were performed and did not yield any bacterial colonies.

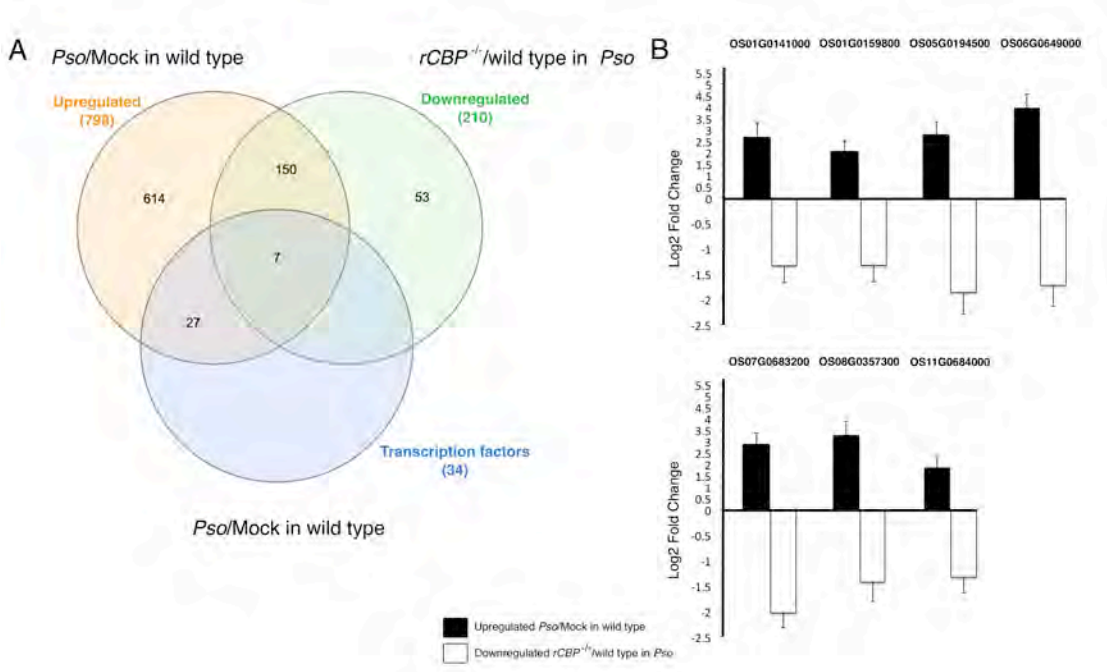


Figure 4.9. rCBP-dependent repressor transcription factors (TFs) in rice-*Pso* pathosystem

(A) Venn diagram showing the identification of seven putative rCBP targets in the regulation of basal defense system in locally infected tissues. (B) Log2 fold changes in seven repressor transcription factors showing up-regulation in wild type infected plants and down-regulation in *rCBP*$^{-/-}$ infected mutants. Two independent RNA-sequencing samples in both treatment and condition are presented. Bars in B indicate standard error bars at *P* adjusted value < 0.01.

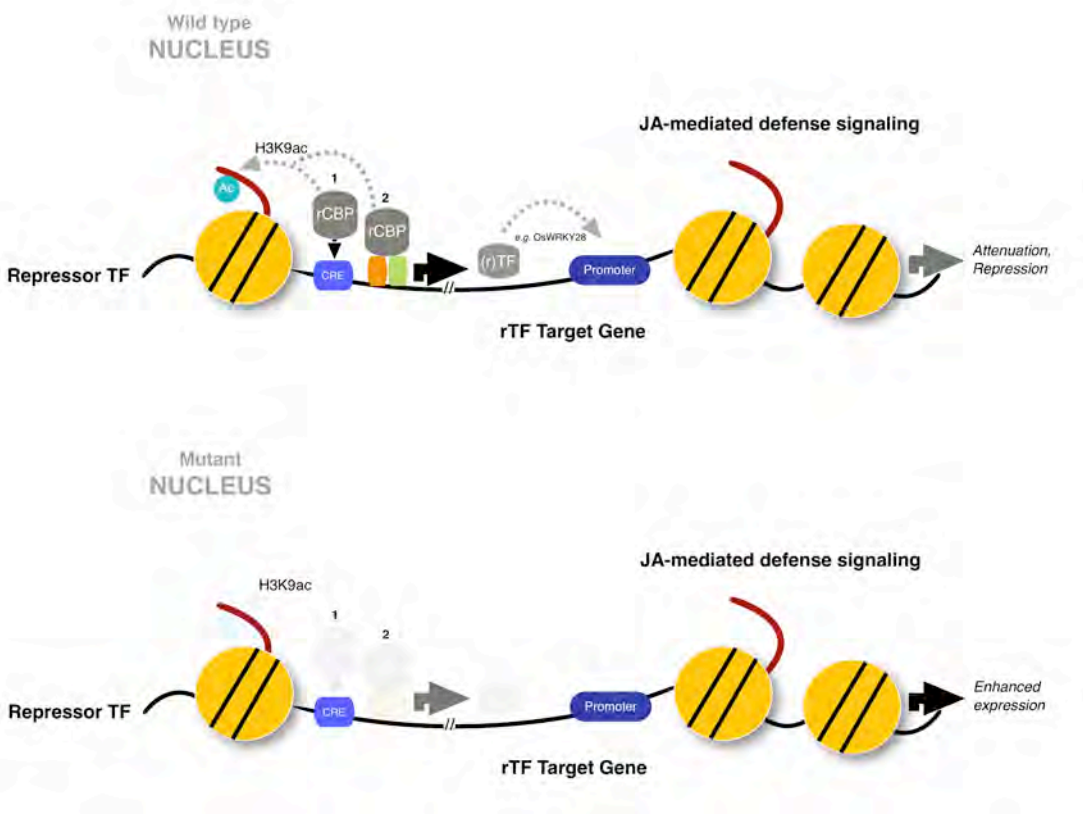


Figure 4.10. A possible model for rCBP-dependent basal immune defense mechanism in rice during *Pso* infection

In the wild type, rCBP regulates repressor transcription factors (rTF) that in turn negatively regulate JA-mediated signaling defense pathway genes and gene networks. There are two possible ways rCBP can be targeted to genes: (1) rCBP can bind to CREB transcription factors that can recognize CRE *cis* elements; or (2) rCBP can bind to other transcription factors associated with the rTF gene promoter. Upon recognition, rCBP possibly acetylates surrounding H3K9 sites to activate the rTF. The expression of rTF down-regulates JA-mediated defense gene. On the other hand, mutants do not express rCBP, which leads to down-regulation of expression of rTF. JA-mediated defense gene in mutant is activated without the repressive activity of the rTF and thus confers resistance.

Table 4.1. Oligonucleotides used for RT-qPCR.

Primer Name	Forward (5'-3')	Reverse (5'-3')
ACT1	TCCATCTTGGCATCTCTCAG	TGGCTTAGCATTCTTGGGTC
HAC701/rCBP	TGGCGGTGCTTGGTTTGCCT	ACGGGCACGGGTATGACATCGT
rCBP-3	TCCAACCACGTTTCAAGGAT	GTACGGAAATTCCCCAGGAT
rCBP-4	TCCAACCACGTTTCAAGGAT	CTTGCACATACATGGCGAAC

Table 4.2. Repository information of deposited local tissue RNA-sequencing and related files

File name	Type	Reference No.	Database
1_11_9-5_Mock1plus	.bam file	TBD	TBD
1_11_9-5_Mock1minus	.bam file	TBD	TBD
1_11_9-5_Pso6plus	.bam file	TBD	TBD
1_11_9-5_Pso6minus	.bam file	TBD	TBD
3_6_9-5_Mock1plus	.bam file	TBD	TBD
3_6_9-5_Mock1minus	.bam file	TBD	TBD
3_6_9-5_Pso1plus	.bam file	TBD	TBD
3_6_9-5_Pso1plus	.bam file	TBD	TBD
Up_regulated_in_psovsmock_wildtypeONLY	.csv file	TBD	TBD
Down_regulated_in_psovsmock_wildtypeONLY	.csv file	TBD	TBD
Up_regulated_mtvswt_in_psoONLY	.csv file	TBD	TBD
Down_regulated_mtvswt_in_psoONLY	.csv file	TBD	TBD

*To be deposited (TBD): Files will be deposited to OIST Institutional Repository & Research Data Archive after the final version of the thesis is completed. Plus (wild type); Minus (mutant).

Table 4.3. Summary of total RNA-sequencing data in local tissues

File name	Total Reads[1]	Mapped Reads[1]	% Mapped
1_11_9-5_Mock1plus	70, 690, 605	68, 735, 549	97.2
1_11_9-5_Mock1minus	72, 816, 919	70, 926, 649	97.4
1_11_9-5_Pso6plus	52, 183, 901	50, 842, 183	97.4
1_11_9-5_Pso6minus	80, 050, 844	78, 036, 770	97.5
3_6_9-5_Mock1plus	74, 398, 971	72, 210, 799	97.1
3_6_9-5_Mock1minus	84, 570, 915	81, 886, 941	96.8
3_6_9-5_Pso1plus	82, 187, 395	79, 813, 459	97.1
3_6_9-5_Pso1plus	76, 959, 170	74, 640, 024	97.0

[1] Left and right reads were combined.

Concordant pair alignment rate for all files are above 94%.

Table 4.4. Putative repressor transcription factors interacting with rCBP to repress JA-mediated defense signaling during *Pso* infection

Gene ID	Family	Description
OS01G0141000	AP2-EREBP	B3 DNA binding domain containing protein, expressed, putatively OsRAV2, related to ABI3/VP1-2, AP2/EREBP129
OS01G0159800	bHLH	basic helix-loop-helix, putative, expressed, putatively OsbHLH035
OS05G0194500	NAC	no apical meristem protein, putative, expressed, putatively ONAC085
OS06G0649000	WRKY	OsWRKY28 - Superfamily of TFs having WRKY and zinc finger domains, expressed
OS07G0683200	NAC	no apical meristem protein, putative, expressed, putatively ONAC103, OsNAC18
OS08G0357300	bZIP	bZIP transcription factor domain containing protein, expressed, putatitvely OsbZIP65
OS11G0684000	MYB	MYB family transcription factor, putative, expressed, putatively OsJAmyb, JA-regulated transcription factor

Chapter 5

Systemic gene expression in rice-*Pso* pathosystem

Summary:

Rationale:

The purpose of this chapter is to investigate gene expression of systemic or distal tissues in wild type and *rCBP*$^{-/-}$ mutant three days after local infection with *Pseudomonas syringiae* pv. *oryzae*. This aim was addressed by performing RNA-sequencing on systemic or distal tissues excluding the roots.

Results:

This chapter presents two major results:

1. Systemic gene expression in wild type plants in rice-*Pso* pathosystem has substantial amount of differentially expressed genes (DEGs), although it is significantly less than the DEGs in locally infected tissues.
2. Mutation in *rCBP* gene resulted in the reduction of expressed genes as compared to wild type indicating a possible role of rCBP-dependent regulation in systemic defense responses in rice.

RESULTS

To explore the nature of systemic gene expression in rice-*Pso* pathosystem, I performed RNA-sequencing on systemic or distal tissues of wild type and *rCBP*$^{-/-}$ mutant plants. Genomewide transcriptome analysis in wild type samples treated with *Pso* showed that 24 genes were differentially expressed, and among them were eight genes that were common to both local and systemic tissues (**Fig. 5.1A; Table 5.1; Table 5.2**). Comparative analysis also showed that although there were substantial expression changes in systemic tissues, the number of DEGs was highly reduced in systemic tissues than in local tissues (**Fig. 5.1A**). Among the eight DEGs, three of them showed altered transcriptional expression from local to systemic tissues (**Fig. 5.1B**). These genes are terpene synthase (OS04G0344100), putative cytochrome P450 (OS09G0275400), and mannose-specific jacalin-related lectin/OsJAC1 (OS12G0247700), all with putative functions in response to pathogen infection (**Table 5.3**). These genes showed increased up-regulation of expression in the locally infected tissues and down-regulation in systemic or distal tissues, indicating that these genes among others are coordinately regulated in response to pathogen attack. These results also indicate that these genes are mostly activated locally in the infection site and that any altered expression in distal tissues may facilitate systemic acquired resistance. To investigate the nature of co-expressed genes with the three genes showing altered expression, I performed correlation analysis in RiceXPro platform and found that correlated genes with terpene synthase, cytochrome P450, and OsJAC1 are mostly defense response genes (**Fig. 5.2**). It is also interesting to note that each input gene apparently is correlated to genes having similar features as the other input genes, thus indicating that they are possibly regulated in similar defense pathway. Heat map of correlated genes of the three input genes were up-regulated mostly in leaves, pre-reproductive structures, and specific reproductive plant parts suggesting that these genes are activated locally and systemically in leaf tissues and may well overlap with vegetative-

reproductive transition (**Fig. 5.2**). Gene network of *OsJAC1* (OS12G0247700) gene also showed up-regulation in inflorescence and anther reproductive parts possibly implying a reproductive role via JA-mediated defenses (**Fig. 5.2c**). To compare the effect of *rCBP* mutation on the difference of expression of DEGs, I analyzed the MA-plots of systemic tissues of wild type and *rCBP*$^{-/-}$ mutants under *Pso* infection. My results show that the number of genes with significant expression (*i.e.* those that are in red) was diminished in the mutant background (**Fig 5.3**). This also demonstrates the possible role of *rCBP* in systemic signaling in rice-*Pso* pathosystem. Overall, these results indicate that systemic tissues in *Pso*-challenged wild type plants have augmented transcriptional gene expression that possibly aims to 'prime' distal tissues on the onset of secondary pathogen attack. This also indicates that *rCBP* might potentially regulate systemic defenses through an unknown mechanism at distal non-infected site in preparation for future infection episodes.

DISCUSSION

Defense priming phenomenon is an induced sensitized state of a plant that allows a more rapid and robust activation of defense responses upon secondary chemical or pathogen challenge compared to a non-primed plant (Balmer et al., 2015; Conrath et al., 2015). It is an integral component of resistance-inducing mechanism termed as systemic acquired resistance (SAR), a wide-spectrum state of enhanced defense in whole plant upon recognition of microbe-derived molecules from pathogens. Here, I demonstrate that a number of genes in systemic or distal tissues of wild type *Pso*-infected plants in rice-*Pso* pathosystem are differentially expressed and a fraction of these expressed genes is similarly regulated in local tissues for three days after infection (**Fig. 5.1A; Table 5.3**). An infection period of three days is deemed sufficient to induce and measure systemic gene expression in plants. This result had also been identically observed previously in genes and gene networks of systemic tissues in several plant pathosystems (Penninckx et al., 1996; Schenk et al., 2003; Sun et al., 2011). Of the 24 genes that showed differential expression in systemic tissues, eight genes were common to both local and systemic tissues. Three of them, terpene synthase, cytochrome P450, and jacalin-related lectin/OsJAC1, are oppositely regulated in local and systemic tissues indicating that these genes are mostly utilized in locally infected sites and that their down-regulation at systemic tissues may mean modulation of defense signaling responsible for their expression. It is interesting to note that these three genes have been shown to be regulated by JA (Devoto and Turner, 2003; Lannoo and Van Damme, 2014; Singh and Sharma, 2015), thus my results suggest that systemic defense in rice-*Pso* pathosystem is predominantly JA-dependent and is well consistent with the features found in the basal defense mechanism. However, these results are limited to systemic gene expression only and there is a need to fully address through experimental analysis the features of defense priming

in this pathosystem. Secondary infection on systemic leaves of locally infected plants has not been performed yet to elucidate the components of defense priming in rice-*Pso* pathosystem. Priming experiment is warranted in this pathosystem as hypersensitive response with programmed cell death was observed in the locally-infected tissues (Shah, 2009). This indicates that sufficient dosage of *Pso* was utilized that could lead to systemic acquired resistance.

I also demonstrate here the reduction in number of differentially expressed genes (DEGs) in *rCBP*$^{-/-}$ mutant background as compared to wild type upon *Pso* infection (**Fig. 5.3**). Singh et al. (2014) showed that HAC1-dependent pathway executes defense priming against bacterial pathogen only when plants have been exposed to several abiotic stresses. However, my results show that rCBP-dependent regulation of defense is not limited to basal defense in the locally infected site, but may also regulate systemic and eventually priming defense via JA-mediated pathways. The diminished number of DEGs may indicate that rCBP through JA-defense pathway orchestrates systemic gene expression that may lead to the development of a 'primed state' and systemic acquired resistance. My results further indicate that rCBP-dependent regulation of rice innate immunity is mostly reliant on JA hormone signaling in rice-*Pso* pathosystem, although our genomewide analysis also indicates overlap of expressed genes and gene networks regulated by SA- and other defense related plant hormones.

FIGURES & TABLES

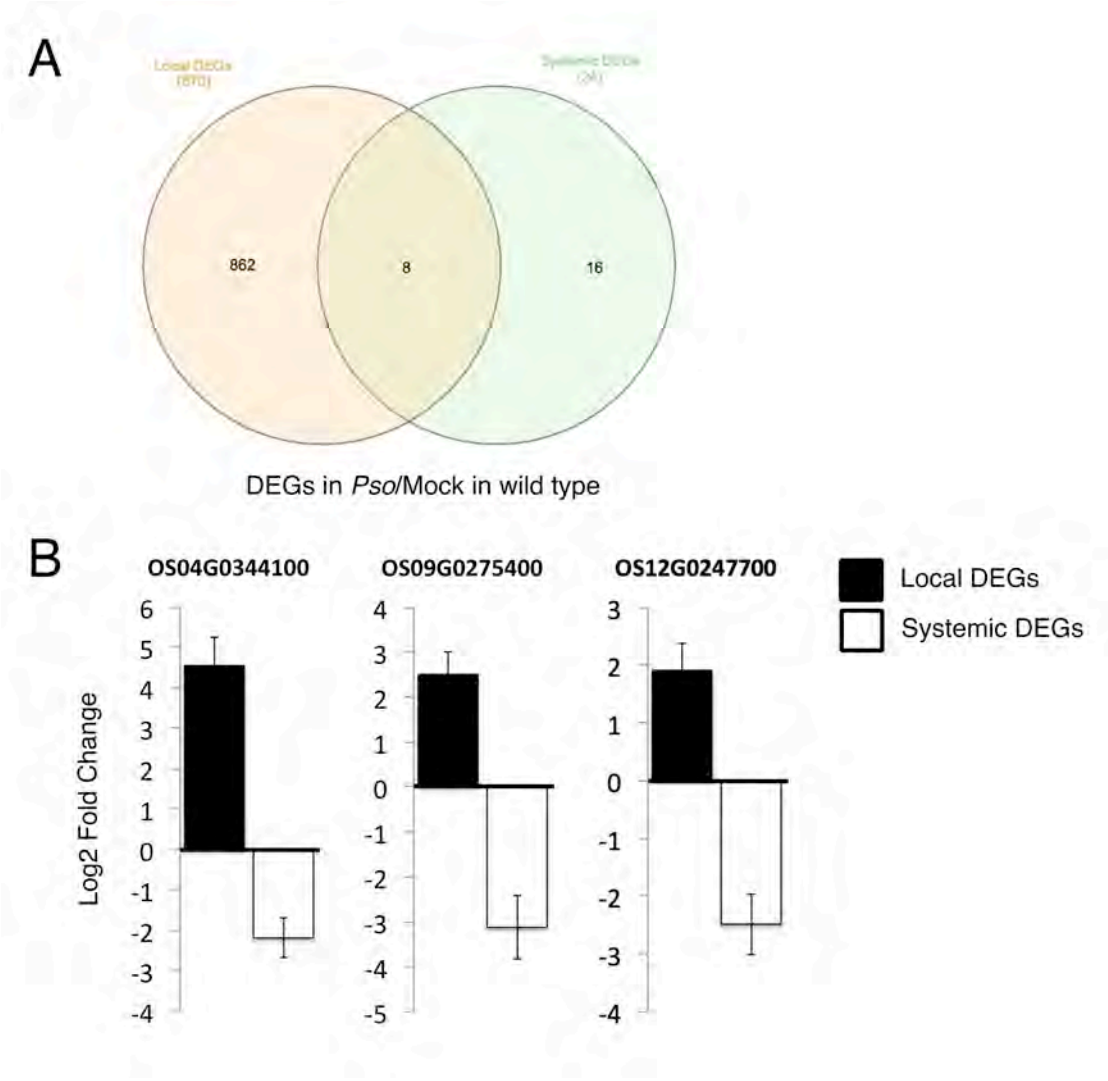
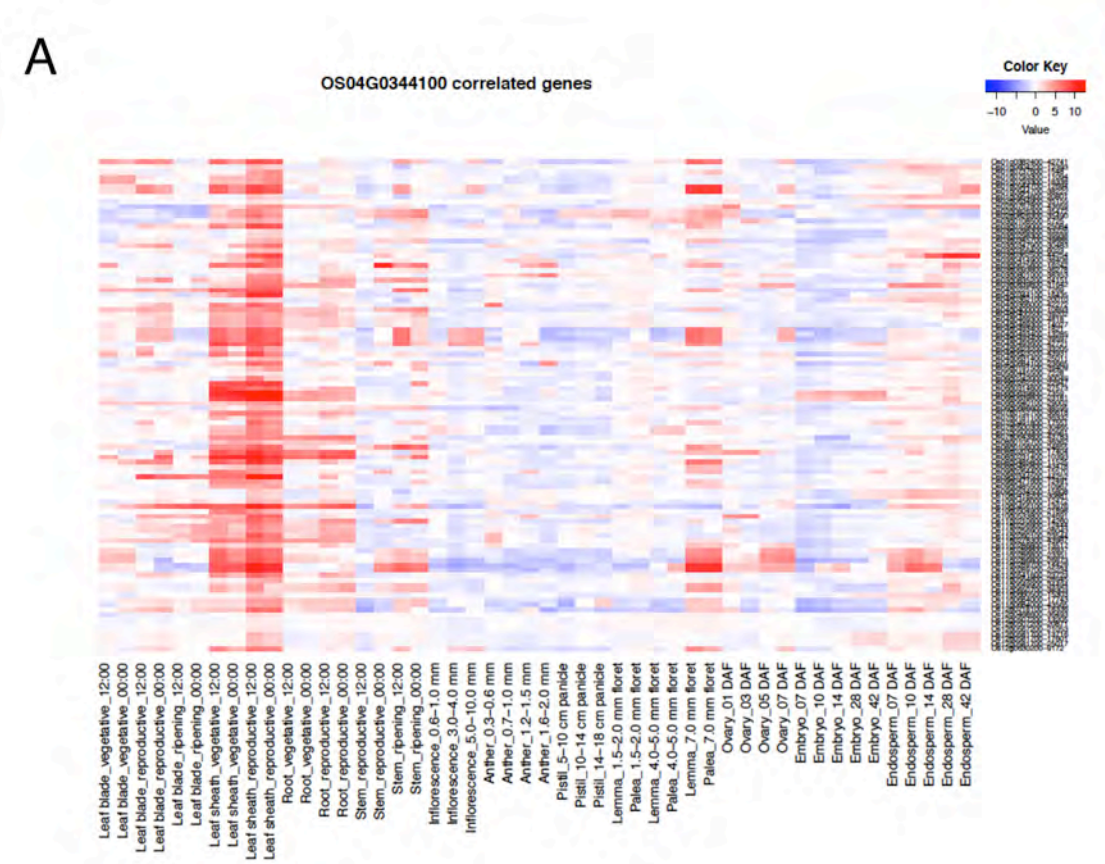


Figure 5.1. Systemic gene expression analysis in rice-*Pso* pathosystem

(A) Differentially expressed genes (DEGs) in the local (870) and systemic (24) tissues of plants infected with *Pseudomonas syringiae* pv. *oryzae* (*Pso*). Eight DEGs were found to be common in both local and systemic tissues. (B) Three of the eight DEGs were oppositely expressed in local and systemic tissues, respectively shown in log2 fold changes. For local tissue analysis, two independent RNA-sequencing samples in both treatments (e.g. mock and *Pso*) are presented. For systemic tissue analysis, two independent RNA-sequencing samples in mock treatment and three in *Pso* treatment are presented. Bars in B indicate standard error bars at *P* adjusted value < 0.01.

A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Stem_ripening_12:00
Stem_ripening_00:00
Inflorescence_0.6–1.0 mm
Inflorescence_3.0–4.0 mm
Inflorescence_5.0–10.0 mm
Anther_0.3–0.6 mm
Anther_0.7–1.0 mm
Anther_1.2–1.5 mm
Anther_1.6–2.0 mm
Pistil_5–10 cm panicle
Pistil_10–14 cm panicle
Pistil_14–18 cm panicle
Lemma_1.5–2.0 mm floret
Palea_1.5–2.0 mm floret
Lemma_4.0–5.0 mm floret
Palea_4.0–5.0 mm floret
Lemma_7.0 mm floret
Palea_7.0 mm floret
Ovary_01 DAF
Ovary_03 DAF
Ovary_05 DAF
Ovary_07 DAF
Embryo_07 DAF
Embryo_10 DAF
Embryo_14 DAF
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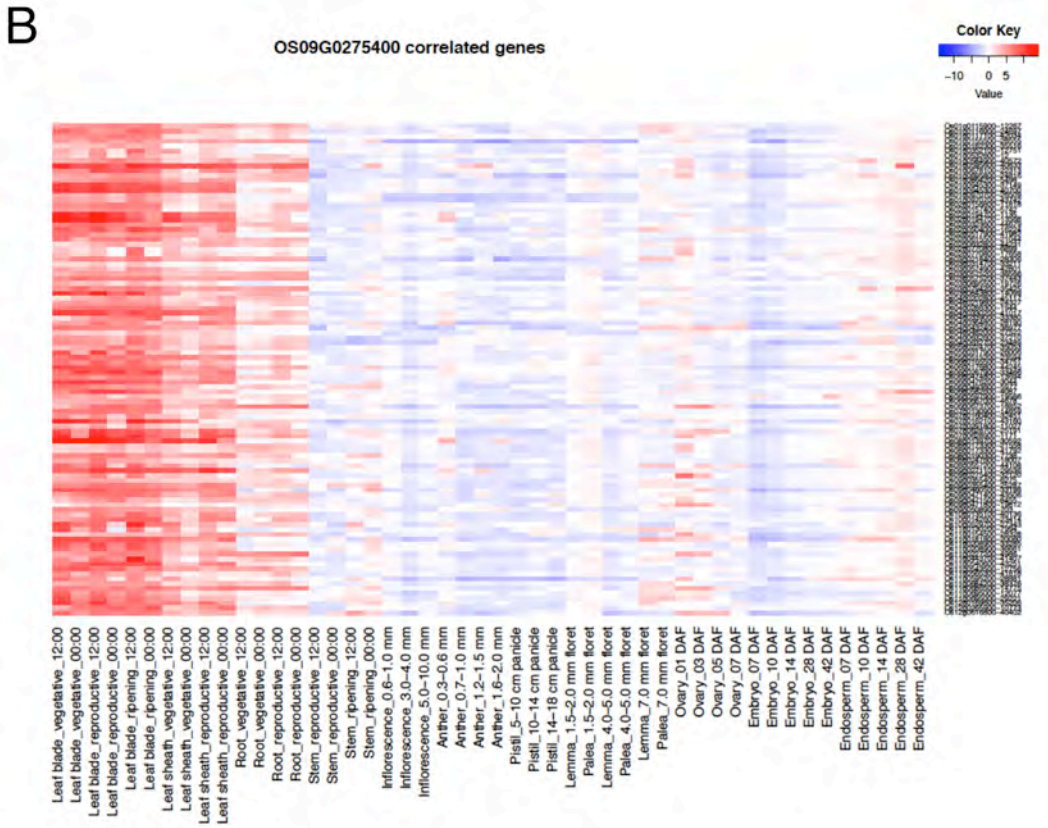
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Stem_ripening_12:00
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Inflorescence_5.0–10.0 mm
Anther_0.3–0.6 mm
Anther_0.7–1.0 mm
Anther_1.2–1.5 mm
Anther_1.6–2.0 mm
Pistil_5–10 cm panicle
Pistil_10–14 cm panicle
Pistil_14–18 cm panicle
Lemma_1.5–2.0 mm floret
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Palea_7.0 mm floret
Ovary_01 DAF
Ovary_03 DAF
Ovary_05 DAF
Ovary_07 DAF
Embryo_07 DAF
Embryo_10 DAF
Embryo_14 DAF
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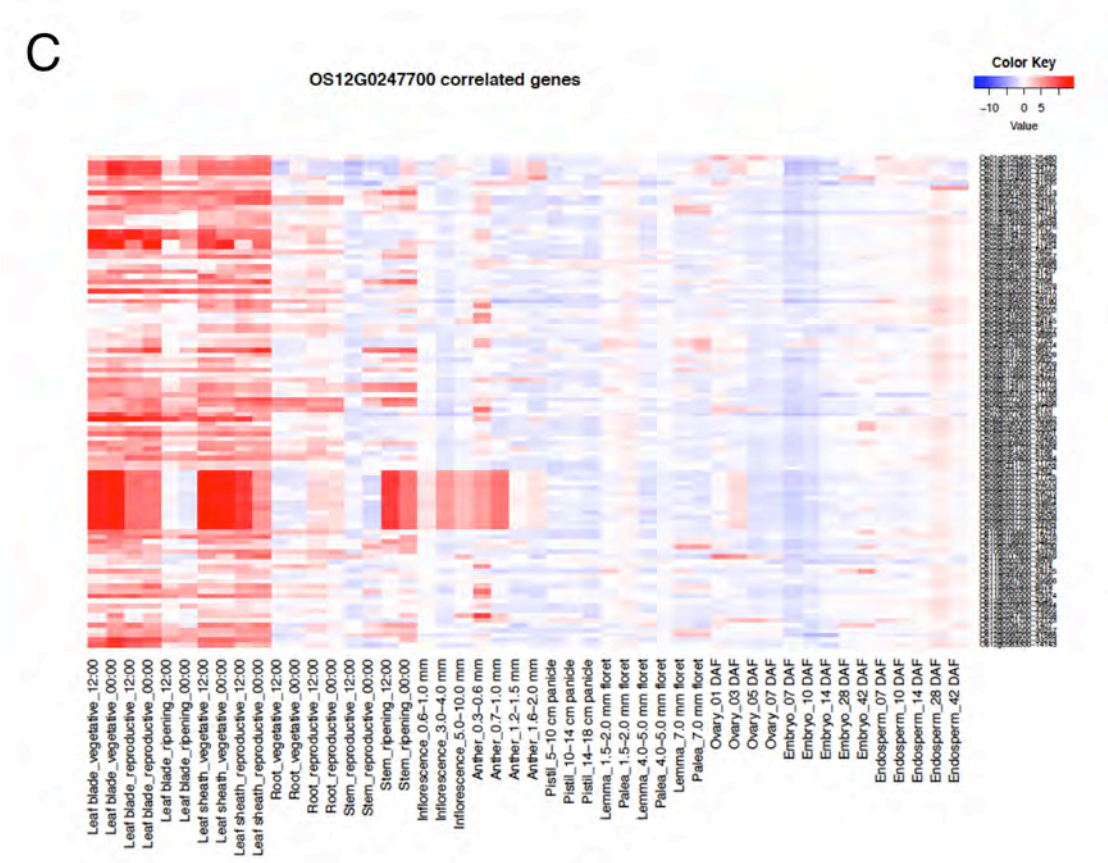

Figure 5.2. Correlation analysis of candidate genes regulated in both local and systemic tissues in rice-*Pso* pathosystem

Spatio-temporal expression of genes (A) OS04G0344100, (B) OS09G0275400, and (C) OS12G0247700 in various tissues/organs of rice during entire growth stages in the field.

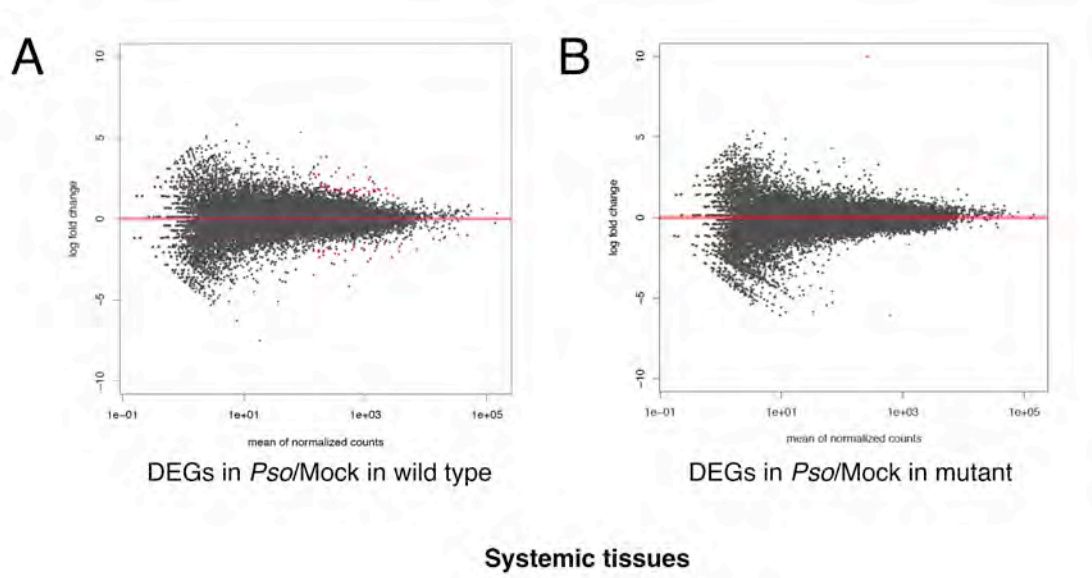

Figure 5.3. MA-plot of differentially expressed genes (DEGs) in systemic tissues of wild type and mutant *rCBP* plants infected with *Pseudomonas syringiae* pv. *oryzae* (*Pso*)

(A) and (B) plots showing differences in measurements of differentially expressed genes (DEGs) in wild type and mutant conditions under infection. *P* adjusted value is set at <0.01. For wild type and mutant samples, two independent RNA-sequencing samples in mock treatment and three in *Pso* treatment are presented, respectively.

Table 5.1. Repository information of deposited systemic tissue RNA-sequencing and related files

File name	Type	Reference No.	Database
1_11_9-5_Mock1plus_Sys	.bam file	TBD	TBD
1_11_9-5_Mock1minus_Sys	.bam file	TBD	TBD
1_11_9-5_Pso6plus_Sys	.bam file	TBD	TBD
1_11_9-5_Pso6minus_Sys	.bam file	TBD	TBD
3_6_9-5_Mock2plus_Sys	.bam file	TBD	TBD
3_6_9-12b_Mock2minus_Sys	.bam file	TBD	TBD
3_6_9-5_Pso1plus_Sys	.bam file	TBD	TBD
3_6_9-12b_Pso1minus_Sys	.bam file	TBD	TBD
3_6_9-5_Pso4plus_Sys	.bam file	TBD	TBD
3_6_9-12b_Pso4minus_Sys	.bam file	TBD	TBD
DEGs_psovsmock_in_wildtypeONLY	.csv file	TBD	TBD
DEGs_psovsmock_in_mutantONLY	.csv file	TBD	TBD

*To be deposited (TBD): Files will be deposited to OIST Institutional Repository & Research Data Archive after the final version of the thesis is completed. Plus (wild type); Minus (mutant).

Table 5.2. Summary of total RNA-sequencing data in systemic tissues

File name	Total Reads[1]	Mapped Reads[1]	% Mapped
1_11_9-5_Mock1plus_Sys	41, 742, 224	40, 662, 040	97.4
1_11_9-5_Mock1minus_Sys	40, 942, 744	39, 972, 258	97.6
1_11_9-5_Pso6plus_Sys	35, 867, 459	34, 962, 735	97.5
1_11_9-5_Pso6minus_Sys	33, 050, 143	32, 139, 673	97.2
3_6_9-5_Mock2plus_Sys	36, 795, 701	35, 876, 713	97.5
3_6_9-12b_Mock2minus_Sys	38, 831, 858	37, 955, 174	97.7
3_6_9-5_Pso1plus_Sys	38, 581, 325	37, 578, 745	97.4
3_6_9-12b_Pso1minus_Sys	36, 350, 179	35, 459, 239	97.5
3_6_9-5_Pso4plus_Sys	38, 075, 149	36, 853, 181	96.8
3_6_9-12b_Pso4minus_Sys	39, 466, 964	38, 332, 318	97.1

[1] Left and right reads were combined.

Concordant pair alignment rate for all files are above 94%.

Table 5.3. Differentially expressed genes in both local and systemic tissues in rice-*Pso* pathosystem

Gene ID	Description
OS01G0702000	Bifunctional nuclease 1
OS02G0674233	Putative uncharacterized protein
OS04G0344100	Putative uncharacterized protein; terpene synthase family
OS05G0555600	Glutamate synthase 2 [NADH], chloroplastic
OS07G0187400	ATPase-like protein
OS09G0275400	Putative cytochrome P450
OS12G0190000	VTC2, putative, expressed
OS12G0247700	Expressed protein; Mannose-specific jacalin-related lectin/OsJAC1

The genes in boldface are significantly up-regulated in local tissues and down-regulated in systemic tissues.

Chapter 6
Materials and Methods

Biological samples and plant growth conditions. *Oryza sativa* ssp. *japonica* cv. Nipponbare (wild type) plants, transgenic and mutant lines were grown in commercial soil (Kumiai, JA Okinawa) at 30°C day/25°C night temperatures under a 12-h-light/12-h-dark photoperiod. The lighting was supplied by white light at an intensity of 31,000 lx. Relative humidity was at 70%.

Leaf disc assay (PAMP-triggered immunity). Flagellin, a well-known inducer of plant innate immunity, specifically of PTI was used to test which rice HATs respond to flagellin treatment. Leaf disc assay (modified from Heese et al., 2007; Park and Ronald, 2012) was performed on fully expanded 30-d-old wild-type leaf samples and treated with synthetic flagellin peptide (ADI, Inc.) at different time periods and concentrations. Briefly, leaves were cut into about 5 mm sizes and floated on the water for 24-h in growth chamber to remove the symptoms of wounding stress. Leaves were then treated with PAMP solution in water at 15 ml falcon tubes with rotation (Corning Science). After treatment, paper towel-dried leaves were frozen in liquid nitrogen.

Isolation and screening of *rCBP* transgenic and mutant lines. To generate RNAi-*rCBP* knockdown transgenic lines, 5'- and 3'-region blunt-end fragments of *rCBP* gene and GFP-control were amplified and TOPO® cloned into pENTR™ TOPO® vector to produce the TOPO® entry vector. The entry vector was recombined with pANDA destination vector using Gateway LR Clonase Enzyme Mix (Thermo Fisher Scientific) to produce the *rCBP*-pANDA expression vector (Miki et al., 2005; Miki and Shimamoto, 2004). To generate *rCBP* CRISPR/Cas9 knockout mutant lines, two sgRNA *rCBP*-specific target sites were obtained

from CRISPR-P website and were used to synthesize primers for pRGEB31 (stable system) (Xie and Yang, 2013) (**Table 2.2**). Briefly, the vectors were digested with BSA I, while primers were phosphorylated and annealed to produce a DNA oligo duplex. The digested vectors were ligated to DNA oligo duplex using T4 ligase. RNAi and CRISPR/Cas9 vectors were introduced into *Agrobacterium* EHA105 and rice calli were transformed using the standard *Agrobacterium*-mediated transformation procedure. For RNAi screening, PCR and RT-PCR genotyping using *gus linker* primers were used to detect the presence and expression of the *gus linker* in T0 and T1 generations (**Table 2.1**). For CRISPR/Cas9 T0 and T1 screening, leaf samples were collected and genomic DNA was extracted using a standard CTAB protocol. Then, PCR-RFLP assay utilizing BseLI restriction enzyme (Thermo Fisher Scientific) was used to detect the CRISPR/Cas9-engineered mutations on *rCBP* targets (**Table 2.2**). Positive lines detected by PCR-RFLP assay were further analyzed by sequencing for INDEL mutations using S2 and S5 forward primers (**Table 2.2**). Four DNA amplicons per line were cloned and sequenced to determine the zygosity of the lines. Positive lines containing either monoallelic or biallelic mutations were phenotyped for embryonic lethality and effective grain production by examining and counting the mature grains produced in each panicle. Biallelic mutations were found in a few T0 and T1 lines.

Gene expression analysis. Total RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen) or Maxwell® 16 LEV Plant RNA Kit (Promega). cDNA was synthesized using Primescript II 1st Strand cDNA Synthesis Kit (Takara) according to manufacturer's instructions. RT-PCR and RT-qPCR assays were performed on three biologically independent samples or as indicated. RT-qPCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara) and was calculated following Pfaffl (2001) by averaging the values relative to *ACT1* control gene. Primer sequences are listed in **Table 2.1 and Table 4.1**.

Bioinformatics analysis. To draw the p300/CBP-family protein domain architecture, the conserved protein domains from representative taxa were searched using NCBI's CD-Search against three databases namely CDD v3.11- 45746 PSSMs, Pfam v27.0 – 14831 PSSMs, and SMART v6.0 – 1013 PSSMs (Marchler-Bauer et al., 2011). Protein domain structures were drawn using Prosite's MyDomains image creator adapted primarily from CDD v3.11 database. Other databases were used to estimate the start and end range of the domain as shown by the yellow demarcation lines. To draw the multiple sequence alignment of members of rice CBP family, an R package called msa was used (Bodenhofer et al., 2015). Amino acid sequences used to make the fasta file were derived from The Rice Annotation Project Database (RAP-DB). To predict the lysine acetyltransferase and deacetylase-specific site acetylation based on amino acid sequences, acetylation set enrichment-based method (ASEB) was used on rice canonical histone H3 amino acid sequence (Wang et al., 2012a). A predefined acetyltransferases and deacetylases including the CBP/p300, GCN5/PCAF, TIP60/MYST1/2/3/4, HDAC1/HDAC2/HDAC3, and SIRT1 were used to predict a statistically probable acetylation site in rice H3 sequences based on known human data acetylated and deacetylated protein sites as catalyzed by these enzymes. To verify the effect of CRISPR/Cas9-mediated mutation on *rCBP* gene, coding sequences of *rCBP* were obtained from The Rice Annotation Project Database (RAP-DB). INDELS were deposited into these coding sequences and were translated into protein amino acid sequences using ExPASy's Translate web tool (Artimo et al., 2012). To perform Gene Ontology (GO) analysis, gene lists were submitted to Enrichment Analysis web tool of the Gene Ontology Consortium (Consortium, 2000, 2015, 2017). To summarize and visualize gene ontology results from the Enrichment Analysis web tool, gene ontology categories and p-values were submitted to REVIGO web tool with allowed similarity of medium (0.7) and were subsequently modified using the provided R scripts at dispensability values of 0.15 for down-regulated and 0.01 for

up-regulated genes (Supek et al., 2011). To create the Venn diagram, gene sets were submitted to InteractiVenn web tool using *unions by list* to obtain overlapping components of input datasets (Heberle et al., 2015). The identified transcription factors in the set were rechecked againts The Rice Stress-Responsive Transcription Factor Database (SRTFDB) and Rice TF Database of the Rice Phylogenomics Database. To perform the correlation analysis, input genes were submitted to RiceXPro platform to identify correlated genes based on the expression database over entire growth stages of rice in natural field conditions (Sato et al., 2011; Sato et al., 2013).

Rice histone protein and natural biological peptide extraction. Late vegetative phase (30~40-day-old) plants were hyperacetylated by treating with a final concentration of 1μM Trichostatin A (TSA; Cell Signaling Technology) and 100 μM Nicotinamide (NAM; Sigma Life Science) for 48 h before collection. Whole plants were snap frozen in liquid nitrogen and homogenized using mortar and pestle until powdered form. Histone proteins were isolated from sonicated nuclei extracts using EpiQuik™ Total Histone Extraction Kit (Epigentek) with minor modifications. Samples were then resuspended in Milli-Q water through buffer exchange using Amicon® Ultra 3K device (Millipore) with 3,000 Nominal Molecular Weight Limit (NMWL) cutoff and concentration was determined using Direct Detect™ Assay-free Green Cards (Millipore) resulting in a yield ranging from 0.2 - 1.1 μg/μl. Histones were either utilized for EpiQuik™ Histone H3 Modification Multiplex Assay Kit (Colorimetric; Epigentek) to identify the histone modifications or propionylated for mass spectrometric analysis (Garcia et al., 2007; Maile et al., 2015; Meert et al., 2015). Histone proteins were propionylated twice (*i.e.* double propionylation means propionylated once in protein form and again in peptide form) using propionic anhydride (Sigma-Aldrich) and were digested

with trypsin/Lys-C mix (Promega). Samples were acidified using 0.1% formic acid (FA; Fisher Scientific) in Milli-Q water in preparation for loading into LC-MS analysis.

LC-MS/MS. A Dionex UltiMate 3000 RSLCnano system combined with an electrospray ion source to the mass spectrometer was used for exploratory and targeted proteomics. Peptides were separated on C18 column using HPLC solvent A (0.1% v/v formic acid, 1% v/v acetonitrile in HPLC grade water) and Solvent B (0.1% v/v formic acid, 98% v/v acetonitrile in HPLC grade water) and analyzed using a hybrid Quadrupole-Orbitrap (Q-Exactive Plus, Thermo Scientific). For data-dependent acquisition (DDA)-mass spectrometry, peptides were loaded into C18 column (Zorbax 300SB-C18; 0.3 x 150 mm; 3.5 μm; Agilent Technologies) at 3 μl per minute and separated by a linear gradient: 1-5% solvent B for 2 min, followed by a ramp of 5-35% B in 50 min, then 35-45% B in 2 min, wash at 75% B in 5 min, and re-equilibration at 1% B for a total run of 70 min. Full-scan mass spectra were collected at 70,000 resolution in a mass range of 250-1500 m/z with a target value of 1e6, while the MS/MS spectra were recorded at 17,500 resolution (Higher Energy Collisional Dissociation or HCD). For parallel reaction monitoring (PRM)-mass spectrometry, a mass inclusion list for histone H3 peptides (**Table 3.1**) generated from the analysis of synthetic peptides was specified for targeted identification of *in vivo* histone modifications. Biological natural peptides were loaded similarly into C18 column and separated by the same conditions as above. MS/MS spectra were collected at 17,500 resolution with a target value of 2e5 and isolation window of 2.0 m/z.

Fmoc-based solid-phase synthesis of peptide. Intavis ResPep SL automated peptide synthesizer was used for solid-support synthesis of non-acetylated and acetylated rice histone H3 and H4 peptides. Fmoc-protected amino acids (Watanabe Chemical IND., LTD.) were

used to produce the synthetic peptides using standard automated Fmoc protocols, with 0.5 M of each amino acid in NMP (N-Methyl-2-pyrrolidone) and 0.5 M of HBTU (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) coupling reagent in DMF (dimethylformamide). The amino acid was deprotected using a solution of 20% piperidine in DMF and Fmoc-Rink Amide resin (Intavis) was used for solid phase peptide synthesis. The synthesis was performed on a 2 μmol scale (96-well plates) and the synthesized peptides were cleaved from the resin using 100% TFA/TIPS/H_2O (90:5:5). The peptides were precipitated from the solution by adding -30°C cold tert-butyl methyl ether and was kept at -30°C overnight. The ether solution was discarded, and the precipitated peptides were subsequently washed with ether and dried under vacuum. Finally, doubly propionylated (*i.e.* double propionylation here means propionylated twice in peptide form) synthetic peptides were analyzed using LC-MS/MS.

Western blot analysis. An equal amount of total histone proteins from *rCBP* RNAi transgenic lines were separated in 10-20%/15% SDS-PAGE (ePAGELmini gel, Atto) for 50 min at constant voltage. Proteins were transferred to 0.2 μm nitrocellulose membrane (Trans-Blot Turbo transfer pack, Bio-Rad) using Trans-Blot Turbo Transfer Systems (Bio-Rad) and immunodetected with antibodies (**Table 3.2**) on a fluorescent image analyzer (LAS 3000, Fujifilm).

Pathogenesis assay. Seeds were surfaced sterilized and imbibed in sterile water in the dark for 72 h before sowing on Murashige and Skoog (MS) basal medium (Sigma Life Science). After 10 days, the plantlets were transferred to soil and were grown for another 18 days until infection. *Pseudomonas syringiae* pv. *oryzae* (*Pso*) (MAFF No. 301530, NIAS Genebank) was grown in Luria-Bertani (LB) broth (Sigma-Aldrich) at 28°C until OD = 0.2 and was

resuspended in 10 mM $MgCl_2$. The fourth leaf counting from the first true leaf was infected with *Pso* using needleless syringe injected from the lower surface of the leaf. *Pso* leaf infiltration was performed 10 cm from the tip of the leaf and was done 3x with approximately 1 cm space between the infiltration sites. Infected plants were temporarily maintained outside the growth chamber for 2 h to allow drying of the infected sites before returning to the chamber. Infected leaf samples were collected 3 days after *Pso* inoculation utilizing only the tissues comprising the spaces between the infiltration sites. These tissues were grounded in sterile 10 mM $MgCl_2$ and the grounded tissue suspensions were transferred to LB agar medium for incubation. The infected local tissues were assayed with minor modifications as detailed in Liu et al. (2015). The remaining samples of the fourth leaf were used for RNA-sequencing analysis.

Systemic tissue analysis. Whole plants excluding the locally infected leaves and roots were collected at the same time as the infected leaves and were sent for RNA-sequencing analysis.

Salicylic acid (SA) priming. Seed priming with SA was done by treating the surface-sterilized rice seeds with 500 μM salicylic acid in 70% ethanol for 3 days in the dark at 4°C. Alternatively, 1mM sodium salicylate in sterile water was sprayed twice to mature (prior to flowering) plants 24 h before *Pso* infection to induce SA-mediated defense signaling.

RNA-sequencing. Total RNA was isolated from mock- and *Pseudomonas syringiae* pv. *oryzae* (Pso)-treated $rCBP^{+/+}$ segregated wild type and $rCBP^{-/-}$ mutant with Maxwell 16 LEV Plant RNA Kit (Promega) run on the Maxwell 16 Instrument (Promega) and/or mirVana miRNA Isolation Kit (Invitrogen by Thermo Fisher Scientific). To remove the contaminating genomic DNA from RNA samples isolated using the mirVana miRNA Isolation Kit, RNA was treated with DNase I (RNA free) (Nippon Gene) following the manufacturer's

instructions. Samples were submitted to OIST Sequencing Center for RNA quality checking, library preparation, and paired-end mRNA-sequencing (PE mRNA-seq).

Data analysis. For DDA-mass spectrometry analysis, raw MS files were processed using Proteome Discoverer version 1.4 (Thermo Scientific). Proteins were identified using a combination of SEQUEST and Mascot database search algorithms. MS/MS spectra were searched with dynamic modifications of propionyl (N-term), propionyl (K), acetyl (K), acetyl (protein-N-term), and methylation. The precursor mass tolerance was set at 20 ppm, while the fragment mass tolerance was at 0.02 Da. Tryptic specificity was strict with maximum allowable missed cleavages of 2. Percolator was used for PSM (Peptide Spectrum Match) validation to achieve an estimated FDR <0.01. For PRM-mass spectrometry, area-under-the-curve values for peptide peaks were calculated using Pinpoint software version 1.3 (Thermo Scientific). Pinpoint was also used to validate the retention times and relative intensities of five transitions of each histone modifications in RNAi-transgenic lines to wild type samples. For RNA-sequencing analysis, high quality reads were trimmed in order to remove sequencing bias and adapter effects. Trimmed reads were then mapped to the *Oryza sativa* spp. *japonica* genome (Os-Nipponbare-Reference-IRGSP-1.0) using Tophat (Trapnell et al., 2009; Trapnell et al., 2012). Custom R scripts were used to generate the RNA count table necessary to analyze the differentially expressed genes (DEGs). Differential expression analysis was performed using DESeq2 package involving count normalization, dispersion estimation, and differential expression test (Love et al., 2014). DEGs were selected at Benjamini-Hochberg adjusted p value of < 0.01.

Data visualization. Visualization of the data was performed using Microsoft Excel for Mac 2011 (version 14.7.1), Numbers (ver 3.5.3), heatmaply package, functions from *gplots* package, and *ggplot2* package.

Data Repository. For DDA-mass spectrometry, raw MS files were deposited to PRIDE (Proteomics Identifications Database) via ProteomeXchange (PRIDE Archive) (**Table 3.3**). For PRM-mass spectrometry, raw files were deposited to PeptideAtlas (peptideatlas.org) (**Table 3.3**). For RNA-sequencing, data were deposited to DNA Databank of Japan (DDBJ) (**Table 4.2; Table 5.1**).

Published article related to this dissertation

1. Espinas NA*, Saze H, and Saijo Y*. 2016. Epigenetic control of defense signaling and priming in plants. *Front. Plant Sci.* 7: 1201. doi: 10.3389/fpls.2016.01201

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