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Exploring novel regulators and enzymes in salicylic acid-mediated plant defense

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Citation

Zhou, Y. (2018, May 9). *Exploring novel regulators and enzymes in salicylic acid-mediated plant defense*. Retrieved from https://hdl.handle.net/1887/62028

Version:	Not Applicable (or Unknown)
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Title: Exploring novel regulators and enzymes in salicylic acid-mediated plant defense
Issue Date: 2018-05-09

Chapter 1

General introduction

Sessile plants have evolved elaborate defense strategies to deal with the survival crises caused from the dynamically changing environments, including pathogen infections from bacteria, viruses, and fungi. Those defense responses require quick and massive transcriptional reprogramming, hormone synthesis and enzymatic modifications, in addition to the structural barriers preventing pathogens access to the plants cells and tissues. The defense response launched in plants after attack by biotrophic pathogens is activated by an endogenously produced small phenolic compound: salicylic acid (SA). By contrast, defense against necrotrophic pathogens, which destroy host tissues and feed on the contents, relies on signaling molecules jasmonates (JAs) and ethylene (ET) (Glazebrook, 2005; Pieterse *et al.*, 2009).

SA is a key component of local and systemic defense in plants, particularly systemic acquired resistance (SAR) (Vlot *et al.*, 2009). SAR is not just specific to the local site of infection, but also throughout the whole plant, providing a broad spectrum and long-lasting resistance (Malamy *et al.*, 1990; Fu and Dong, 2013). This process is associated with the induction of *PATHOGENESIS-RELATED* (*PR*) genes in both local and systemic tissues (non-infected tissue) (van Hulten *et al.*, 2006). Activation of the SAR response and expression of *PR* genes fail in plants in which SA signaling is interfered upon infection. On the other hand, constitutive expression of *PR* genes and enhanced resistance occurs in plants that over-produce SA. Together, this indicates that SA is a crucial intermediate in the SAR signaling pathway (Verberne *et al.*, 2000; Pieterse *et al.*, 2009). Besides the role in plant defense, SA is a necessary molecule involved in various physiological responses, including thermogenesis, seed germination, cell growth, respiration, stomatal closure, responses to abiotic stresses and disturbing JA/ET signaling pathways (Vlot *et al.*, 2009; Rivas-San Vicente and Plasencia, 2011; Robert-Seilaniantz *et al.*, 2011; Boatwright and Pajerowska-Mukhtar, 2013).

SA biosynthesis

SA is synthesized from chorismate, the final product of the shikimate pathway in the chloroplasts, which is also the precursor for the aromatic

amino acids in plants (tryptophan, phenylalanine, and tyrosine) (Herrmann, 1995; Maeda and Dudareva, 2012). Chorismate is converted into SA via two distinct biosynthesis pathways. The first pathway, which has been reported initially, relies on phenylalanine ammonia lyase (PAL). The second pathway catalyzes the conversion of chorismate into SA via the intermediate isochorismate and depends on isochorismate synthase (ICS) (Figure 1). However, neither of these branches has been conclusively elaborated (Dempsey *et al.*, 2011; Boatwright and Pajerowska-Mukhtar, 2013; Dempsey and Klessig, 2017).

PAL pathway

The PAL pathway involves a series of chemical reactions catalyzed by many enzymes (Figure 1). The initial committed step in this pathway is chorismate mutase (CM) that catalyzes the conversion of chorismate to prephenate. Arabidopsis contains three CMs: AtCM1, AtCM2, and AtCM3 (Eberhard *et al.*, 1996; Mobley *et al*., 1999;Westfall *et al.*, 2014). AtCM1 and AtCM3 contain a putative plastid-targeting signal while AtCM2 is predicted to be localized in the cytosol. Analogues of the plastidial CM1 and cytosolic CM2 have also been reported in other plants, such as *Nicotiana silvestris* and *Petunia hybrida* (d'Amato *et al*., 1984; Goers *et al*., 1984; Singh *et al*., 1986;Colquhoun *et al.*, 2010).

It has been found that prephenate can be converted into phenylalanine (Phe) via either arogenate or via phenylpyruvate. In the arogenate route, prephenate is transaminated by prephenate aminotransferase (PPA-AT) to generate arogenate (Graindorge *et al*., 2010; Maeda *et al*., 2011; Dornfeld *et al*., 2014). In a next step, arogenate is decarboxylated and dehydrated by the catalytic action of chloroplast-targeted arogenate dehydratase (ADT) to form the aromatic amino acid Phe (Cho *et al*., 2007; Tzin and Galili, 2010; Bross *et al*., 2017). Previous studies demonstrated that Phe is predominantly synthesized from arogenate in the chloroplasts, while a cationic amino-acid transporter (PhpCAT) has been identified in Petunia to be localized in the chloroplast envelop and involved in exporting Phe to the cytosol (Maeda *et al*., 2010; Maeda *et al*., 2011; Widhalm *et al*., 2015).

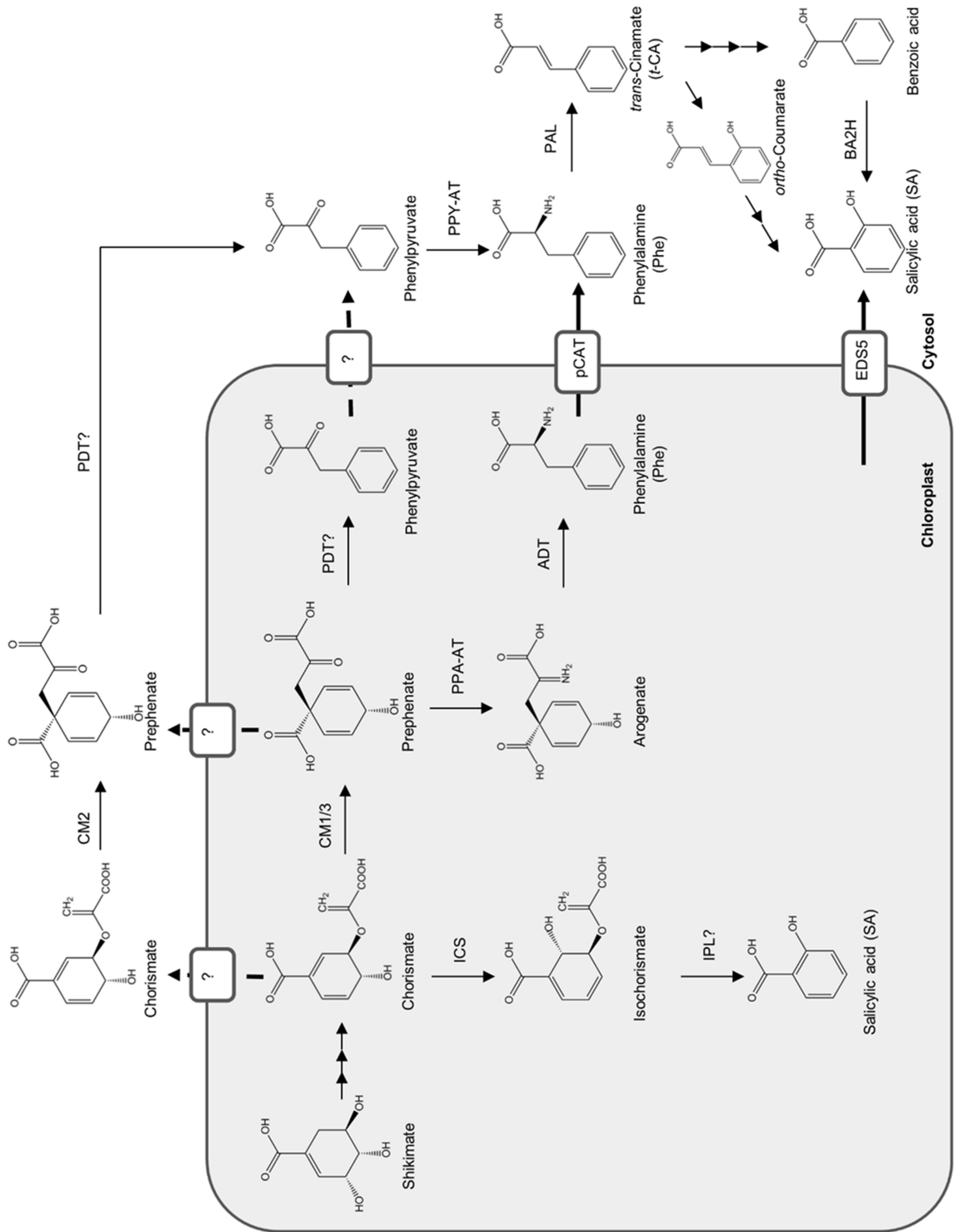
Chorismate
CM2
Prehenate
PDT?
Phenylpyruvate
PPY-AT
Phenylalamine
(Phe)
PAL
trans-Cinamate
(t-CA)
ortho-Coumarate
Benzoic acid
BA2H
Salicylic acid (SA)
Shikimate
Chorismate
CM1/3
Prephenate
PDT?
Phenylpyruvate
?
ICS
PPA-AT
ADT
Phenylalamine
(Phe)
pCAT
Isochorismate
Arogenate
IPL?
Salicylic acid (SA)
EDS5
Chloroplast
Cytosol

Figure 1. Two possible pathways of salicylic acid (SA) biosynthesis in plants. Enzyme names are given next to the arrows. Question marks indicate that the enzyme responsible for the indicated conversion has not yet been definitively identified. White boxes with question marks indicate unknown transport steps. Abbreviations: CM, chorismate mutase; PPA-AT, arogenate aminotransferase; ADT, arogenate dehydratase; PDT, prephenate dehydratase; PPY-AT, phenylpyruvate aminotransferase; pCAT, plastidial Phe transporter; PAL, phenylalanine ammonia lyase; BA2H, benzoic acid-2-hydroxylase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase.

The alternative pathway contributes to Phe biosynthesis in a reverse order. Prephenate is first dehydrated/ decarboxylated into phenylpyruvate by prephenate dehydratase (PDT). The phenylpyruvate pathway exists in most microoganisms and is carried out by a bi-functional chorismate mutase/prephenate dehy-dratase (PheA) (Fischer *et al*., 1993; Romero *et al*., 1995; Prakash *et al*., 2005). Then phenylpyruvate is converted to Phe by phenylpyruvate aminotransferase (PPY-AT). Tzin *et al*. (2009) over-expressed a bacterial PheA in Arabidopsis, which resulted in significantly elevated Phe production, implicating that plants possess a functional PPY-AT capable of converting phenylpyruvate to Phe. It has been described that Petunia utilizes a cytosolic PPY-AT to convert phenylpyruvate to Phe, suggesting Phe biosynthesis is not limited to chloroplasts (Yoo *et al*., 2013).

Phe that is transported into the cytosol or synthesized in the cytosol serves as building blocks for proteins, but also as a precursor of numerous downstream metabolites in plants (Tzin and Galilia, 2010; Vogt, 2010). Arabidopsis contains at least four PALs (PAL1-PAL4) that are responsible for converting Phe to *trans*-cinnamic acid (*t*-CA) and ammonia (Raes *et al*., 2003; Huang *et al*., 2010). The *pal1 pal2* double mutant and *pal* quadruple mutant are stunted and sterile (Rohde *et al*., 2004; Huang *et al*., 2010). The quadruple mutant accumulates substantially reduced levels of SA and exhibit enhanced susceptibility to virulent bacteria (Huang *et al*., 2010).

Early radiolabeling experiments revealed that *t*-CA is converted to SA via the potential intermediates ortho-coumaric acid or benzoic acid (BA), depending on the plant species and growth conditions. Production of radioactive SA was observed via ortho-coumaric acid, by feeding ^{14}C-

labeled Phe or *t*-CA via ^{14}C-labeled benzoic acid to the leaves of *Primula acaulis* and *Gaultheria procumbens*, implying both pathways are active in SA biosynthesis (El-Basyouni *et al*., 1964; Ellis and Amrhein, 1974). The ortho-coumaric acid pathway was favored in young tomato seedlings that were infected with *Agrobacterium tumefaciens* (Chadha and Brown, 1974). However, radioactive tracer results suggested that SA is mainly synthesized via BA in the PAL branch pathway, for instance in tobacco, cucumber, and rice (Yalpani *et al*., 1993; Meuwly *et al*, 1995; Silverman *et al*., 1995; Ribnicky *et al*., 1998). BA is converted to *t*-CA either by intermediates benzaldehyde (BD) or benzoyl-CoA and presumably to SA, catalyzed by BA 2-hydroxylase (BA2H) (León *et al*., 1995; Wildermuth, 2006; Ibdah *et al*., 2009; Dempsey *et al*., 2011; Widhalm and Dudareva, 2015).

IC pathway

Lower amounts of SA were produced than expected upon infection/ induction in plants based on the radio-label incorporation studies and *pal* quadruple mutant is still able to accumulate SA after infection, implying that plants possess alternative SA biosynthesis pathway(s) (Figure 1) (Chada and Brown, 1974; Yalpani *et al*, 1993; Mauch-Mani and Slusarenko, 1996; Coquoz *et al*., 1998; Huang *et al*., 2010). In some bacteria, SA was found to be produced by two enzyme-catalyzed steps via ICS and isochorismate pyruvate lyase (IPL) (Serino *et al*., 1995; Mercado-Blanco *et al*., 2001; Gaille *et al*., 2002). In *Y. enterocolitica* and *M. tuberculosis*, a bi-functional enzyme salicylate synthase (SAS) carries out the direct conversion of chorismate to salicylate (Kerbarh *et al*., 2005; Zwahlen *et al*., 2007).

The first plant ICS was identified in elicited cell cultures of *Catharanthus roseus*. The presence of a chloroplast transit peptide at the N-terminus suggested that this ICS is probably a plastidic enzyme (van Tegelen *et al*., 1999). Large amounts of SA were synthesized via a bacterial IC pathway in both tobacco and Arabidopsis, after introducing bacterial ICS and IPL or single SAS into the chloroplasts (Verberne *et al*., 2000; Mauch *et al*., 2001). Two putative *ICS* genes in the Arabidopsis genome were identified that provide powerful proof of an endogenous IC to SA

pathway in plants (Wildermuth *et al*., 2001). The *ics1* (*sid2*) mutant plants accumulate very low levels of SA after pathogen inoculation or UV irradiation (an alternative stimulus for SA accumulation) and are more susceptible to infection (Nawrath and Métraux, 1999; Nawrath *et al*., 2002). In addition, in *sid2* mutant, the SAR molecular marker gene *PR1*, downstream of SA accumulation, is induced at very low levels (Nawrath and Métraux, 1999; Wildermuth *et al*., 2001). The *ICS1* encoded protein shares a high homology with ICS2 (83% of homology at the amino acid level). Both proteins contain a predicted chloroplast transit peptide and are localized in the chloroplasts (Strawn *et al*., 2007; Garcion *et al*., 2008).

Even though ICS1 and ICS2 have highly similar structural characteristics and ICS activity, their function and expression patterns are divergent in Arabidopsis (Macaulay *et al*., 2017). UV-treated *ics1* single mutant barely accumulates SA, but *ics2* single mutant can still produce SA levels comparable to WT Arabidopsis after UV treatment (Garcion *et al*., 2008). *ICS1* and *ICS2* are constitutively expressed at low levels in regular growth conditions according to microarray data analysis (Zimmermann *et al*., 2005). *ICS1* is induced by pathogen infection and exogenous SA, however, the *ICS2* transcript is not detected after pathogen infection or SA treatment (Nawrath and Métraux, 1999; Hunter *et al*., 2013). ICS1 contributes more than 90% of pathogen-induced SA amounts (Wildermuth *et al*., 2001; Garcion *et al*., 2008). These data suggest that ICS1 is responsible for the major portion of SA biosynthesis in plants. Isochorismate is also an essential precursor for the biosynthesis of phylloquinone, which is a pivotal component for photosystem I (PSI) (Gross *et al*., 2006). Dramatically reduced phylloquinone production along with a striking visual unhealthy phenotype in *ics1 ics2* double mutant and decreased phylloquinone amounts in *ics1* or *ics2* single mutant suggest that both ICS1 and ICS2 are indispensable for phylloquinone biosynthesis and complementary for each other in this pathway (Garcion *et al*., 2008).

So far, in higher plants with sequenced genomes, *Glycine max* also encodes two *ICS* genes which are 95% identical to each other at the

amino acid level (Yuan *et al*., 2009; Shine *et al*., 2016). Other plants, such as *Catharanthus roseus*, *Nicotiana benthamiana*, *Populus trichocarpa*, and *Solanum lycopersicum*, contain one *ICS* gene (van Tegelen *et al*., 1999; Upplapati *et al*., 2007; Catinot *et al*., 2008; Yuan *et al*., 2009).

The role of the PAL and IC pathways as provider of SA is significantly different between plant species. For example, the basal and pathogen-induced SA levels in the pal quadruple mutant of Arabidopsis were only about 25% to 50% of wild-type levels (Huang *et al*., 2010). On the contrary, PAL and IC pathways are equally important for pathogen-induced SA biosynthesis in soybean (Shine *et al*., 2016). In tobacco, the IC pathway plays a dominant role in SA-accumulation after exposure to UV or to pathogen stress (Catinot *et al*., 2008).

However, an IPL in charge of converting isochorismate to SA has not been identified from plants. Arabidopsis expressing salicylate hydroxylase (nahG) fused to a chloroplast transit sequence failed to produce SA after pathogen infection or UV exposure treatment, inferring that SA is initially located in the chloroplasts (Fragnière *et al*., 2011). So, the unidentified IPL is plausibly localized in the chloroplasts. The Arabidopsis genome does not contain any genes encoding proteins similar to the bacterial IPL PchB (Chen *et al*., 2009a). Arabidopsis peptide deformylase (PDF1B) localized in the chloroplasts (Dirk *et al*., 2001; Giglione *et al*., 2000) and PmsB, an IPL of *Pseudomonas fluorescens* (Mercado-Blanco *et al*., 2001), share 19% identity and 56% similarity at the amino acid level, indicating PDF1B might be involved in SA biosynthesis.

Another important gene in the IC pathway is *ENHANCED DISEASE SUSCEPTIBILITY5* (*EDS5*), encoding a member of the multidrug and toxin extrusion (MATE) transporter family, which is responsible for exporting SA from chloroplasts to cytosol (Serrano *et al*., 2013). *eds5* mutant exhibits enhanced susceptibility to pathogen infection and is unable to mount the SAR response and *PR1* gene expression, probably due to limited accumulation of SA in the cytosol (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999). When *EDS5* is constitutively

expressed at a very low level under normal growth conditions, makes that the plant responds strongly to pathogen inoculation and UV treatment (Nawrath *et al*., 2002). The expression of *EDS5* is also induced by exogenous SA and plants constitutively expressing *EDS5* show enhanced SA levels and heightened resistance to the yellow strain of *Cucumber mosaic virus* [CMV(Y)], indicating that EDS5 is involved in a positive feedback regulation loop by SA (Nawrath *et al*., 2002; Ishihara *et al*., 2008). In plants, SA is suggested to be synthesized in the chloroplasts as well as isochorismate (Garcion *et al*., 2008; Fragnière *et al*., 2011). Confocal microscopy results confirmed that EDS5 is targeted to the chloroplast envelop (Serrano *et al*., 2013; Yamasaki *et al*., 2013). In *eds5* mutant, over-produced SA is trapped in the chloroplasts (Serrano *et al*., 2013).

Regulation of SA biosynthesis

The plant's defense response against biotrophic pathogens relies on SA accumulation. However, over-produced SA in Arabidopsis or high dose treatment with SA of Arabidopsis resulted in strongly dwarfed plants, reduced seed production, or even an infertile phenotype (Mauch *et al*., 2001; Heidel *et al*., 2004; Ishihara *et al*., 2008; Chandran *et al*., 2014). It is potentially due to SA-repressed expression of the auxin-related genes that interferes with normal plant development (van Hulten *et al*., 2006; Wang *et al*., 2007; Heidel *et al*., 2004). SA accumulation is tightly regulated by an intricate genetic modulatory network to balance the regular growth and emergent responses to pathogen attacks.

Regulators involved in SA accumulation

Mutant screening has revealed that *EDS1* and *PHYTOALEXIN DEFICIENT4* (*PAD4*) encoding lipase-like proteins are involved in the earliest steps of SA accumulation (Feys *et al*., 2001). EDS1, as a basal resistance regulator, interacts with PAD4 and other resistance-associated proteins and forms cytoplasmic and nuclear protein complexes (Feys *et al*., 2001; Feys *et al*., 2005; Bhattacharjee *et al*., 2011; Heidrich *et al*., 2011). Moreover, *ICS1* and *EDS5* expression upon infection depends on *EDS1* and *NON-RACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*), encoding a plasma membrane-localized

protein (Nawrath *et al*., 2002; Glazebrook *et al*., 2003). How exactly their activation influences the expression of *ICS1* and *EDS5* remains unknown. *EDS1* and *NDR1* expression is negatively regulated by SIGNAL RESPONSIVE1 (SR1), a calmodulin (CaM)-binding transcription factor, indicating that Ca^{2+} signaling is highly related to SA accumulation upon infection (Du *et al*., 2009; Nie *et al*., 2012).

Transcription factors (TFs) in the regulation of SA biosynthesis

1) CaM-binding TFs

Two key regulators, SAR DEFICIENT1 (SARD1) and CAM-BINDING PROTEIN 60-LIKE G (CBP60g), have been identified that positively regulate the transcription of *ICS1*, *EDS5*, *EDS1*, and *PAD4* (Zhang *et al*., 2010a; Wang *et al*., 2011; Truman and Glazebrook, 2012; Sun *et al*., 2015). CBP60g belongs to the CBP60 CaM-binding protein family and binds to CaM at its N-terminal region (Wang *et al*., 2009). Notably, SARD1 is not a CaM-binding protein, but shares 39% identity with CBP60g at the amino acid level (Wang *et al*., 2009; Zhang *et al*., 2010a). In the *sard1 cbp60g* double mutant, induction of *ICS1* and *PR1* expression, and SA synthesis are dramatically reduced (Zhang *et al*., 2010a; Wang *et al*., 2011). *ICS1* expression and SA accumulation are partially compromised in *sard1* and *cbp60g* single mutant, suggesting SARD1 and CBP60g are partially redundant for activating *ICS1* expression (Zhang *et al*., 2010a; Wang *et al*., 2011).

CBP60a also belongs to CBP60 CaM-binding protein family but has the CaM-binding domain at the C-terminus. In *cbp60a* mutant, ICS expression is elevated as well as SA amount compared with WT plants in the presence of pathogens. *cbp60a* mutant demonstrates reduced pathogen growth, suggesting CBP60a is a negative regulator of immunity in contrast to CBP60g and SARD1 (Truman *et al*., 2013).

2) WRKY and TCP TFs

The WRKY subfamily of transcription factors is characterized by the conserved amino acid sequence WRKYGQK and the zinc-finger-like domain, which has a high affinity for binding the W-box motif (TTGACC/T) (Eulgem *et al*., 2000). In Arabidopsis, the WRKY TF family

consists of 74 members, many of them have been reported to be involved in the SA signaling pathway (Eulgem and Somssich, 2007; Rushton *et al*., 2010). WRKY28 was shown to bind two W-box core motifs in the *ICS1* promoter and is able to trans-activate expression of an *ICS1:GUS* (glucuronidase) reporter gene in Arabidopsis protoplasts, suggesting WRKY28 may act as a positive regulator of induced *ICS1* expression (van Verk *et al*., 2011a). WRKY28 might be regulated through phosphorylation by CPKs (Ca^{2+}-dependent protein kinase) to activate the expression of *ICS1* (Gao *et al*., 2013).

TCP8 and TCP9, transcription factors of the TCP (teosinte branched1/ cycloidea/pcf) family, were found to directly bind to the promoter of *ICS1* and redundantly function as positive regulators of *ICS1* during the immune response (Wang *et al*., 2015). Moreover, TCP8 interacted with SARD1, NAC019 (a transcriptional repressor of *ICS1* expression) and WRKY28, inferring that TCPs may coordinate those TFs to regulate the expression of *ICS1* during pathogen infection. Unlike TCP8/9, TCP21 designated as CCA1 HIKING EXPEDITION (CHE), a component of the circadian clock, is active not only on the systemic induction of *ICS1* and SA synthesis upon infection, but also regulates the circadian expression of *ICS1* and rhythmic accumulation of SA (Goodspeed *et al*., 2012; Zheng *et al*., 2015). NTM1-LIKE9 (NTL9), preferentially expressed in guard cells, and characterized at the same time as CHE, binds to the *ICS1* promoter and is required for stomatal immunity (Zheng *et al*., 2015).

3) TFs involved in SA-JA/ET crosstalk

It is generally believed that SA and JA/ET defense pathways act antagonistically (Glazebrook, 2005). Coronatine (COR) produced by *Pseudomonas syringae*, a mimic of jasmonoyl-L-isoleucine (JA-Ile), binds to the COI1-JAZ complex (Zheng *et al*., 2012). Three homologous NAC TFs (petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2), NAC019, NAC055, and NAC072, which are induced by MYC2 released from the COI1-JAZ complex, repress the transcription of *ICS1* via binding the NAC core-binding sites (CACG tetramer) in the promoter of *ICS1* and reduce SA accumulation (Tran *et al*., 2004; Zheng *et al*., 2012). In addition, transcription factors ENTHYLENE INSENSITIVE3 (EIN3)

and ENTHYLENE INSENSITIVE3-LIKE1 (EIL1) that mediate ethylene signaling, are capable of binding the *ICS1* promoter and repress *ICS1* expression. In *ein3 eil1* double mutants, SA and *PR1* accumulate constitutively in the absence of pathogen attack (Chen *et al*., 2009b). The TFs involved in *ICS1* and *EDS5* transcription are summarized in Figure 2.

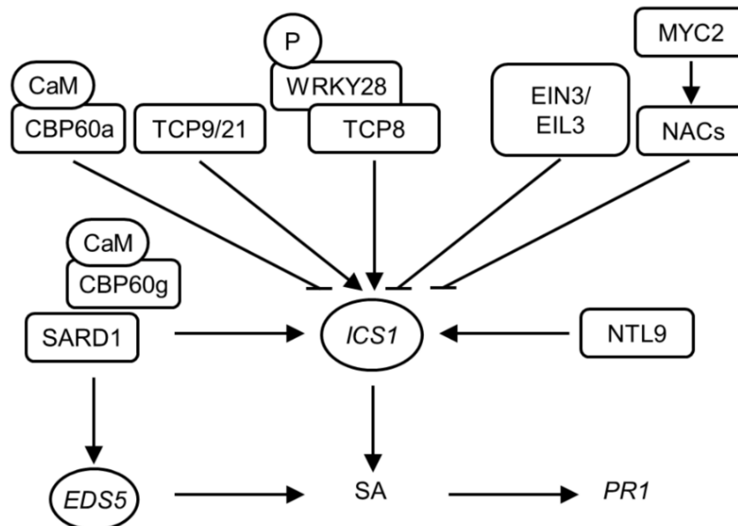

Figure 2. An overview of the roles of transcription factors in *ICS1* and *EDS5* regulation. The CaM-binding transcription factor CBP60g and its homolog SARD1 function redundantly for positive transcription of *ICS1* and *EDS5*. CBP60a is a negative regulator of *ICS1* transcription, respectively. WRKY28, regulated through phosphorylation by CPKs, interact with TCP8 and positively regulate expression of *ICS1*. TCP9, TCP21 and NTL9 also contribute to *ICS1* expression. In contrast, EIN3, EIL3 and NACs repress *ICS1* transcription represents cross talk between SA and JA/ET signaling pathways.

Metabolic enzymes

Genetic screens for mutants revealed that *avrPphB susceptible3* (*pbs3*) mutants displayed reduced disease resistance (Warren *et al*., 1999). Further investigation showed that both total SA amounts and *PR1* expression were dramatically compromised in these mutants, while exogenous SA treatment restored the total amount of endogenous SA and *PR1* expression (Jagadeeswaran *et al*., 2007; Nobuta *et al*., 2007). *PBS3* (also designated as *GH3.12*), encoding a member of the GH3 protein family of acyl-adenylate/thioester-forming enzymes, is induced by exogenous SA, as well as *ICS1* and *EDS5* (Staswick *et al*., 2002; Nobuta *et al*., 2007). PBS3 can specifically conjugate amino acids to 4-

substituted benzoates, while SA as a competitive inhibitor of 4-HBA conjugation inhibits its enzymatic activity (Okrent *et al*., 2009; Westfall *et al*., 2012). In the stress-treated *pbs3* mutant, decreased SA but elevated SA-Asp levels were observed, indicating that PBS3 presumably functions as an inhibitor of SA metabolism from SA to SA-Asp (Dempsey *et al*., 2011; Chen *et al*., 2013).

enhanced Pseudomonas susceptibility 1 (*eps1*) mutant showed a similar phenotype as the *pbs3* mutant: compromised resistance to both virulent and avirulent strains of *P. syringae* and reduced SA accumulation (Zheng *et al*., 2009). The reduction of resistance and pathogen-induced *PR1* gene expression in *eps1* mutant can be recovered by exogenous SA (Zheng *et al*., 2009). EPS1, a member of the BAHD acyltransferase superfamily, together with PBS3 have been suggested to function as IPL (Zheng *et al*., 2009). By a large-scale genetic screen for mutants, new mutants (*sln and isn*) have been identified that show altered SA accumulation and defense resistance upon infection in the *npr1* mutant background, suggesting the presence of additional regulators or enzymes of the SA signaling pathway (Ding *et al*., 2015).

SA Metabolism

Once accumulated in the cytosol via either the PAL or IC biosynthesis pathways, SA can be modified to various derivatives of which most are inactive. These modifications include glucosylation, methylation, amino acid conjugation, sulfonation and hydroxylation (Dempsey *et al*., 2011). The large amount of SA that accumulates in a short time in the cytosol is harmful to the cells, and therefore SA is converted to SA-O-β-glucoside (SAG) or salicylate glucose ester (SGE) by pathogen-inducible SA glucosyltransferase (SAGT) (Vlot *et al*., 2009; Dempsey *et al*., 2011). Non-toxic SAG is transported and stored in the vacuole to serve as a storage form that can be hydrolyzed to SA if needed (Loake and Grant, 2007). In tobacco, SA is converted to volatile methyl salicylate (MeSA) that serves as a mobile signal that translocates from the infected leaf to the systemic tissues where it is converted back to SA by SA-BINDING PROTEIN2 (SABP2) to induce SAR (Seskar *et al*., 1998; Park *et al*., 2007).

SA signaling pathway

Enhanced endogenous SA after pathogen infection is accompanied by the establishment of the SAR response, and associated with induction of defensive *PR* genes, which are considered as marker genes for SAR. Many studies have revealed that the positive regulator NON-EXPRESSER OF PR GENE1 (NPR1), also referred to as NON-INDUCIBLE IMMUNITY1 (NIM1), plays a central role in the signaling pathway downstream of SA that leads to the induction of SAR (Wu *et al*., 2012; Pajerowska-Mukhtar *et al*., 2013). The NPR1 paralogs NPR3 and NPR4 are SA receptors with different binding affinities that mediate degradation of NPR1 through their interaction with NPR1 (Fu *et al*., 2012, 2013).

The *npr1* mutant was identified through a screening for Arabidopsis mutants insensitive to either SA or the SA analog 2, 6-dichloroiso-nicotinc acid (INA). It displays a reduced expression of *PR* genes (Cao *et al*., 1994; Cao *et al*., 1997). NPR1 comprises a BTB/POZ domain, an ankyrin repeat domain, both of which mediate protein-protein interactions, and a nuclear localization signal (NLS) in the C-terminal part (Mou *et al*., 2003). In the absence of SA, NPR1 is predominantly sequestered in the cytosol as an oligomeric complex though intermolecular redox-sensitive disulfide bonds (Kinkema *et al*., 2000; Mou *et al*., 2003). Upon SA accumulation, NPR1 is reduced to monomers and translocated into the nucleus, resulting in defense resistance gene expression (Mou *et al*., 2003; Tada *et al*., 2008). On the other hand, elevated expression of ICS1 and toxic levels of SA in the *npr1* mutant, indicate that NPR1 acts as a negative regulator of *ICS1* expression and SA accumulation (Wildermuth *et al*., 2001; Zhang *et al*., 2010b).

Due to the absence of a DNA-binding domain in NPR1, in the nucleus, the expression of *PR* genes is activated by NPR1 via specific interaction with diverse families of TFs (Després *et al*., 2000; Pieterse and van Loon, 2004). NPR1 interacts with several members of the TGA family of TFs, which are characterized by a basic leucine zipper domain and directly

bind to *PR* gene promoters at the *as-1* elements (Gatz, 2013). In addition to TGAs, *PR* expression may be regulated by interactions between NPR1 and NIM1-INTERACTING (NIMIN) proteins, which might play a negative role in expression of NPR1-regulated genes (Pajerowska-Mukhtar *et al*., 2013).

Moreover, WRKY TFs are also involved in the regulation of *PR* genes, either indirectly or directly. Several WRKY TFs have been identified to bind the *PR1* promoter (Wang *et al*., 2006; Eulgem and Somssich, 2007). Tobacco NtWRKY12 is able to activate *PR-1a* expression via binding to the promoter region of *PR-1a* and interaction with TGA2.2 (van Verk *et al*., 2008; van Verk *et al*., 2011b). Husain (2012) found that Arabidopsis WRKY50 and WRKY28 bind to the *PR1* promoter and both of them presumably function as positive regulators of *PR1* expression. WRKY50 could interact with TGA2 and TGA5 and synergistically enhance *PR1:GUS* expression in Arabidopsis protoplasts.

Thesis Outline

The isochorismate (IC) pathway localized in the chloroplasts is the major contributor for SA to mediate the defense response in Arabidopsis. In this pathway, ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), a member of the multidrug and toxin extrusion (MATE) transporter family, localized in the chloroplast envelope is responsible for SA transport from the chloroplasts to the cytosol. Several transcription factors have been implicated in the expression of *EDS5*, including the positive regulators SARD1 and CBP60g. The first objective in this thesis focused on the transcription factors for the regulation of *EDS5* in the SA signaling pathway. The IC pathway of SA biosynthesis is not well defined yet. The second objective was to investigate the missing isochorismate pyruvate lyase (IPL) activity in Arabidopsis.

Chapter 2 describes that transcription factors AtERF-1, WRKY11 and WRKY28 bind to the promoter of *EDS5*, *ICS1* and *PR1*. Yeast one-hybrid (Y1H) screening led to the identification of AtERF-1 and WRKY11 as regulators of *EDS5*. Together with WRKY28, which was previously found to bind to and activate the promoter of *ICS1*, they act as potent activators of *ICS1* and *PR1* expression. In addition, we showed that

WRKY28 also binds to the *EDS5* promoter. *aterf-1* mutant plants showed elevated *PR1* expression. Constitutive expression of WRKY28 in plants resulted in enhanced expression of *PR1*. These results indicate that AtERF-1 acts as negative regulator of *PR1* and WRKY28 positively regulates *PR1* expression.

In **Chapter 3**, several proteins that were reported to mediate SA biosynthesis in Arabidopsis or have amino acid sequence similarity with bacterial proteins involved in SA biosynthesis were co-expressed with the bacterial isochorismate synthase *EntC* gene in *E. coli*. SA measurements were carried out by using a SA-responsive biosensor. The results implicate that co-expression of Arabidopsis PBS3 with EntC leads to SA biosynthesis in *E. coli*.

To investigate potential Arabidopsis proteins with IPL activity, an *E. coli* SA biosensor was developed based on the SalR regulator/*salA* promoter combination from *Acinetobacter* sp ADP1 controlling the expression of the reporter *luxCDABE* operon, which was described in **Chapter 4**. cDNA library screening using this biosensor resulted in a number of candidate genes possibly encoding the missing Arabidopsis IPL.

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