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

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

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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 glauca* 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 envelope and involved in exporting Phe to the cytosol (Maeda *et al.*, 2010; Maeda *et al.*, 2011; Widhalm *et al.*, 2015).

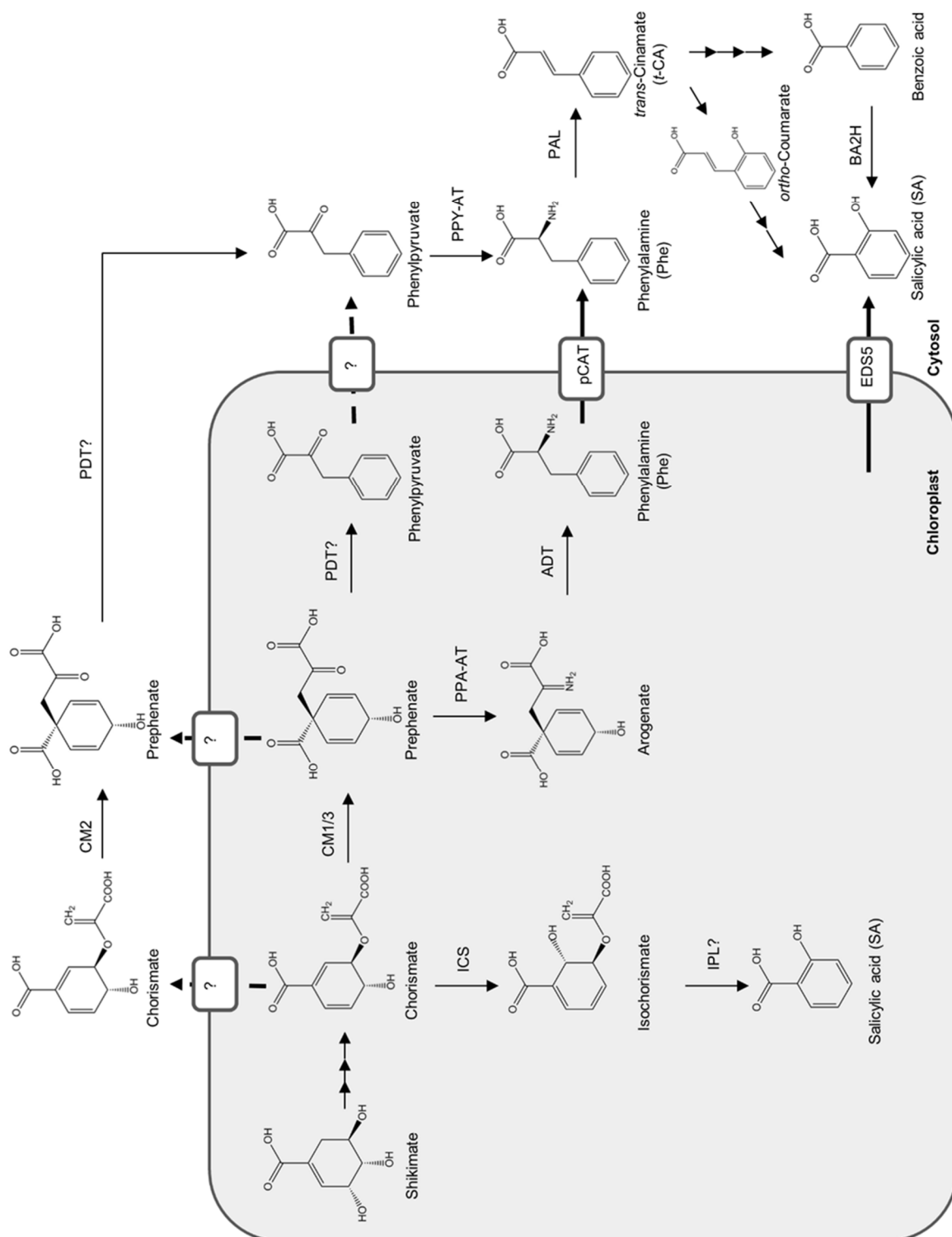


Figure 1. Two possible pathways of salicylic acid (SA) biosynthesis in plants. Enzyme names are given next to the arrows. Question marks indicate that the enzyme responsible for the indicated conversion has not yet been definitively identified. White boxes with question marks indicate unknown transport 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 microorganisms and is carried out by a bi-functional chorismate mutase/prephenate dehydratase (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 ¹⁴C-

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 phyloquinone, which is a pivotal component for photosystem I (PSI) (Gross *et al.*, 2006). Dramatically reduced phyloquinone production along with a striking visual unhealthy phenotype in *ics1 ics2* double mutant and decreased phyloquinone amounts in *ics1* or *ics2* single mutant suggest that both *ICS1* and *ICS2* are indispensable for phyloquinone 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 (Fragrière *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 isochlorogenic acid (Garcion *et al.*, 2008; Fragnière *et al.*, 2011). Confocal microscopy results confirmed that *EDS5* is targeted to the chloroplast envelope (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 Ca²⁺ 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 (Ca²⁺-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.

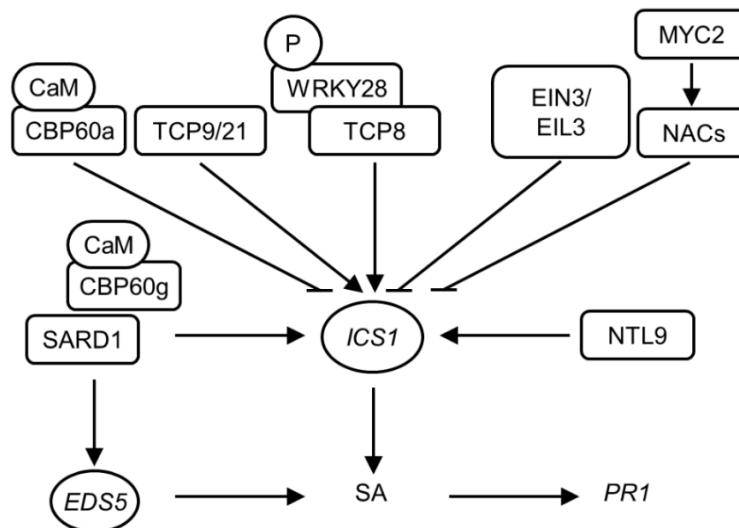


Figure 2. An overview of the roles of transcription factors in *ICS1* and *EDS5* regulation. The CaM-binding transcription factor CBP60g and its homolog SARD1 function redundantly for positive transcription of *ICS1* and *EDS5*. CBP60a is a negative regulator of *ICS1* transcription, respectively. WRKY28, 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. The arrow from SA to *PR1* 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-EXRESSER 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-dichloroisonicotinic 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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Chapter 2

AP/ERF and WRKY transcription factors involved in the coordinated regulation of the salicylic acid signaling pathway in *Arabidopsis thaliana*

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Abstract

Salicylic acid (SA) is an indispensable plant hormone, associated with local and systemic defense against biotrophic pathogens, which rely on living plant tissue for nutrients. *ENHANCED DISEASE SUSCEPTIBILITY5* (*EDS5*) encodes a protein that has homology to bacterial multidrug and toxin extrusion (MATE) antiporters and functions upstream of SA biosynthesis in Arabidopsis. By yeast one-hybrid screening we identified two transcription factors (TFs), AtERF-1 and WRKY11, which directly bind to the promoter sequence of *EDS5*. We also detected WRKY11 and WRKY28 associated with identical W-box sequences in the promoter region of *EDS5* *in vitro* assays. Moreover, AtERF-1 and WRKY11 were identified as candidate regulators of *ISOCHORISMATE SYNTHASE1* (*ICS1*) that encodes the key enzyme in SA biosynthesis, as well as *PATHOGENESIS-RELATED1* (*PR1*) that encodes a widely used marker for SA-mediated response. Significant elevation in the expression of *PR1* was observed in *aterf-1* mutants, indicating that AtERF-1 functions as a negative regulator of *PR1* expression. Meanwhile, enhanced *PR1* expression in WRKY28 overexpression plants infers that WRKY28 positively regulates *PR1* expression.

Introduction

Upon detection of an attacking pathogen, plants mobilize elaborate defense responses. Separate defense strategies have evolved against biotrophic and necrotrophic pathogens that use distinct but interacting signaling pathways mediated by small signal molecules produced during initial stages of the infection. For the defense response launched after attack by biotrophic pathogens, genetic data from *Arabidopsis* have led to a signal transduction model in which SA plays a central role. By contrast, defense against necrotrophic pathogens is controlled by signaling molecules jasmonates (JAs) and ethylene (ET). Generally, SA and JA/ET defense pathways act antagonistically (Glazebrook, 2005; Pieterse *et al.*, 2009)

The biosynthesis of pathogen-induced SA depends on either of two distinct pathways, one involving phenylalanine ammonia lyase (PAL), the other depending on isochorismate synthase (ICS), but both of the pathways are derived from substrate chorismate (Verberne *et al.*, 2000; Wildermuth *et al.*, 2001; Dempsey *et al.*, 2011). In *Arabidopsis*, the ICS pathway occurs in the chloroplasts and is the major contributor for SA accumulation (Strawn *et al.*, 2007; Garcion *et al.*, 2008; Fragnière *et al.*, 2011). *EDS5*, encoding a member of the multidrug and toxin extrusion (MATE) transporter family located in the chloroplast envelope, is required for exporting SA from the chloroplasts to the cytoplasm (Nawrath *et al.*, 2002; Ishihara *et al.*, 2008; Serrano *et al.*, 2013; Yamasaki *et al.*, 2013). *eds5* mutant is unable to mount the SAR response due to lack of sufficient SA in the cytosol, while plants constitutively expressing *EDS5* showed enhanced SA levels and elevated resistance to the yellow strain of *Cucumber mosaic virus* [CMV(Y)] (Nawrath *et al.*, 2002; Ishihara *et al.*, 2008).

In non-infected cells, SA is present only at very low levels. The expression of *ICS1* and *EDS5* is rapidly activated upon pathogen attack and leads to enhanced SA accumulation, subsequently. The accumulation of the signal molecule SA in infected leaves, as well as in non-infected leaves, triggers a long-lasting protection against infection known as systemic acquired resistance (SAR). This process is accompanied by

increased expression of a large number of *PR* genes and relies on the activation of NPR1 (Mou *et al.*, 2003; Durrant and Dong, 2004; Pajerowska-Mukhtar *et al.*, 2013).

In spite of the fact that SA accumulation is vital for the SAR response, high constitutive levels of SA in *Arabidopsis* or *Arabidopsis* mutants constitutively expressing SA-inducible defenses are detrimental to plant development, owing to SA-mediated repression of auxin-related genes, which results in a strongly dwarfed and infertile phenotype (Mauch *et al.*, 2001; Heidel *et al.*, 2004; van Hulten *et al.*, 2006; Wang *et al.*, 2007; Ishihara *et al.*, 2008). This indicates that in order to balance the regular growth and emergent responses to pathogen attacks, the genes involved in SA biosynthesis and the SAR responsive genes are under tight regulation.

As mentioned above, *EDS5* is rapidly induced after infection and results in increased *EDS5* mRNA levels preceding SA accumulation. In addition to this early, SA-independent response, *EDS5* is induced by exogenous SA, suggesting that the gene is also under late transcriptional control, likely mediated by other transcription factors. In previous studies, two pathogen-induced transcription factors, SAR DEFICIENT1 (SARD1) and CALMODULIN BINDING PROTEIN 60-LIKE G (CBP60g), were identified that directly bind and regulate the expression of *ICS1* and *EDS5* (Zhang *et al.*, 2010; Wang *et al.*, 2011; Sun *et al.*, 2015). In addition, atypical DP-E2F-LIKE 1 (DEL1) protein functions as a negative regulator of *EDS5* (Chandran *et al.*, 2014).

In the present study, we focused on transcription factors involved in *EDS5* gene regulation for unravelling the control of the SA homeostasis network in plants. By using DNA fragments of the *EDS5* gene promoter as bait in a yeast one-hybrid assay, we have identified three transcriptional regulators, i.e. AtERF-1, an APETALA2/ETHYLENE RESPONSE FACTOR (AP/ERF) domain transcription factor, WRKY11 and WRKY28, which both belong to transcription factor of WRKY family. Furthermore, we confirmed that AtERF-1 and WRKY11 play a role in *ICS1* and *PR1* gene expression as well as WRKY28. Thus, AtERF-1 and WRKY11 that have been shown to participate in the JA/ET-signaling pathway (Journot

-Catalino *et al.*, 2006; Pré *et al.*, 2008), were identified here as regulators in the SA signaling pathway, indicating that a sophisticated network between these two pathways exists.

Results

Transcription factors *AtERF-1* and *WRKY11* bind to the *EDS5* promoter

To identify TFs involved in regulation of gene expression of *EDS5*, we carried out a yeast one-hybrid screen through inserting promoter fragments in front of the yeast *His-3* reporter gene as baits and integrating the fused sequences into the genome of yeast strain Y187. After the initial screen and rescreen of individual clones, the region II fragment (-805 to -536 bp of *EDS5* promoter in Y187-II) resulted in 54 independent candidates that were able to grow on medium lacking histidine. Sequencing of those candidates revealed that cDNA sequences corresponding to *AtERF-1* (At4g17500) and *WRKY11* (At4g31550) were in frame with the GAL4 activation domain (AD) and resulted in transcription of *His-3* gene expression via binding to the -805 to -536 bp region of the *EDS5* promoter (Figure 1). In the previous work, *WRKY28* (At4g18170) has been identified as binding the promoters of *ICS1* and *PR1*, which were closely co-expressed with *EDS5* (van Verk *et al.*, 2011, Hussain, 2012; Aoki *et al.*, 2016). We examined *WRKY28* for binding to the promoter of *EDS5* using the same Y1H assay. The results indicated that *WRKY28* binds to the same promoter fragment as *AtERF-1* and *WRKY11*.

AtERF-1, a member of AP2/ERF-family of TFs, is highly induced upon inoculation with diverse pathogens and rapidly responds to exogenous JA and ethephon treatment (Oñate-Sánchez and Singh, 2002; Atallah, 2005; Thilmony *et al.*, 2006). *AtERF-1* has a predicted nuclear localization signal (NLS) in the C-terminal region (amino acids 221 to 237) and expression of an *AtERF-1*-GFP fusion construct in Arabidopsis protoplasts resulted in a strong GFP signal in the nuclei, suggesting that *AtERF-1* is localized to the cell nucleus (Figure S1) which is consistent with the result from Rioja *et al.* (2013).

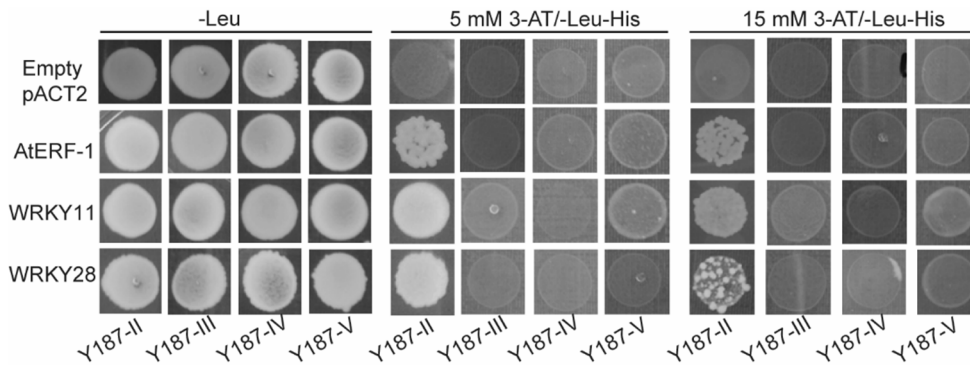


Figure 1. AtERF-1, WRKY11 and WRKY28 proteins bind to fragment II (-805 to -536 bp upstream of the transcription start site) of the *EDS5* promoter in yeast one-hybrid assay. The coding regions of AtERF-1, WRKY11 and WRKY28 cloned in the pACT2 vector or empty pACT2 (negative control) were transformed into bait-target yeast strains. Transformants were spotted on minimal synthetic defined (SD)-glucose medium lacking leucine (Leu) or Leu and histidine (His) and supplemented with the indicated concentrations of 3-amino-1,2,4-triazole (3-AT).

Characterization of AtERF-1, WRKY11 and WRKY28 binding sites in the *EDS5* promoter

AtERF-1 characteristically binds to consensus GCC box (5'-GCCGCC-3') (Allen *et al.*, 1998; Shoji *et al.*, 2013), while WRKY11 and WRKY28 are specifically bind to the consensus *cis*-acting W box element [5'-TTGAC(C/T)-3'] (Eulgem *et al.*, 2000; Rushton *et al.*, 2010). Region II (-805 to -536 bp) of the *EDS5* promoter does not contain a typical GCC box, but possesses three W boxes (Figure 2a). To establish whether AtERF-1, WRKY11 and WRKY28 indeed bind the *EDS5* promoter, we performed electrophoretic mobility shift assays (EMSAs) using the radioactively labeled fragment region II as probe to verify the results from the Y1H assays. Recombinant proteins were expressed in *E. coli* and purified by His tag or GST tag affinity chromatography. As shown in Figure 2a, the full-length AtERF-1 protein was unable to bind to the promoter fragment, which contradicted the results from the Y1H assays. However, a GST-tagged partial AtERF-1 derivative (AtERF-1-BD) containing the DNA-binding domain was able to interact with the *EDS5* promoter *in vitro*. WRKY11 and WRKY28 were able to bind to the probe and displayed shifted bands, which was consistent with the Y1H assays.

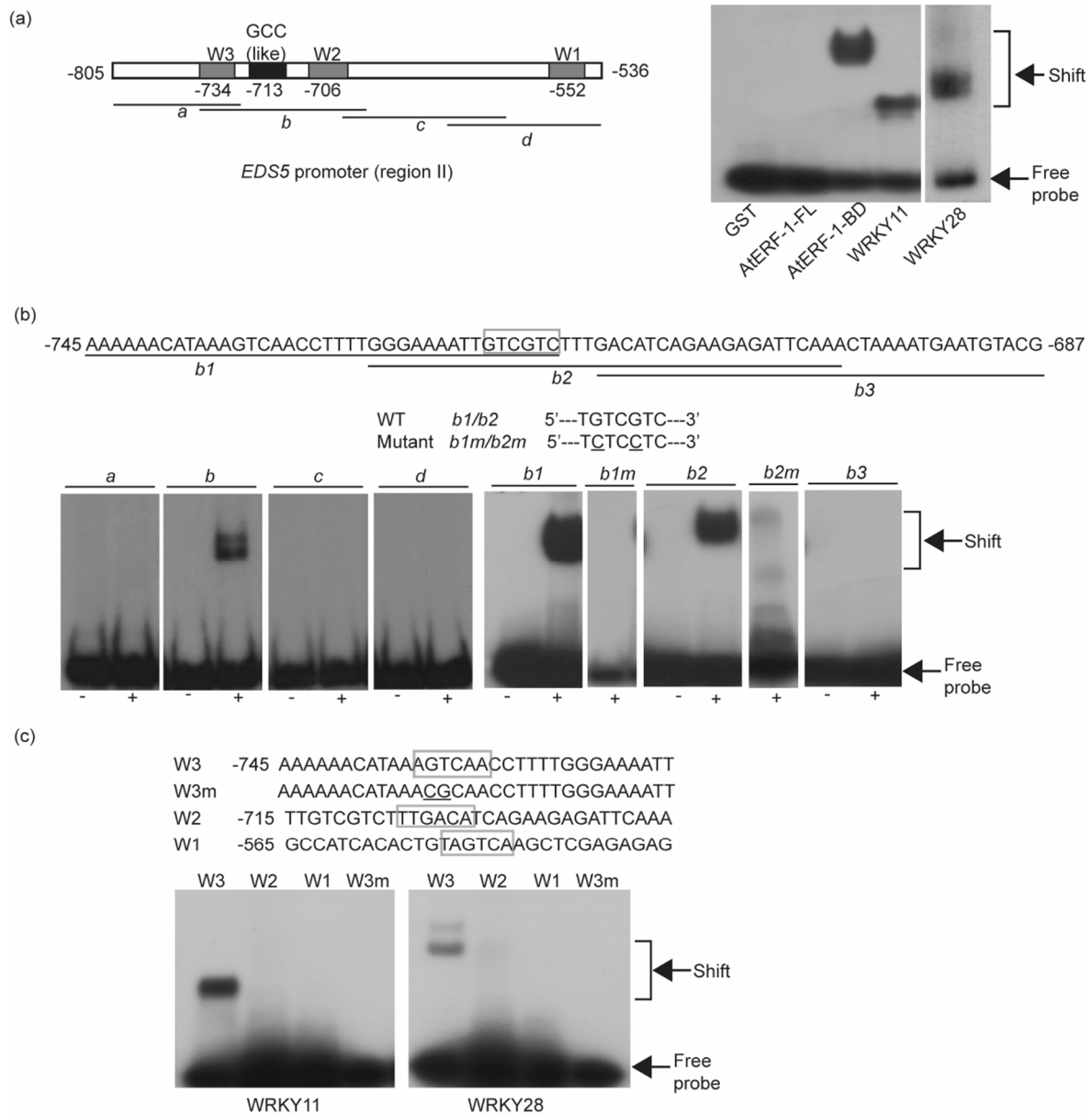


Figure 2. AtERF-1, WRKY11 and WRKY28 proteins bind to the *EDS5* promoter *in vitro*. The positions of the shifts and free probes are indicated by arrows. (a) Schematic representation of wild-type (WT) promoter fragments. The promoter region -805 to -536 bp was divided into four overlapping sub-fragments (a, b, c, d) of around 80 nucleotides each. EMSAs were performed with the ^{32} P-labeled fragment -805 to -536 bp of the *EDS5* promoter used as probe and His-tagged full length proteins AtERF-1 (AtERF1-FL), WRKY11 and WRKY28 or GST-tagged C-terminal part of AtERF-1 (AtERF-1-BD). (b) Radioactively labeled fragments (a, b, c, d) as indicated in (a) were used as probes to carry out EMSAs with AtERF1-BD. Three overlapping fragments derived from fragment b (b1, b2, b3) and mutated fragment b1 (b1m) and b2 (b2m) were labeled and used as probes for *in vitro* binding. Lanes marked with the minus (-) sign were loaded with control GST protein binding mixtures. A GCC-like *cis*-element is indicated by the box. (c) WRKY11 and WRKY28 proteins bind to the same W-box sequence in the *EDS5* promoter. WT and mutated W box

fragments (W3m) as listed were used as probes. The W-box is indicated by a rectangle in each probe. Mutant nucleotides are underlined.

Next, we investigated overlapping subfragments of promoter fragment II as probes (*a*, *b*, *c*, *d*) to narrow down the binding site of AtERF-1-BD in the promoter (Figure 2b). Only the *b* probe was recognized by AtERF-1. Subfragment *b* was further divided into three smaller fragments each containing 35 to 40 base pairs (*b1*, *b2*, *b3*). Band shifts were observed with the *b1* and *b2* probes. A GCC (like) box (GTCGTC) is present in the overlapping part of *b1* and *b2*. Mutation of the GCC (like) box in *b1* eliminated the binding of AtERF-1-BD (Figure 2b), while mutant *b2m* did not form a band shift indicating that the corresponding sequence GTCGTC (-713 to -708 bp from the transcription start site) is essential for binding to the AtERF-1-BD *in vitro*.

As shown in Figure 2c, the W box (W3) at position -734 to -729 bp was specifically involved in WRKY11 and WRKY28 binding to the promoter. Interestingly, a double shift was produced with the wild-type probe, while with the mutant probe both shifted bands were lost. This might suggest that W3 supports the binding of two different W box-WRKY28 complexes.

AtERF-1 and WRKY11 proteins bind to the promoters of SA biosynthesis gene *ICS1* and SA responsive gene *PR1*

In addition to *EDS5*, *ICS1* encoding the isochorismate synthase in the SA biosynthesis pathway is required for SA accumulation and *PR1* is critical for the SAR response (Wildermuth *et al.*, 2001; Glazebrook, 2005). Because *EDS5*, *ICS1* and *PR1* expression are co-regulated, we examined AtERF-1 and WRKY11 for binding to the promoters of *ICS1* and *PR1* using EMSAs (Figure 3). The 960 bp *ICS1* promoter contains one GCC-box-like sequence (GTCGTC) at position -591 to -585 bp identical to the GCC-like box in the *EDS5* promoter, as well as three W boxes (Figure 3a). The *PR1* promoter contains one classical GCC box and three W boxes (Figure 3b). AtERF-1-BD protein was able to bind to the promoters of *ICS1* and *PR1*, respectively (Figure 3c). EMSAs with ³²P-labeled wild-type and mutant versions of W boxes of *ICS1* and *PR1*

promoter fragments suggested that WRKY11 binds to the W3 box of the *ICS1* promoter and the W2 box of the *PR1* promoter (Figure 3d).

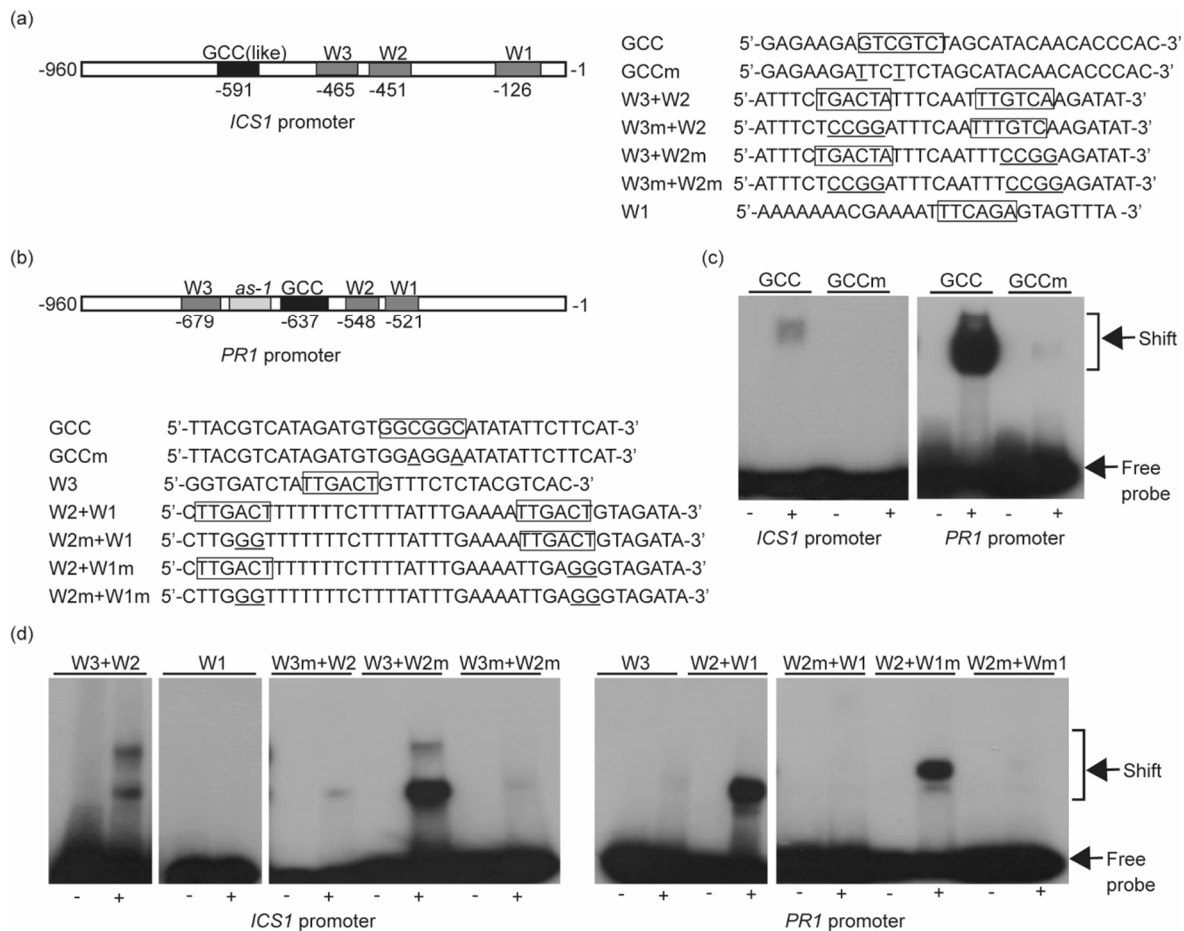


Figure 3. AtERF-1 and WRKY11 proteins bind to the promoters of SA biosynthesis gene *ICS1* and SA-responsive gene *PR1* *in vitro*. The positions of the shifts and unbound free probes are indicated by arrows. Lanes marked with minus (-) signs were loaded with binding mixtures with control GST protein. (a) WT and mutant *ICS1* promoter fragments. The GCC box and W boxes are indicated by rectangles in the probes. (b) WT and mutant *PR1* promoter fragments. The GCC box and W boxes are indicated by rectangles in the probes. (c) AtERF-1 protein binds to GCC boxes indicated at (a) and (b) in the *ICS1* and *PR1* promoter. (d) WRKY11 protein binds to a single W box in the *ICS1* and *PR1* promoters. Binding sites are indicated in (a) and (b). Mutant nucleotides are underlined.

Role of AtERF-1 and WRKY11 in *EDS5*, *ICS1* and *PR1* gene expression in protoplasts

To investigate the effects of transactivation of *EDS5*, *ICS1* and *PR1* promoters by AtERF-1 and WRKY11, Arabidopsis protoplasts were co-transfected with *GUS* reporter gene constructs fused to 1 kb promoter regions of the respective genes together with effector plasmids carrying the *AtERF-1*, *WRKY11* or *WRKY28* genes under the control of the

CaMV 35S promoter (Figure 4). *EDS5* and *ICS1* promoter-directed *GUS* expression were enhanced approximately 6-fold and 8-fold, respectively, by AtERF-1 compared to the controls (Figure 4a). As shown in Figure 4b, WRKY11 was able to trans-activate the *ICS1:GUS* reporter gene 5-fold and the *PR1:GUS* reporter gene 6-fold. However, WRKY11 did not significantly increase *EDS5:GUS* gene expression. Co-transfection of *EDS5:GUS* with *35S:WRKY28* increased *GUS* expression 20-fold over the control level (Figure 4c). All *GUS* expression levels were significantly lower with mutant promoter constructs. Together these observations indicate that AtERF-1 trans-activated the expression of *EDS5* and *ICS1* through the specific binding sites in the promoters *in vivo*. In addition, WRKY11 was an efficient activator of *ICS1:GUS* and *PR1:GUS* reporter gene expression in Arabidopsis protoplasts via specific binding to the W3 and W2 box in the promoters, respectively, while WRKY28 positively regulates *EDS5:GUS* expression by binding to the W3 box in the *EDS5* promoter.

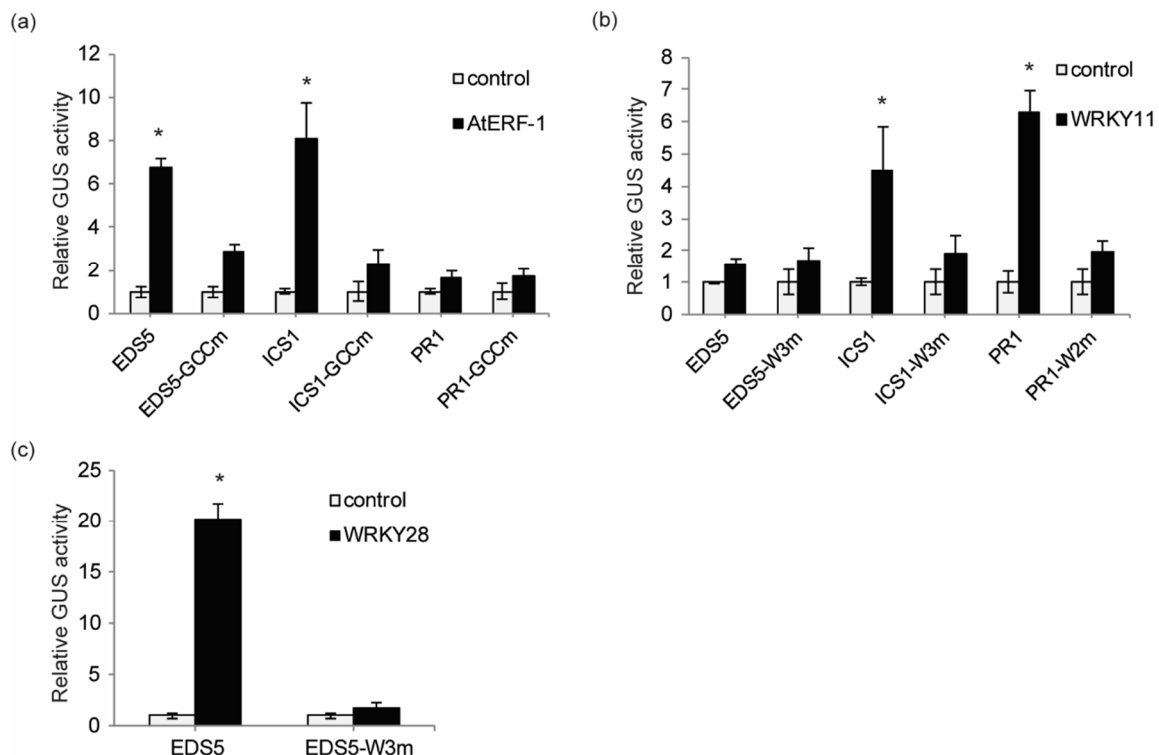


Figure 4. Effects of AtERF-1, WRKY11 and WRKY28 on the expression of *EDS5:GUS*, *ICS1:GUS* and *PR1:GUS* in Arabidopsis protoplasts. (a) AtERF-1 trans-activation *EDS5*, *ICS1* and *PR1* promoter. Protoplasts were co-transformed with wild-type-*GUS* or mutant (m)-*GUS* construct (1 µg) and effector plasmid containing *35S:AtERF-1* (6 µg) or empty

pRT101 vector (control). The mutant GCC boxes (GCCm) in *EDS5*, *ICS1* and *PR1* promoter identified in Figure 2 and 3 were used to generate the mutation constructs. (b) WRKY11 trans-activation of *ICS1:GUS* and *PR1:GUS*. Protoplasts were co-transformed with wild-type-GUS or mutant (m)-GUS construct (2 µg) and effector plasmid containing 35S:*WRKY11* (6 µg) or empty pRT101 vector (control). The mutant W boxes (Wm) in *EDS5*, *ICS1* and *PR1* promoter identified in Figure 2 and 3 were used to generate the mutation constructs. (c) Transactivation of *EDS5:GUS* gene expression by WRKY28. Protoplasts were co-transformed with wild-type *EDS5:GUS* or mutant (Wm)-GUS construct (2 µg) and effector plasmid containing 35S:*WRKY28* (6 µg) or empty pRT101 vector (control). The mutant W box in the *EDS5* promoter identified in Figure 2 was used to generate the mutation construct. The GUS values obtained with empty pRT101 were set to 1. Asterisks indicate significant differences in GUS activity in comparison to the controls ($P < 0.01$). The bars represent means $\pm$ SE ($n=3$). Experiments were repeated three times with similar results.

Gene expression patterns in plants

GUS activities in protoplasts indicated that AtERF-1, WRKY11 and WRKY28 act as positive regulators in SA-mediated gene expression. To investigate gene expression patterns in response to SA, Arabidopsis wild-type (Col-0) seedlings were treated with SA. *ICS1* and *EDS5*, were expressed at detectable levels in plants (Figure 5a). *EDS5* and *PR1* were significantly induced after spraying with SA, particularly *PR1*. *EDS5* expression levels reached a peak between 12h to 24h after SA application and returned to baseline levels at 48h after the application. A modest induction was observed with the genes *ICS1* and *AtERF-1* upon SA treatment (Figure 5a, Atallah, 2005). However, *WRKY11* and *WRKY28* were not responsive to SA treatment (Dong et al., 2003).

To investigate whether AtERF-1 and WRKY28 regulate defense gene expression, *aterf-1* mutant plants, 35S:*AtERF-1* transgenic Arabidopsis plants constitutively expressing *AtERF-1* (AtERF-1 OE) and 35S:*WRKY28* transgenic plants overexpressing *WRKY28* (WRKY28 OE) were analyzed. RNA gel blot analyses revealed that the *aterf-1* mutant containing a T-DNA insertion in the 5'-UTR region of the *AtERF-1* gene expressed the gene at reduced levels in comparison to WT plants (Figure 5b). The expression of *EDS5* and *ICS1* was hardly changed in the mutant or in the overexpression lines. The *PR1* gene was highly expressed in *aterf-1* mutant and WRKY28 OE compared to WT plants and 35S:*GUS* (GUS) plants without SA treatment. However, the *PR1* expression level was barely changed in *AtERF-1* overexpression lines

(Figure 5c). Together, these results indicate that AtERF-1 negatively affects basal level of *PR1* gene expression and WRKY28 stimulates basal level of *PR1* expression in plants.

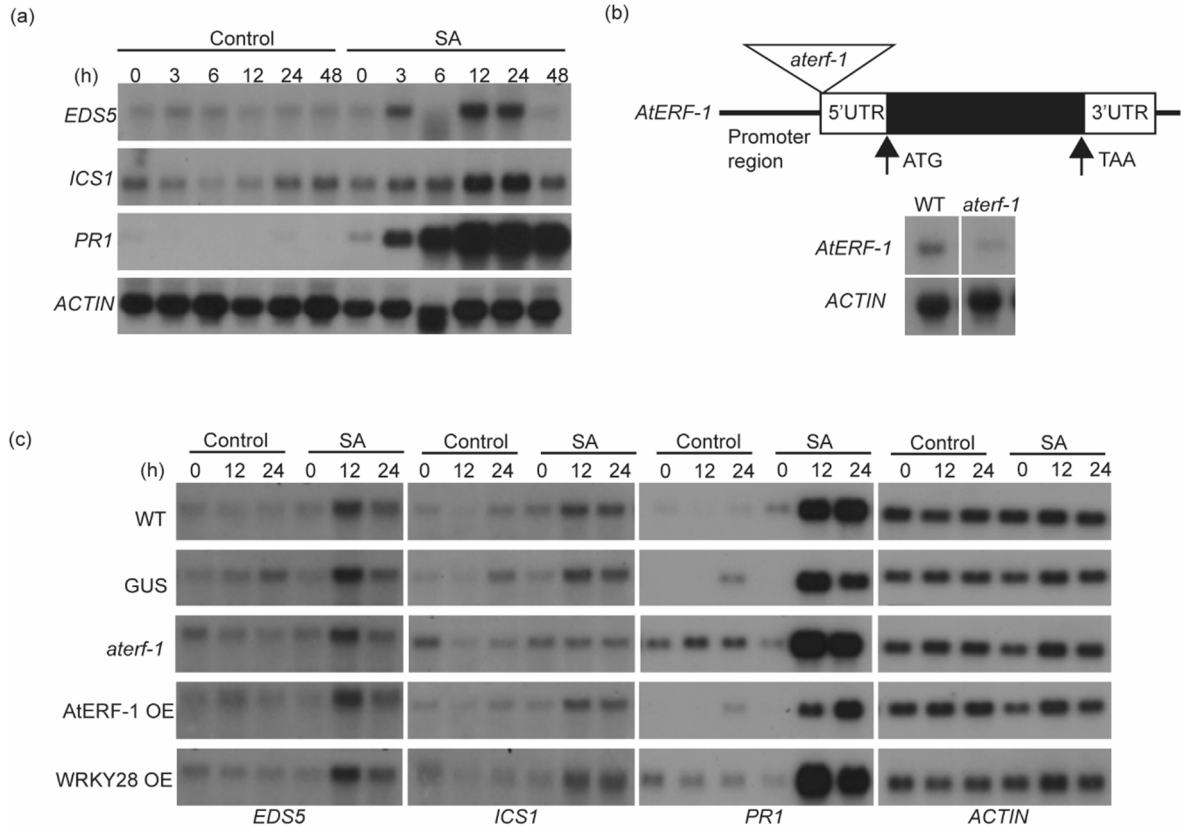


Figure 5. Analysis of gene expression in Arabidopsis wild-type plants (Col-0), 35S:GUS (GUS), *atarf-1*, transgenic plants 35S:AtERF-1 (OE) and 35S:WRKY28 (OE). Total RNA was isolated from three to four-week-old Arabidopsis seedlings treated with water (control) or 2 mM SA for the number of hours indicated. *ACTIN* served as a control for RNA loading. (a) Gene expression in wild-type plants after water (control) or 2 mM SA treatment. (b) Diagrammatic representation of the T-DNA insertion in *AtERF-1* (SALK_036267). The position of the T-DNA insertion in the *atarf-1* mutant is indicated by the triangle, while the coding region of the gene is indicated by the black box. The white boxes are untranslated regions (UTR). (c) *EDS5*, *ICS1* and *PR1* gene expression in Arabidopsis *atarf-1* mutant and transgenic plants. Panels hybridized with the same probe were on the same blot and exposed to film for the same time to enable direct comparison. *ACTIN* expression levels were used to correct for differences in loading.

SA accumulation in plants after UV treatment

To determine whether AtERF-1 or WRKY28 plays a role in SA biosynthesis, we further analyzed the induction of SA accumulation by treating the *atarf-1* mutant, AtERF-1 OE and WRKY28 OE plants with UV light according to Nawrath *et al.* (1999). SA quantification was

carried out by a SA-responsive biosensor *Acinetobacter* sp. ADPWH *_lux* (Huang *et al.*, 2005; Huang *et al.*, 2006). As shown in Figure 6, UV-induced free SA and total SA accumulation in WRKY28 OE are significantly higher than in WT and GUS plants. In contrast, neither the *aterf-1* mutant nor AtERF-1 OE plants have significantly different levels of SA accumulation upon UV exposure or in the non-induced state, compared to WT plants and GUS lines.

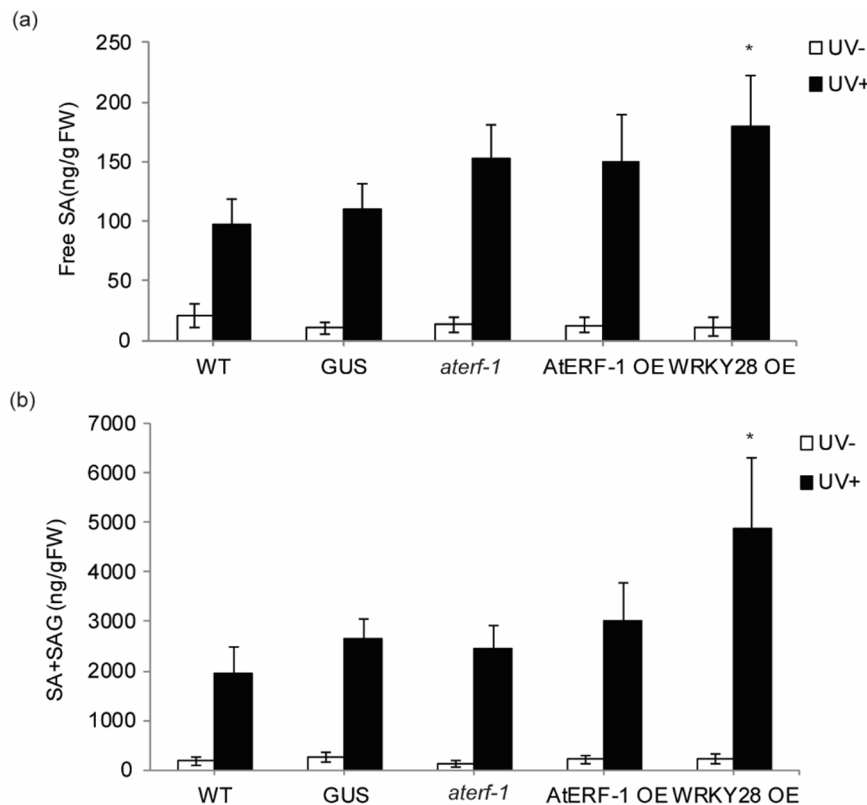


Figure 6. Quantification of total SA in leaves of wild-type plants (Col-0), *aterf-1*, 35S:GUS (GUS), 35S:AtERF-1 (AtERF-1 OE) and 35S:WRKY28 (WRKY28 OE) transgenic lines. Four-week-old seedlings were treated with UVc light for 20 min and leaf tissues were collected after 48 hours. **(a)** Quantification of free SA in leaves. **(b)** Quantification of total SA (SA+SAG) in leaves. The values represent the average of six treated plants ($\pm$ SE). Asterisks indicate significant differences in SA content in comparison to WT and GUS plants ($P < 0.05$). The experiments were repeated twice with similar results. FW indicates fresh weight.

Discussion

The signaling molecule SA is a key regulator in plant disease defense and acts in a complex network of cross talk with the JA/ET signaling pathways. *EDS5*, encoding a transporter involved in exporting SA from the chloroplasts to the cytoplasm and situated downstream of SA

synthesis, is crucial for SA accumulation upon pathogen infection. While previously it was shown that AtERF-1 and WRKY11 are involved in the JA signaling pathway (Journot-Catalino *et al.*, 2006; Pré *et al.*, 2008), in this study, we identified AtERF-1, WRKY11 and WRKY28 as putative trans-regulators of *EDS5* by yeast one-hybrid assay. Interestingly, these transcription factors were shown to be also involved in regulation of *ICS1* and *PR1* expression.

The involvement of AtERF-1, WRKY11 and WRKY28 in the regulation of *EDS5* expression were supported by the results of *in vitro* promoter binding studies (Figure 2a). The fragment (-805 to -536 bp) of the *EDS5* promoter contains three classical W boxes, which have high affinity for proteins of the group of WRKY transcription factors (Eulgem *et al.*, 2000; Rushton *et al.*, 2010). In our gel shift experiments, WRKY11 and WRKY28 specifically bound to the same W box sequence (TTGACT) in the *EDS5* and *PR1* promoters (Figure 2c; Hussain, 2012). It is possible that WRKY11 and WRKY28 compete for this binding site. AtERF-1, an AP2/ERF domain transcription factor, has GCC box-specific binding activity, which is sensitive to changes in the GCC box sequence (Fujimoto *et al.*, 2000; Shoji *et al.*, 2013). Full-length AtERF-1 was unable to bind the *EDS5* promoter in EMSAs, which contradicted the results from the Y1H assay (Figure 1). However, the C-terminal part of AtERF-1 (AtERF-1-BD) containing the DNA-binding domain efficiently bound to the probe. This could be related to the fact that the extended N-terminal sequence of AtERF-1 is absent in other members of the family (Rioja *et al.*, 2013) and might indicate that this region interacts with other transcription factors.

The function of AtERF-1 is far from being elucidated. In previous studies, expression of *AtERF-1* is rapidly responsive to JA with a peak 15 min after treatment and is induced by SA as well (Atallah, 2005; Caarls *et al.*, 2017). Constitutive overexpression of *AtERF-1* in plants leads to high expression of the JA marker gene *PDF1.2* due to non-specific stress effects, whereas the expression of *PDF1.2* is not activated when AtERF-1 is transiently or inducibly expressed (Pré *et al.*, 2008). By examining the regulatory effects of AtERF-1, we demonstrated that

AtERF-1 binds to the promoters of *EDS5*, *ICS1* and *PR1* directly *in vitro* and acts as positive regulator of *EDS5:GUS* and *ICS1:GUS* expression when over-expressed in Arabidopsis protoplasts, while it has no effect on expression of *PR1:GUS* (Figure 4a). However, accumulation of *EDS5* and *ICS1* mRNA is virtually unchanged in *aterf-1* mutant and *AtERF-1* overexpressing plants (Figure 5c). *PR1* expression in *aterf-1* was higher compared with Col-0 plants (Figure 5c), which is inconsistent with results reported by Caarls *et al.* (2017). Our results suggest that AtERF-1 negatively influences *PR1* expression. In plants, SA is the key signaling molecule mediating defense against infections and is produced in low concentrations to regulate physiological functions as well (Rivas-San Vicente and Plasencia, 2011; Spoel and Dong, 2012). We speculate that AtERF-1 might act as a transcriptional activator of *EDS5* and *ICS1*, depending on the interaction with other TF(s) to synthesize SA for plant developmental requirements and act as a repressor on *PR1* expression when SA becomes elevated in non-pathogen infection situations. The region between approximately -700 and -600 bp upstream of the transcription start site of the *PR1* gene is crucial for inducible gene expression (Lebel *et al.*, 1998). In this region, transcription factor TGA2 binds to the *activating sequence-1* (*as-1*) and interacts with NPR1 and AtWRKY50 to regulate *PR1* expression (Kinkema *et al.*, 2000; Rochon *et al.*, 2006; van Verk *et al.*, 2008; Hussain, 2012; Pajerowska-Mukhtar *et al.*, 2013). When AtERF-1 binds to this promoter region, it might interfere with the binding or activities of these transcription factors to modulate the expression of *PR1*. So far, various TFs, including TGAs, NPR1, WRKYs and AtERF-1, have been identified that bind to a relatively simple inducible gene promoter, indicating that *PR1* gene expression depends on an intricate interplay (combinations and interactions) of many transcription factors.

WRKY11, a member of the IId subfamily of WRKY proteins, acts as negative regulator of basal resistance to bacterial pathogens and regulates Induced Systemic Resistance (ISR) through activating the JA signaling pathway (Eulgem *et al.*, 2000; Journot-Catalino *et al.*, 2006; Jiang *et al.*, 2016). WRKY11 seems to have similar transcriptional activities as WRKY17, which is reflected by a 72% identity with WRKY17

at the amino acid level. After inoculation with *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000, the double mutant *wrky11wrky17* showed significantly enhanced *PR1* expression (Journot-Catalino *et al.*, 2006). We found that WRKY11 binds to the *EDS5* promoter in Y1H screenings and confirmed the binding sites of WRKY11 in the *EDS5*, *ICS1* and *PR1* promoters by EMSAs (Figure 1, 2 and 3). We tested the effect of WRKY11 in transient transfection assays (Figure 4b). We demonstrated that WRKY11 trans-activate *ICS1:GUS* and *PR1:GUS* expression in protoplasts, while *EDS5:GUS* activation is not observed. These results suggest that WRKY11 might play a role as an essential hub regulator in SA and JA signaling pathway.

WRKY28 belongs to the IIc subfamily of WRKY proteins has been shown to bind to the promoters of *ICS1* and *PR1* by EMSAs and induce their expression in transfection assays (Eulgem *et al.*, 2000; van Verk *et al.*, 2011; Hussain, 2012). According to Truman and Glazebrook (2012), the relationship between WRKY28 and *ICS1* may allow fine-tuning of the regulatory network. Recently, WRKY28 has been reported to interact strongly with TCP8, a transcription factor from the TEOSINTE BRANCHED1/CYCLOIDEA/PCF (TCP) family, to regulate the expression of *ICS1* (Wang *et al.*, 2015). Constitutive expression of *WRKY28* in *Arabidopsis* did not lead to *PR1* expression when plants were cultivated in MA medium (Hussain, 2012). In our experiments, we determined that WRKY28 binds to the *EDS5* promoter via the consensus W box motif and trans-activates *EDS5:GUS* expression in protoplasts (Figure 1, 2 and 4c). The RNA gel blot results demonstrated that with plants in soil, constitutive expression of *WRKY28* leads to *PR1* expression in non-treated plants and elevates *PR1* expression after SA treatment, in comparison to WT plants (Figure 6). Notably, *EDS5* and *ICS1* were not induced by SA treatment when wild-type plants were cultivated in MA medium indicating different gene expression patterns between liquid growth medium and soil. WRKY28 did not activate either *EDS5* or *ICS1* gene expression when over-expressed, which is consistent with the results reported by Truman and Glazebrook (2012). However, elevated SA content was observed in *WRKY28* over-expressing plants after UVc light exposure (Figure 6), which might

suggest that WRKY28 activates *EDS5* and *ICS1* gene expression in stress conditions. Alternatively, WRKY28 might require activation like phosphorylation by CPKs (Ca²⁺-dependent protein kinase) (Gao *et al.*, 2013) to activate the expression of *ICS1* and *EDS5*.

The research described in this study provides more information on the regulation of SA-mediated defense responses. Our findings are summarized in the model of Figure 7. Upon pathogen attack, *ICS1* and *EDS5* are activated for production and accumulation of SA. The transcriptional regulation of *ICS1* and *EDS5* might involve a positive feedback regulation loop by SA, because the expression of *ICS1* and *EDS5* was induced by exogenous SA (Figure 5a). AtERF-1, induced by SA and JA, represses the expression of *PR1*. WRKY28 positively regulates *PR1* expression, and may also positively modulate *ICS1* and *EDS5* expression, depending on the conditions. Expression of the genes encoding the LOX2 and AOS enzymes involved in the JA biosynthesis pathway is regulated positively and redundantly by WRKY11 and WRKY17 (Turner *et al.*, 2002; Journot-Catalino *et al.*, 2006). WRKY11 alone might be a positive regulator of *ICS1* and *PR1* in protoplasts. Whether WRKY11 functions alone or in combination with other transcription factors to activate or repress gene expression in plants is presently unknown.

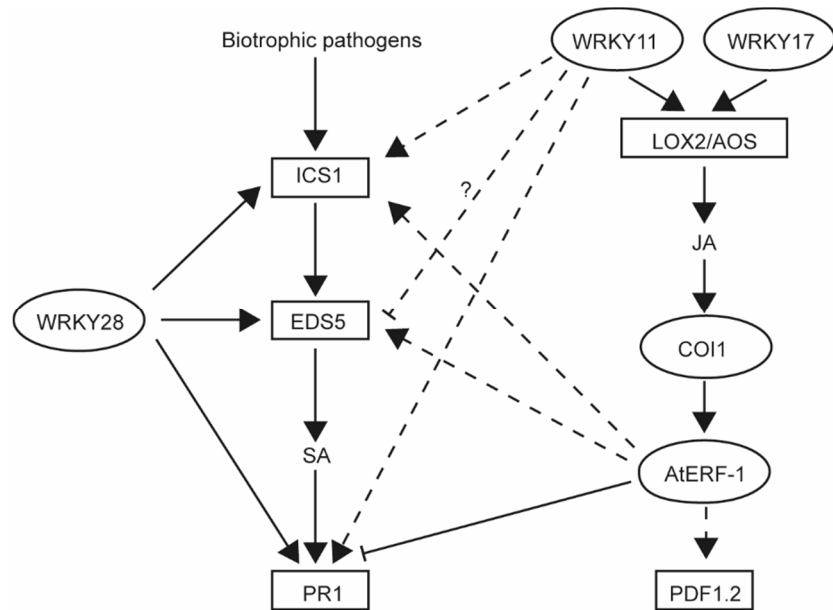


Figure 7. Model for the involvement of AtERF-1, WRKY11 and WRKY28 in SA and JA signaling pathways in Arabidopsis. Upon infection with biotrophic pathogens, SA level is enhanced in the cytosol by the activation of ICS1 and EDS5 and leads to the expression of *PR1*. AtERF-1 represses *PR1* gene expression and is hypothesized to activate *ICS1* and *EDS5* gene expression (indicated by the dashed lines). WRKY11 and WRKY17 redundantly activate *LOX2* and *AOS* expression. WRKY11 might play an activator in the regulation of *ICS1* and *PR1* (indicated by the dashed lines). The exact role of WRKY11 in the *EDS5* gene regulation remains unclear and awaits further analyses (indicated by the dashed line and question marker). WRKY28 is a positive regulator of *PR1*, *ICS1* and *EDS5* gene expression. On the other hand, the expression of *AtERF-1* depends on receptor *COI1* from JA signaling pathway. AtERF-1 indirectly activates JA/ET responsive defense gene *PDF1.2* (indicated by the dashed lines).

Materials and methods

Plant growth conditions and treatments

The following *Arabidopsis thaliana* lines were used: ecotype Columbia (Col-0) plants, homozygous *aterf-1* (SALK_036267) mutant plants, *AtERF-1* overexpression plants (Atallah, 2005) and *WRKY28* overexpression plants (Hussain, 2012) in the Col-0 genetic background. Seeds were surface-sterilized in a closed container with chlorine gas for 3-4 hours and sown on MS medium supplemented with hygromycin (20 mg/L) for overexpression plants. After stratification for 3 days at 4°C, seeds were cultivated at 21°C in a growth chamber with 16 h light/8 h dark for two weeks.

For treatment, seedlings were transferred to soil and grown for 7-10 days. Seedlings were sprayed with 2 mM SA (Sigma-Aldrich) dissolved in water (pH 5.8). For the control treatment, seedlings were sprayed with water and harvested at the same time points as the SA-treated plants.

Yeast one hybrid (Y1H) assay

Y1H screening was performed as described earlier (Ouwerkerk and Meijer, 2001). Briefly, Arabidopsis overlapping fragments of the *EDS5* promoter corresponding to the regions -805 to -536 bp (region II), -575 to -326 bp (region III), -363 to -106 bp (region IV) and -160 to -1 bp (region V) upstream of the transcription start site were obtained from genomic DNA. Collinear dimers of each promoter fragments were cloned in front of the *His-3* gene in pHIS3N/X plasmid. The promoter/*His-3* sequences were cloned in pINT1 vector and integrated into the genome of yeast strain Y187, resulting in strains Y187-II, Y187-III, Y187-IV and Y187-V, respectively. A cDNA library was prepared from an equal mixture of RNA from stems, leaves, roots and flowers of mature ecotype *Landsberg erecta* plants and fused to the GAL4 activation domain in vector pACT2 (Memelink, 1997). The cDNA library was introduced into the target-bait strains. Transformants were initially screened on minimal synthetic defined (SD)-glucose medium with or without 5 mM 3-amino-1,2,4-triazole (3-AT; the concentration was optimized for each strain) and lacking leucine and histidine (SD-LH). The prey fragments of the positive colonies were sequenced.

Full-length *AtERF-1*, *AtWRKY11* and *AtWRKY28* were amplified and cloned into pACT2. For re-transformations, bait plasmids were transformed into the target-bait strains and plated on SD-L medium. Empty pACT2 was used as a negative control. After cultivation for 7 days at 30°C, transformants were grown for 16 hours in liquid SD-L medium and 10 µL of 100-fold dilutions were plated on solid SD-LH medium containing increasing concentrations of 3-AT. The cells were allowed to grow for 7 days at 30°C. Primers used for plasmid construction are presented in Table S1.

Heterologous Expression of proteins in *E. coli* and EMSA

The coding regions of *AtERF-1*, *AtERF-1*-C-terminal, *WRKY11* and *WRKY28* were PCR-amplified from cDNA as described above. The coding regions were cloned into vector pET-16h (a derivative of pET-16b) (Langeveld *et al.*, 2002) and proteins were produced with C-terminal His tags. The C-terminal region of *AtERF-1* ($\Delta 105$) was cloned into vector pGEX-KG (Guan and Dixon, 1991) and protein was produced with a N-terminal GST tag. These proteins were expressed in *E. coli* strain BL21 (DE3) pLysS (Novagen). His-tagged proteins were purified with Ni-NTA Agarose (Qiagen) and GST-tagged protein was obtained with Glutathione-Sepharose 4B (GE Healthcare). The purified proteins were dialyzed overnight against a buffer containing 50 mM HEPES-KOH (pH 7.0), 200 mM KCl and 10% glycerol and stored at -80°C. Fusion proteins were analyzed by 15% SDS-PAGE.

The EMSA assays were performed as described according to Memelink (2013). Promoter fragment of region II and four overlapping fragments of region II were cloned into pJET1.2 (Fermentas) and excised by *Bgl*II and *Bam*HI. Other DNA probes for EMSA assays were obtained by slowly cooling down mixtures of equimolar amounts of complementary oligonucleotides from 95°C to room temperature. The fragments were labelled by filling the overhangs with Klenow fragment of DNA polymerase I and [α -³²P]-dCTP.

EMSA reaction mixtures contained 5,000-10,000 cpm of labeled DNA probe, 0.2-1 µg purified protein, 0.5 µg poly(dAdT)-poly(dAdT), 25 mM HEPES-KOH (pH 7.2), 100 mM KCl, 0.1 mM EDTA and 10% glycerol in a total volume of 10 µL. After incubation for 30-60 min at room temperature, the mixtures were loaded onto 5% polyacrylamide gel in 0.5×Tris-Borate-EDTA buffer and electrophoresed. Subsequently, the gel was dried on Whatman DE81 paper and autoradiographed.

Arabidopsis protoplast transactivation assays and microscopic analysis

For effector plasmids, PCR-amplified fragments of *AtERF-1*, *WRKY11* and *WRKY28* were cloned into pRT101 downstream of the *CaMV* 35S promoter (Töpfer *et al.*, 1987). For reporter constructs, the -960 to -1 bp

region upstream of the transcriptional start site of *EDS5*, *ICS1* and *PR1* were cloned in front of the GUS coding region in vector pT7:GUS (Gallie *et al.*, 1991). Protoplasts prepared from Arabidopsis ecotype Col-0 cell suspension (van Verk *et al.*, 2008) were co-transformed with 1-2 µg of reporter plasmids and 6 µg of effector plasmids. As a control, co-transformed reporter plasmids and empty pRT101 were used. The protoplast transformation was performed according to Schirawski *et al.* (2000). The protoplasts were harvested 18 hours after transformation and frozen in liquid nitrogen. GUS activity was determined as described (van der Fits and Memelink, 1997) and triplicate experiments were normalized against total protein concentration (Zhou, 2014).

For AtERF-1 subcellular localization, the coding region of *AtERF-1* was fused at the C-terminus to the coding region of GFP in plasmid pTH2 (Chiu *et al.*, 1996). Protoplasts were transformed with the plasmids and images of transfected protoplasts were acquired with a Leica DM IRBE confocal laser scanning microscope 488nm (excitation) and a band pass emission filter of 500-550nm. Used primers are presented in Table S1.

RNA extraction and Northern blot analyses

Total RNA was extracted from frozen Arabidopsis leaves grown in soil for 3-4 weeks after spraying with water or 2 mM SA by phenol/chloroform buffer (25% v/v phenol, 25% v/v chloroform, 175 mM glycine, 24 mM NaOH, 170 mM NaCl, 20 mM EDTA and 2% w/v SDS). The mixture was centrifuged, and the aqueous phase was extracted with 0.5 mL phenol. The upper aqueous phase from phenol extraction step was extracted with 0.5 mL chloroform. The RNA was overnight precipitated by 2 M lithium chloride, washed with 70% ethanol. Vacuum dried RNA was resuspended in water and stored at -20°C. RNA-blot analyses were performed as previously described (Memelink *et al.*, 1994). DNA fragments for probes were amplified from Arabidopsis cDNA by PCR. The primers used can be found in Table S1.

UVc light treatment, SA extraction and measurement in plants

Four-week-old Arabidopsis plants were exposed to UVc light (254 nm) as previously described (Nawrath and Métraux, 1999). Plant SA extra-

ction and measurement were performed with a bacterial biosensor *Acinetobacter* sp. ADPWH_*lux* according to Huang *et al.* (2006) and Marek *et al.* (2010)

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Supporting Information

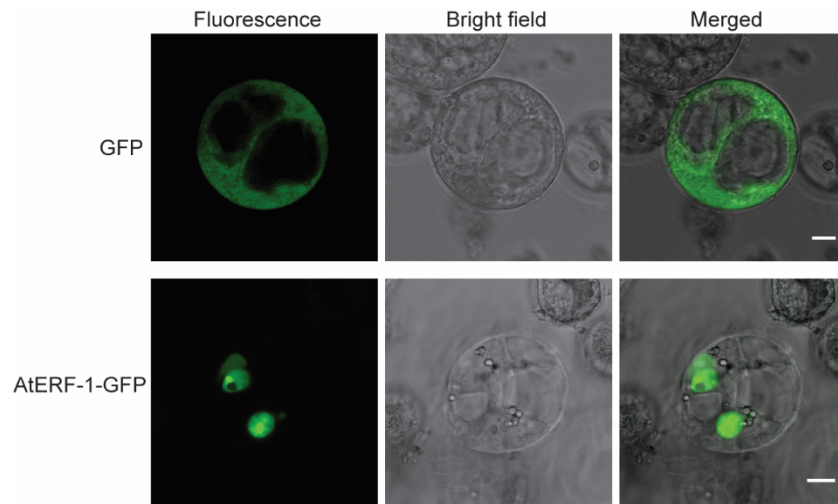


Figure S1. Nuclear localization of AtERF-1. AtERF-1-GFP construct was transiently expressed in Arabidopsis cell suspension protoplasts. GFP signals were observed using confocal microscopy. Scale bar = 10 μ m.

Table S1. Primers used in this study.

Primer Name	F(forward) /R(Reverse)	Sequence (5'-3')*
AtERF-1-	F	GAG <u>CC</u> ATGGAGATGTCTGATGACGGCGGATTC
pACT2	R	GAGGGATCCTTATAAAACCAATAAACGAT
WRKY11-	F	GAG <u>CC</u> ATGGAGATGGCCGTCTGATCTAATGCG
pACT2	R	GAGGGATCCTCAAGCCGAGGCAAACACTAA
WRKY28-	F	GAGGGATCCGGATGTCTAATGAAACCAGAG
pACT2	R	GGGGAGCTCAAGGCTCTTGCTTAAAGA
AtERF-1-	F	GAGCATATGTCGATGACGGCGGATTC
pET16h	R	GAGGAATTCTTATAAAACCAATAAACGA
WRKY11-	F	GAGCATATGGCCGTCTGATCTAATGC
pET16h	R	GAGGGATCCTCAAGCCGAGGCAAACACT
WRKY28-	F	GAGCATATGATGTCTAATGAAACCAGAGA
pET16h	R	GAGGGATCCTCAAGGCTCTTGCTTAAAGA
AtERF-1-C-	F	GAGGAATTCATATGCGTAGCTCTTTCCCG
pGEX-KG	R	GAGCTCGAGTTATCGAACCAGGGTCGGGT
AtERF-1-	F	GAGCTCGAGATGTCTGATGACGGCGGATTC
pRT101	R	GAGGGATCCTTATAAAACCAATAAACGAT
WRKY11-	F	GTCAGGTACCATGGCCGTCTGATCTAATGCGT
pRT101	R	GAGGGATCCTCAAGCCGAGGCAAACACTAA
WRKY28-	F	GAGCTCGAGATGTCTAATGAAACCAGAGA
pRT101	R	GAGGGATCCTCAAGGCTCTTGCTTAAAGA
AtERF-1-pTH2	F	GTCCCATGGTGATGTCTGATGACGGCGGATT
	R	GTCCCATGGATAAAACCAATAAACGAT
EDS5-	F	GTCAAAGCTTCCACCATTTTCATTGAACTAACTAC
pT7:GUS	R	CGATGGATCCTTTGAGAAAAATCGGTGAATCTG
ICS1-	F	GTCAAAGCTTCTGGTCTCAAAGAGCCTAAGTG
pT7:GUS	R	CAGTGGATCCTGCAGAAATTCGTAAAGTGTTTC
PR1-pT7:GUS	F	GTCAAAGCTTCTGATTCGGAGGGGTATATGTTATTG
	R	CAGTGGATCCTTTTCTAAGTTGATAATGGTTATTGTT
EDS5-probe	F	CGGTCGTCATCGGCCAAGGAA
	R	GCTCCAGCGATACCTTGTTCCA
ICS1-probe	F	ATGGCTTCACTTCAATTTTCTTCT
	R	TGCAGAGCCGATACCAGCAACGCT
PR1-probe	F	AGATAGCCCAAGATTATC
	R	CGCAGCGTAGTTGTAGTTAGC
AtERF-1-	F	ATATGCGTAGCTCTTTCCCG
probe	R	TTATAAAACCAATAAACGAT
ACTIN-probe	F	CTGTGCCAATCTACGAGGGTT
	R	GGAAACCTCAAAGACCAGCTC

*Restriction sites are underlined

Chapter 3

Heterologous Expression of Arabidopsis PBS3 Generates Elevated SA content in *E.coli*

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Abstract

Upon pathogen infection, plants produce the signaling molecule salicylic acid (SA) to trigger the systemic acquired resistance response. Plants compromised in SA accumulation show enhanced disease susceptibility. Despite of the significance of SA in plant defense, the proposed SA biosynthesis pathways, either via phenylalanine ammonia-lyase (PAL) or via isochorismate synthase (ICS) are not well understood. In *Arabidopsis thaliana* it is assumed that the pathway via ICS, which is located in the chloroplasts, plays the dominant role in the SA production. This pathway, in which isochorismate is an important intermediate, is expected to be similar to the SA biosynthesis pathways in certain bacteria. However, for the *Arabidopsis* pathway, no evidence has yet been obtained for an activity like bacterial isochorismate pyruvate lyase (IPL) that catalyzes the conversion of isochorismate to SA. In this study, we expressed several potential *Arabidopsis* IPL candidates in *E. coli* and evaluated the levels of synthesized SA via the bacterial biosensor *Acinetobacter* sp. ADPWH_*lux*. We found that PBS3, a critical enzyme in SA accumulation and plant defense response, is able to mediate SA accumulation in *E. coli*.

Introduction

Salicylic acid (SA), a small phenolic compound, is one of the most significant endogenous signal molecules involved in plant defense against biotrophic pathogens. During the defense response SA peaks to micromolar concentrations in the first few days after infection, but its amounts decrease later on, otherwise it would cause damage to the plant development (Mauch *et al.*, 2001; Pieterse *et al.*, 2009). The effects of SA can be mimicked by exogenous SA application, which induces expression of pathogenesis-related (PR) defense genes and initiates the systemic acquired resistance (SAR) response (Nawrath and Métraux 1999; Nawrath *et al.*, 2002).

Until now, there is evidence for at least two independent routes for biosynthesis of SA in plants, although neither of these pathways has been fully characterized (Dempsey *et al.*, 2011; Boatwright and Pajero-wska-Mukhtar, 2013). Both pathways originate from chorismate, a product of the shikimate pathway. The first pathway, which occurs in the cytoplasm, proceeds via phenylalanine ammonia-lyase (PAL) and involves several enzyme-catalyzed steps and various intermediates depending on the plant species (Rohde *et al.*, 2004; Huang *et al.*, 2010; Rivas-San Vicente and Plasencia, 2011). The other pathway involves isochorismate synthase (ICS), which converts chorismate into isochorismate in the chloroplasts (Verberne *et al.*, 2000; Wildermuth *et al.*, 2001; Strawn *et al.*, 2007; Garcion *et al.*, 2008). It is expected that in this second pathway an unknown isochorismate pyruvate lyase (IPL) catalyzes the conversion of isochorismate to SA. Considering the sub-cellular localization of ICS in Arabidopsis, it is assumed that in this plant species stress-induced SA synthesis occurs in the chloroplasts (Garcion *et al.*, 2008; Fragnière *et al.*, 2011). EDS5, a multidrug and toxin extrusion (MATE) type transporter, is responsible for the transport of SA from chloroplasts to cytosol (Nawrath *et al.*, 2002; Ishihara *et al.*, 2008; Serrano *et al.*, 2013). In the cytosol, the high amount of SA is toxic to the plant and is therefore metabolized to non-toxic SA 2-O- β -D-glucoside (SAG) by UDP-glucosyltransferases, and is then stored in the

tonoplast (Lim *et al.*, 2002; Song, 2006; Dean and Delaney, 2008; Song *et al.*, 2008).

Although plants possess ICS enzymes, genes encoding proteins similar to the isochorismate pyruvate lyases (IPL) of SA-producing bacteria, like *pchB* from *Pseudomonas aeruginosa* (Gaille *et al.*, 2002) or *pmsB* from *P. fluorescens* (Mercado-Blanco *et al.*, 2001), have not been found. However, available enzymatic and genetic data suggest that a number of proteins could be involved in stress-induced biosynthesis in plants and could be possible candidates for the unidentified plant IPL. Here, we have investigated possible IPL-like activities of a number of these proteins.

Arabidopsis PEPTIDE DEFORMYLASE1B (PDF1B) localized in the chloroplasts (Giglionne *et al.*, 2000; Dirk *et al.*, 2001) and PmsB from *P. fluorescens* share 19% identity and 56% similarity at the amino acid level. Salicylate synthase (SAS), such as Irp9 from *Yersinia intermedia* (Kerbarh *et al.*, 2005) and MbtI from *Mycobacterium tuberculosis* (Harrison *et al.*, 2006; Zwahlen *et al.*, 2007), exhibiting 57% amino acid sequence similarity, can convert chorismate directly to SA. Alignment of the amino acid sequences of Irp9 and MbtI showed 46% and 44% similarity, respectively, with Arabidopsis ANTHRANILATE SYNTHASE α 1 (ASA1) that catalyzes the rate-limiting step of tryptophan synthesis in the chloroplasts (Maeda and Dudareva, 2012). In addition to their IPL activity, bacterial PmsB and PchB display chorismate mutase activity, converting chorismate to prephenate (Gaille *et al.*, 2002). Due to their functional homology, the chorismate mutases chloroplast-localized CM1 and CM3 (Westfall *et al.*, 2014) from Arabidopsis were also tested if they could catalyze the conversion of isochorismate to SA. Finally, since *eps1* and *pbs3* mutant plants show lower accumulation of SA than wild-type Arabidopsis after infection of *P. syringae*, also the cytosolic acyl transferase ENHANCED PSEUDOMONAS SUSCEPTIBILITY1 (EPS1) and the acyl-adenylate/thioester-forming enzyme AVRPPHB SUSCEPTIBLE 3 (PBS3) were included in this study (Jagadeeswaran *et al.*, 2007; Lee *et al.*, 2007; Nobuta *et al.*, 2007; Zheng *et al.*, 2009).

In recent years, biosensors have been developed that enable real-time monitoring of bioluminescence using live microorganisms, such as *Acinetobacter baylyi* ADP1 to specifically detect SA and *E.coli* to detect a range of phenolic compounds (Huang *et al.*, 2005; Shin, 2010). In these bacteria, the luminescence *lux* operon is under control of an SA-regulated promoter. Consequently, the concentration of SA can be quantitatively analyzed by detecting the bioluminescence intensity with a luminescence meter. The bioluminescence biosensor designed by Huang *et al.* (2005), which greatly minimized the time and cost compared to traditional SA measurements, was utilized widely to detect free SA and SAG in large-scale screens of SA *in planta* (Huang *et al.*, 2006; DeFraia *et al.*, 2008; Ding *et al.*, 2015).

In order to verify whether our candidate proteins could convert isochorismate to SA, we performed *Acinetobacter* sp. ADPWH_*lux* (*Acinetobacter*)-based SA measurements of extracts of *E. coli* transformed with expression plasmids carrying the selected Arabidopsis genes. We show that SA is produced in *E. coli* overexpressing Arabidopsis PBS3 together with *E. coli* isochorismate synthase EntC. Furthermore, the combination of PBS3 and his-tagged purified EntC was able to catalyze the conversion of chorismate to SA *in vitro*.

Results

Quantification of SA through bioluminescence of *Acinetobacter*

In order to quantify the amounts of SA produced in *E. coli*, a series of SA concentrations in LC medium was prepared. At relatively low level of SA concentrations (0-50 μ M), the luminescence increased rapidly with increasing SA concentration. At higher SA concentrations, the luminescence continued to increase, but at a lower rate. This might be caused by saturation of the *saIA* promoter (Figure 1a). Since our aim was to check whether the *E. coli* strains expressing the candidate genes could produce SA, we assumed that the SA levels produced would not exceed 50 μ M, allowing a standard reference line of bioluminescence against lower SA concentrations to be used in further experiments. We also determined the luminescence when SA was dissolved in LC medium containing extracts of *E. coli* BL21 (DE3) pLysS. Obviously, the

reference made with the cell extracts showed significantly lower luminescence than when SA was dissolved in LC medium alone (Figure 1b), suggesting that the cell extract represses the induction of luminescence. Therefore, the standard curve made with the cell extract was used for quantifying SA in *E. coli*.

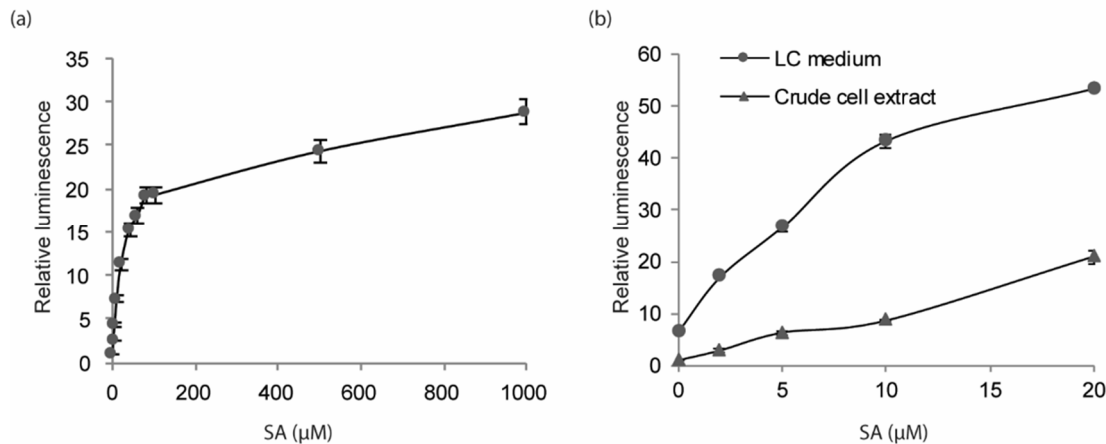


Figure 1. SA standard curves based on luminescence of *Acinetobacter*. (a) SA standards were prepared in LC medium. (b) SA solutions were prepared in LC medium (circles) and in medium containing cell extracts from *E. coli* BL21 (DE3) pLysS (triangles). Luminescence values of uninduced cells incubated with extract were taken as 1. Error bars represent $\pm\text{SE}$ ($n=3$). Experiments were repeated three times with similar results.

Generation of luminescence in *E. coli* over-expressing *EntC*

Several *Arabidopsis* candidate gene products were investigated as to whether they could convert isochorismate into SA (Table 1). In *E. coli*, the *entC* gene encodes ICS, which is expected to provide for a basal supply of isochorismate (Liu *et al.*, 1990). Theoretically, when the candidate gene product is correctly gene product is correctly expressed and

Table 1. Candidate proteins investigated in this study

AGI code	Name	Expression	<i>EntC</i> Expression	Co-expression
AT5G14660	PDF1B	+	+	+
AT5G05730	ASA1	+	+	+
AT5G67160	EPS1	+	+	+
AT3G29200	CM1	+	+	+
AT1G69370	CM3	+	+	+
AT5G13320	PBS3	+	+	+

“+” indicates protein expression was able to be detected by immunoblot analysis.

would have IPL activity, it could catalyze the conversion of isochorismate to SA, which would accumulate in the *E. coli* cells or be extruded into the growth medium. PmsB, being a qualified IPL, was chosen as positive control. Table 1 lists information of the candidates and expression details. After incubation for 3 to 16 h the cells were collected by centrifugation. A sample of the supernatant was collected for measuring SA extruded in the medium. The resuspended cells were sonicated and the soluble fraction was saved for measuring intracellular SA (cytoplasm).

The luminescence measurements in Figure 2 were converted to SA concentrations according to the standard curve of Figure 1b. The bars in the middle show that anhydrotetracycline-induced expression of PmsB resulted in SA accumulation, indicating that PmsB converts endogenous isochorismate to SA, which mostly accumulated in the medium (Figure 2a).

Apparently, PmsB expression in these cells was a bit leaky, as also under non-induced conditions SA was produced to low levels and excreted. The series of bars at the right show that over-expression of EntC further enhanced PmsB-mediated luminescence, suggesting that the basal levels of isochorismate were limiting. In these cells, SA accumulated to high intracellular levels after induction of PmsB. Therefore, the other candidates were tested in cells overexpressing EntC (Figure 2b). Figure 2b shows that only cells expressing PBS3 showed enhanced luminescence, indicating the production of SA, which accumulated both intracellularly and in the medium. The finding that luminescence was only increased when EntC was overexpressed suggests that isochorismate was the substrate for the conversion to SA by IPL-activity of PBS3.

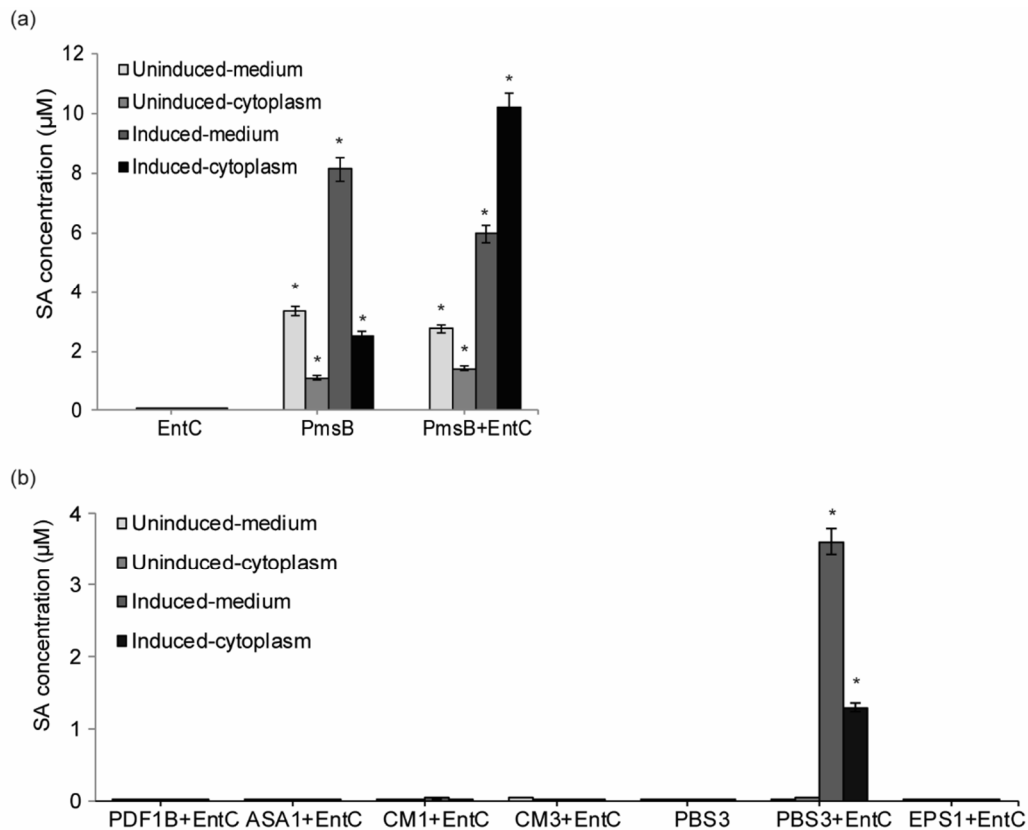


Figure 2. Biosensor-derived accumulation of SA in *E. coli* expressing selected candidate genes. **(a)** SA accumulation in *E. coli* expressing *entC* and positive control *pmsB*. **(b)** SA accumulation in *E. coli* expressing *entC* and candidate genes. Medium indicates luminescence measured in the medium remaining after the cells were removed by centrifugation. Cytoplasm indicates luminescence measured of soluble fraction after sonication of the cells. Bars represent means $\pm$ SE (n=3). Asterisks indicate a significant difference in comparison to *E. coli* expressing *entC* ($P < 0.05$). These experiments were repeated three times with similar results.

SA accumulation in Arabidopsis protoplasts

In order to verify whether PBS3 can convert isochorismate to SA in plants, we performed transient expression assays in protoplasts. The chloroplast targeting sequence (ss) of ribulose biphosphate carboxylase precursor from tobacco, which efficiently targets reporter fusions to the chloroplasts, was fused to the coding regions of both *pmsB* and *PBS3* (Verberne *et al.*, 2000). Arabidopsis protoplasts were co-transformed with plasmids carrying the *ICS* gene and *PmsB*, (ss)*PmsB*, *PBS3* or (ss)*PBS3* genes, each cloned behind the *CaMV* 35S promoter. A series of SA concentrations in Na-acetate buffer were used as the reference to estimate the SA levels in the protoplasts. β -glucosidase

was added directly to the crude extracts to release SA from SAG. Protoplasts co-transformed with *35S::ICS1* and *35S::(ss)pmsB* demonstrated approximately 30-fold higher total content than free SA content. Nevertheless, SA accumulation was not observed in other co-transformation combinations (Figure 3). These results indicate that ICS and IPL are essential in the chloroplasts simultaneously for efficient production of SA.

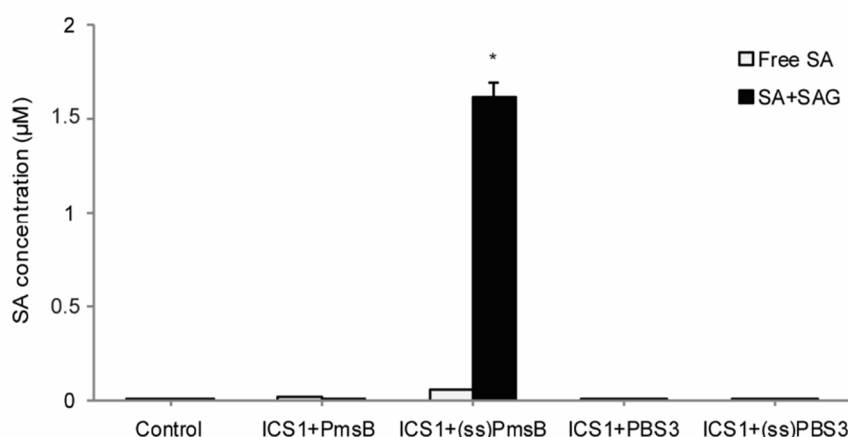


Figure 3. SA accumulation in Arabidopsis protoplasts. Protoplasts transformed with empty pRT101 vector were taken as control. Bars represent means $\pm$ SE (n=6). Asterisk indicates a significant difference in comparison to the control ($P < 0.05$). Experiments were repeated three times with similar results.

PBS3 has isochorismate pyruvate lyase activity

To establish whether PBS3 can catalyze the conversion of isochorismate to SA, we overexpressed and purified recombinant His-tagged EntC, PmsB, PBS3 and GH3.7. The latter protein shares 73% identity and 84% similarity at the amino acid level with PBS3. Analysis of the purified proteins by staining of an SDS-PAGE gel with coomassie brilliant blue showed a single main band in each protein preparation of the expected size (Figure S1). The purified proteins were incubated with chorismate and the production of SA was evaluated by the luminescence observed after incubation with *Acinetobacter*.

As shown in Figure 4a, the SA levels upon addition of chorismate together with purified EntC was slightly higher than with chorismate alone, implying that a small amount of isochorismate was converted to salicylate and pyruvate (DeClue *et al.*, 2006). The combination of

chorismate and purified PmsB displayed a high SA level, suggesting that chorismate was taken up by *Acinetobacter* and converted by the endogenous *Acinetobacter* isochorismate synthase [EC:5.4.4.2] to isochorismate that was subsequently exported and converted to SA by PmsB. When purified EntC was included in the reaction mix, PmsB was able to directly convert the isochorismate produced by EntC, which resulted in further enhanced luminescence and elevated SA accumulation. The reaction mixtures of chorismate with purified GH3.7 in the presence or absence of EntC did not result in enhanced luminescence. Relatively low level of luminescence was observed when purified PBS3 and EntC were tested for conversion of chorismate. The enzymatic activity of PBS3 requires ATP as a cofactor (Okrent *et al.*, 2009). In our results, addition of ATP did not further increase SA accumulation, suggesting ATP is not necessary in the reaction.

Experiments to further support these findings were performed with crude cell extracts from PBS3- and GH3.7-expressing *E. coli* (Figure 4b). The results indicate that much higher SA levels were obtained with crude extracts of PBS3 expressing *E. coli* and that the GH3.7 extract did not show increased SA content, as was the case with the purified protein.

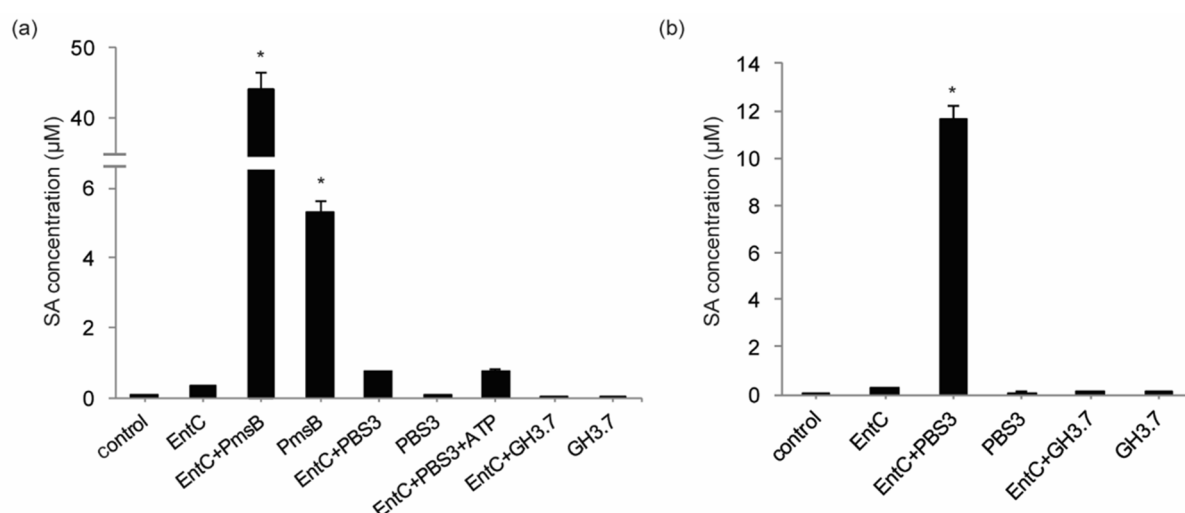


Figure 4. *In vitro* production of SA by proteins expressed in *E. coli*. Conversion of chorismate to SA was evaluated by luminescence observed after incubation with *Acinetobacter*. (a) Chorismate was incubated with different combinations of purified proteins. (b) Chorismate was incubated with different combinations of protein extracts from *E. coli*. Reaction mixture with chorsimate only was taken as control. Error bars represent $\pm$ SE (n=3).

Asterisks indicate a significant difference in comparison to EntC (*P < 0.05). Experiments were repeated three times with similar results.

PBS3 expression is induced by SA

The expression of *ICS1*, *EDS5* and *PR1* involved in the SA signal defense pathway has been shown to be induced by SA (Wildermuth *et al.*, 2001; Nawrath *et al.*, 2002). Therefore, we tested the accumulation of *PBS3* mRNA after exogenous SA treatment (Figure 5). Three hours after application of SA to Arabidopsis leaves, the expression of *PBS3* was higher than before SA was applied and was further enhanced till at least 12 hours after application. *PBS3* mRNA accumulation gradually decreased at later time points (12 and 24 hours). This expression pattern is similar to that of *EDS5* and suggests that the transcriptional regulation of *PBS3* might involve a positive feedback regulation loop by SA.

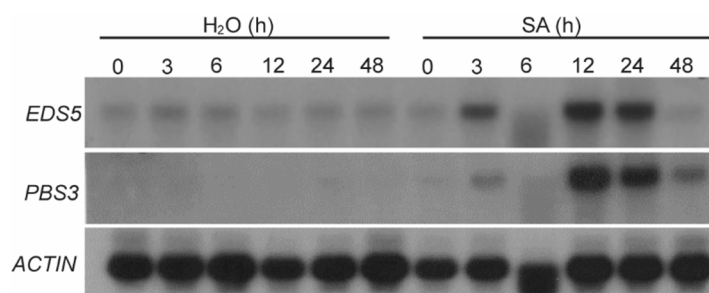


Figure 5. Time course of the expression of *PBS3* after exogenous SA treatment. Col-0 plants were sprayed with 2 mM Na-SA and harvested at the times indicated. Results of *ACTIN* gene expression were used as a loading control. The RNA of sample SA-6h was partially degraded.

Discussion

Until now, the biosynthesis of SA in plants has not been conclusively elucidated. No plant genes encoding an IPL or SA synthase activity have been identified. Several candidate genes of Arabidopsis that might encode an IPL were expressed in *E. coli*. Among the candidates we investigated, significantly increased luminescence values were only observed in *PBS3* over-expressing *E. coli* when isochorismate was over produced (Figure 2).

In our research, a SA responsive biosensor, i.e. chromosomally engineered *Acinetobacter* sp. ADPWH_*lux* strain, was utilized to estimate

the SA content by measuring the increase in luminescence (Huang *et al.*, 2005). The biosensor is specifically induced by concentrations of SA up to 1 μ M but is not responsive to SA analogues like e.g. 4-hydroxybenzoic acid (4-HBA), 3-hydroxybenzoic acid (3-HBA), benzoate or catechol (Huang *et al.*, 2005; DeFraia *et al.*, 2008). We have not investigated why luminescence is reduced when SA is present in *E.coli* crude cell extracts (Figure 1b), but speculate that this may be the result of conversion of SA by bacterial enzymes. Nevertheless, luminescence increases can still be measured in bacterial extracts, allowing its use for SA measurements.

In a previous study, *pmsB* from *P. fluorescens* was shown to be able to use the endogenous substrate isochorismate synthesized by *E. coli* to achieve SA biosynthesis (Mercado-Blanco *et al.*, 2001). In our experiments, SA was detectable in the cell-free growth medium, even though it was produced in the *E. coli* cytosol (Figure 2), which is consistent with the results from Mercado-Blanco *et al.* (2001). We speculate that SA, due to its toxicity, is transported from the cytosol to the medium by membrane transporters. Furthermore, co-expression of *EntC* with *PmsB* did increase measurable accumulation of SA. We investigated several proposed IPL candidates from Arabidopsis, which have similarity with bacterial IPL or SAS (e.g. PDF1B, ASA1) and are mentioned in earlier reports (e.g. CMs, EPS1) (Chen *et al.*, 2009; Dempsey *et al.*, 2011). Our results show that none of these proteins, when expressed in *E. coli*, has IPL activity. Enhanced SA was only observed in *E. coli* cytosol and growth medium when *EntC* and *PBS3* were co-expressed.

Unlike *pmsB*, which is an effective IPL, *PBS3* is not able to efficiently convert the relative low amounts of endogenous isochorismate to SA in *E.coli*. We were unable to detect elevated SA production in *ICS1*-(ss)*PBS3*-transformed protoplasts (Figure 3). This could have several reasons, like insufficient production of substrate, inefficiency of the protoplast-targeted (ss)*PBS3*, etc. The finding that SA production is higher when *PBS3* is present in crude *E. coli* extract, as opposed to the purified *PBS3*, could suggest that the enzyme needs co-factors such as

NAD(P)H, metal ions, etc. for optimal activity. We have not investigated this further.

PBS3 (*GH3.12*), was previously identified through screening for *Arabidopsis* mutants that displayed reduced resistance to infection with avirulent pathogens (Warren *et al.*, 1999). The mutant was compromised in SA accumulation and activation of defense genes (Nobuta *et al.*, 2007; Ding *et al.*, 2014). Elevated SA-Asp formation in *pbs3* mutants suggested that PBS3 might enhance SA accumulation via suppressing the SA metabolism in the cytosol (Dempsey *et al.*, 2011). *PBS3* encodes an acyl acid–amido synthetase that conjugates amino acids to 4-substituted benzoates. It belongs to the large GH3 family of proteins and is required for regulating plant defense by modulation of plant hormone activity (Okrent *et al.*, 2009; Westfall *et al.*, 2012). Another member of the GH3 family, GH3.11, known as JAR1 catalyzes the formation of jasmonoyl-L-isoleucine (JA-Ile), which is considered as the main bioactive jasmonic acid derivative (Fonseca *et al.*, 2009).

In addition, we confirmed that the *PBS3* expression pattern is similar to that of *EDS5* after application of exogenous SA. Together, these results suggest that PBS3 is active upstream of SA and its gene expression involves positive feedback regulation by SA during the first 12-24 h after infection (Figure 5; Jagadeeswaran *et al.* 2007; Dempsey *et al.* 2011). However, PBS3 is a cytosol localized enzyme (Figure S2), and this is incompatible with the supposed chloroplast localization of SA production (Table 1; Dempsey *et al.*, 2011; Fragnière *et al.*, 2011). One possibility to explain a role for PBS3 in SA biosynthesis is that upon biotic stress, produced isochorismate is transported from the chloroplast to the cytosol by a transporter like EDS5. In this option, PBS3 synthesizes small amounts of SA in the early stage of the defense response. Subsequently, one or more chloroplast localized IPL take over and synthesize SA in much higher yields. Another possibility could be that upon pathogen infection, PBS3 is recruited to the chloroplast to accomplish SA production.

Materials and methods

Plant growth conditions and treatments

Arabidopsis thaliana ecotype Columbia (Col-0) seeds were surface-sterilized with chlorine gas for 3 hours and grown on MS medium after stratification for 3 days at 4°C. After incubation in a growth chamber (16h light/8h dark, 2500 lux) for 14 days, the seedlings were transferred to soil to grow for 7 days until wetting leaves with 2 mM Na-salicylate (pH 5.8) for different times. As controls, leaves of seedlings treated with water were collected.

SA standard curve based on *Acinetobacter*

Series of salicylic acid (Sigma) concentrations were prepared in LC medium, and in LC medium containing a cell extract from sonicated *E. coli* BL21(DE3) pLysS (OD₆₀₀=1.0), containing empty pASK-IBA45plus and anhydrotetracycline (0.2 mg/L) or 0.1 M Na-acetate buffer (pH 5.8). The biosensor *Acinetobacter* was used to measure the SA concentration as described previously (Huang *et al.* 2005). Mixtures containing 20 µL SA solution, 50 µL *Acinetobacter* culture (OD₆₀₀=0.4) and 60 µL LC medium were added in the wells of a 96-well cell culture plate and incubated at 37°C. After 1 hour, luminescence was measured using a Victor light 1420 counter (Perkin-Elmer).

Generation of bacterial expression constructs

The full-length coding region of *entC* was amplified from the genomic DNA of transgenic tobacco plants overexpressing *entC* (Verberne *et al.*, 2000) and cloned into pET-16h (a derivative of pET-16b) and pET-30a(+). Plasmids containing the coding region of chorismate mutases were provided by J. Jez (Westfall *et al.*, 2014). The open reading frames of *PBS3* and *EPS1* were obtained by PCR on cDNA of *Arabidopsis* ecotype Col-0 leaves treated with SA for 24 hours and cloned into expression vectors. For PDF1B and ASA1, expression constructs (PDF1BΔ50 and ASA1Δ60) were generated. Primers and expression vectors are listed in supplementary Table 1.

Determination of SA in extracts of *E. coli* expressing candidate genes

Expression plasmids containing candidate genes were transformed into *E. coli* strain BL21 (DE3) pLysS and the cells were grown in LC medium containing carbenicillin (200 mg/L) or kanamycin (50 mg/L) and chloramphenicol (50 mg/L) until $OD_{600}=0.6$. The cell cultures were induced by isopropyl β -D thiogalactopyranoside (IPTG) (20 mg/L) or anhydrotetracycline (0.2 mg/L) at 28°C or 18°C for 3 to 16 hours. The cells were harvested by centrifugation at 4000 rpm for 20 mins at 4°C. One mL of the supernatants was sampled and stored at -20°C for luminescence activity measurements. The pellets were resuspended in 1 mL TE buffer (10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA), frozen in liquid nitrogen and store at -80°C. After sonication the cell lysates were centrifuged at 14000 rpm for 20 mins at 4°C. Ten μ L of the soluble protein fractions in the supernatants were subjected to electrophoresis on 10% or 15% SDS-polyacrylamide gels and transferred to Protan nitrocellulose (GE Healthcare) by semi-dry electroblotting. Immunoblot analysis was performed as described by Zarei *et al.*, (2011) to confirm the presence of recombinant proteins. The anti-his tag horseradish peroxidase (HRP) conjugated antibodies were purchased from Qiagen.

The rest of the extracts were stored at -20°C for luminescence measurements. To determine the presence of SA, 20 μ L of supernatant after centrifugation of the harvested cells or 20 μ L of the soluble cell extract after sonication were mixed with 50 μ L *Acinetobacter* culture ($OD_{600}=0.4$) and 60 μ L LC medium. Luminescence was measured as described above.

Arabidopsis protoplast transformation

The full-length coding sequences of *ICS* and *PBS3* were obtained from cDNA described above and inserted into vector pRT101 behind the CaMV 35S promoter, respectively. (ss)*PBS3* was made by cloning the plastid signal sequence (ss) of the small subunit of tobacco ribulose biphosphate carboxylase precursor in the upstream of the *PBS3* coding sequence. *pmsB* and (ss)*pmsB* were amplified from the genomic

DNA of transgenic tobacco plant line overexpressing *pmsB* and (*ss*)*pmsB* (Verberne *et al.* 2000) and cloned into vector pRT101. Protoplasts were transformed according to Schirawski *et al.* (2000).

The transformed protoplasts were harvested by centrifugation after incubation for 18 hours at 25°C in the dark and quickly frozen in liquid nitrogen. The pellets were resuspended and vortexed violently in 100 µL Na-acetate buffer (0.1 M, pH 5.8). The cell extracts were collected by centrifugation at 4°C. Forty µL of the cell extracts were treated with 5 U β-glucosidase (Sigma) for 1.5 hours at 37°C. Twenty µL β-glucosidase-treated cell extract was mixed with 50 µL *Acinetobacter* culture (OD₆₀₀ = 0.4) and 60 µL LC medium. The mixture was transferred into a 96-well cell culture plate and incubated at 37°C for 1 hour. The luminescence was then monitored by the luminescence meter. Calibration line based on a series of SA concentrations in Na-acetate buffer was used to estimate the SA levels in the protoplasts. Empty vector pRT101-transformed protoplasts were taken as controls.

For PBS3 subcellular localization, the coding region of PBS3 was fused at the C-terminus to the coding region of GFP in plasmid pTH2. Images of transfected protoplasts were acquired as described before (Chapter 2). Used primers are presented in Table S1.

Crude enzyme extraction and protein purification

His-tagged EntC, PmsB, PBS3 and GH3.7 were expressed in *E. coli* strain BL21 (DE3) pLysS. The full-length coding sequence of *GH3.7* was amplified from Arabidopsis cDNA and inserted into vector pET-16h. To obtain crude enzyme extracts, 250 mL cell cultures were used. The cells were collected by centrifugation, frozen in liquid nitrogen and stored at -80°C until use. The cells were thawed on ice and resuspended in 1 mL reaction buffer (100 mM Tris-HCl pH 7.0, 15 mM MgCl₂, 1 mM DTT, 10% glycerol). After sonication and centrifugation, the supernatants were collected. His-tagged proteins were purified by Ni-NTA agarose chromatography according to the manufacturer's instruction (QIAGEN) and dialyzed against dialysis buffer (100 mM Tris-HCl pH 7.0, 15 mM MgCl₂, 1 mM DTT, 10% glycerol).

IPL activity assay

Reaction mixtures of 150 μ L contained 100 mM Tris-HCl pH7.0, 15 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5 mM chorismate (Sigma), 5 μ g purified EntC protein, 300 μ g crude protein extract or 10 μ g purified protein. The mixtures were incubated at 28°C for 1 hour and centrifuged. Twenty μ L of the supernatants were mixed with 50 μ L *Acinetobacter* culture ($OD_{600}=0.4$) and 60 μ L LC medium. The mixtures were transferred into a 96-well cell culture plates at 37°C for 1 hour after which luminescence was measured by the luminescence meter.

RNA extraction and Northern blot

Total RNA extraction was performed as described before (Chapter 2). RNA-blot analyses were performed as previously described (Memelink *et al.*, 1994). DNA fragments for use as radioactively labeled probes were amplified from Arabidopsis cDNA by PCR. The primers used are listed in Table S1.

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Supporting information

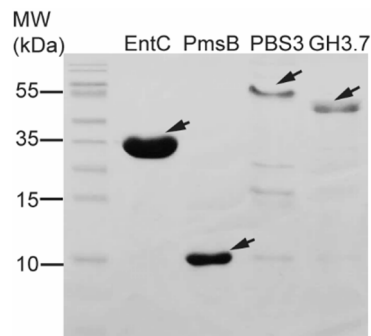


Figure S1. Purified recombinant EntC, PmsB, PBS3 and GH3.7 proteins were separated on a 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Proteins are indicated with the arrows. Molecular weights (kDa) of size markers are indicated at the left.

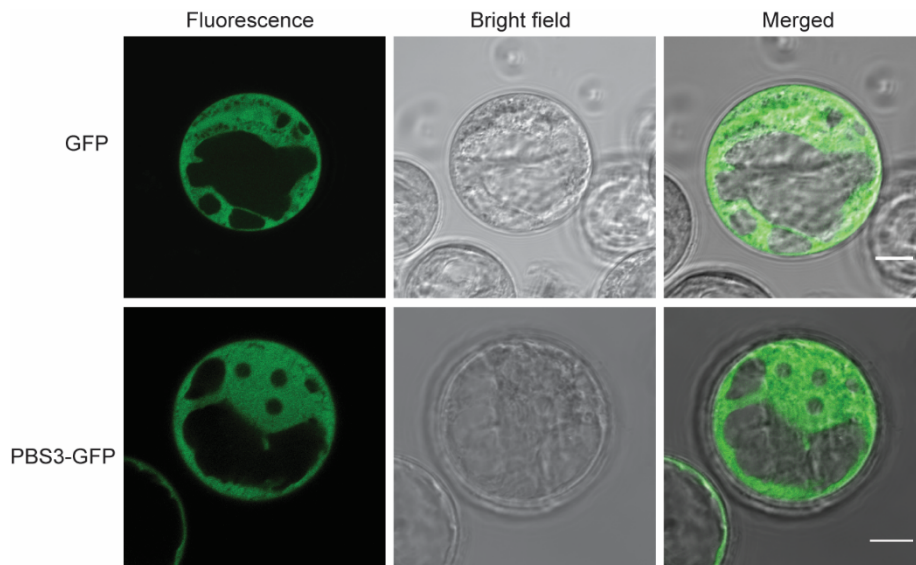


Figure S2. Subcellular localization of PBS3. Arabidopsis protoplasts were transiently transformed with a plasmid expressing PBS3-GFP. Scale bar = 10 μ M.

Table S1. Primers used for cloning in this study

Primer Name	F(Forward) /R(Reverse)	Sequence(5'-3')*
pmsB-pASK- IBA45plus	F R	GCGAATTCGATGCTGCAACCTAAAACCCCT GCCTCGAGCTGACTTGGCCTGCGCCGA
entC-pET-16h	F R	GAGCATATGGATACGTCACTGGC GAGCTCGAGTTAATGCAATCCAAAA
entC-pET-30a(+)	F R	GCGGAGCTCATGGATACGTCACTGGCT GCGGAGCTCATGGATACGTCACTGGCT
PDF1B-pET-16h	F R	GAGCATATGCTGACATCTTCGGTCCGC GAGCTCGAGTCAACGTTTGCCAAAACC
EPS1-pET- 30a(+)	F R	GAGGAATTCATGGAAGAAGAATTGGTGGTA GAGGTCGACTCATACAATGCTTACATATTC
ASA1- pET-16h	F R	CAGGGTACCATGGCTTCAATAGTAAGTGATA CAGGGATCCTCATTTTTTACAAATGCAG
PBS3-pASK- IBA45plus	F R	TTCCGCCGATATGAAGCCAATCTTCGATATCAACGAAAC TTCTGCAGATACTGAAGAATTTTGGCTACACACATG
ICS1-pRT101	F R	GAGGAATTCATGGCTTCACTTCAATT GAGGGATCCTTATTGTGAGAACCCCTT
(ss)pmsB- pRT101	F R	CACTCGAGATGGCTTCCTCAGTTCTTT AGGGATCCTCATGACTTGGCCTGCGCCG
PBS3-pRT101	F R	GAGGGTACCATGAAGCCAATCTTCGATA GAGGGATCCTCAAATACTGAAGAATTTGG
PBS3-pTH2	F R	GAGGTCGACATGAAGCCAATCTTCGATATCAAC GAGGTCGACAATACTGAAGAATTTGGCTACC
(ss)PBS3-Kpn I	F R	CAGGTACCATGGCTTCCTCAGTTCTTT CAGGTACCATGCATTGCACTCTTCC
PBS3-probe	F R	ATGAAGCCAATCTTCGATATCAAC TGTTGCGACTCGAGCAGGCAAGC
ACTIN	F R	CTGTGCCAATCTACGAGGGTT GGAAACCTCAAAGACCAGCTC

*Restriction sites are underlined.

Chapter 4

A genetically engineered *E. coli* biosensor for screening of cDNA libraries for isochorismate pyruvate lyase-encoding cDNAs

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Abstract

Salicylic acid (SA) is an essential hormone for physiological development and induced defense against biotrophic pathogens in plants. The formation of SA derives from chorismate, the end product of the shikimate pathway, via two distinct pathways: the isochorismate pathway (IC) and the phenylpropanoid pathway. The IC pathway proceeds via isochorismate synthase (ICS) and an as of yet unidentified isochorismate pyruvate lyase (IPL). Here, we developed an *E. coli* SA biosensor to screen for IPL activity based on the SalR regulator/*salA* promoter combination from *Acinetobacter* sp ADP1, to control the expression of a reporter *luxCDABE*. The biosensor was responsive to micromolar concentrations of exogenous SA, and to endogenous SA produced after transformation with a plasmid permitting IPTG-inducible expression of bacterial IPL in this biosensor strain. This allowed screening of a cDNA library constructed from turnip crinkle virus (TCV) induced *Arabidopsis thaliana* Di-17 for *Arabidopsis* cDNAs encoding proteins with IPL enzyme activity.

Keywords: Salicylic acid, Isochorismate pyruvate lyase, Screening, *Arabidopsis thaliana*

Introduction

Salicylic acid (SA) is a small phenolic compound present in plants and bacteria. In plants, SA is an essential signaling molecule that mediates defense against infections with biotrophic pathogens and is produced in low concentrations to regulate physiological functions, including flowering induction and seed germination (Rivas-San Vicente and Plasencia, 2011; Spoel and Dong, 2012). Meanwhile, SA functions as a precursor of siderophores in many bacteria, such as pyochelin in *Pseudomonas aeruginosa*, yersiniabactin in *Yersinia pestis* and *Y. enterocolitica*, and mycobactin in *Mycobacterium tuberculosis* (Cox et al. 1981; Crosa and Walsh, 2002; Gaille et al. 2003; Pelludat and Brem, 2003).

The SA biosynthesis pathway in bacteria is well known. Chorismate, the end product of the shikimate pathway, is converted to SA by two enzymes: isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL), encoded by *P. aeruginosa* genes *pchA* and *pchB*, and *P. fluorescens* genes *pmsA* and *pmsB* (Mercado-Blanco et al. 2001; Gaille et al. 2002). In *Y. enterocolitica* and *M. tuberculosis*, salicylate synthase (Irp9 and MbtI) carries out the direct conversion of chorismate to salicylate (Kerbarh et al. 2005; Zwahlen et al. 2007). SA biosynthesis in plants also originates from chorismate, but here two different pathways have been suggested. However, neither of these branches has been conclusively elaborated (Dempsey et al. 2011; Boatwright and Pajerowska-Mukhtar, 2013; Dempsey and Klessig, 2017). The first branch involves the enzyme phenylalanine ammonia-lyase (PAL) which converts phenylalanine to cinnamic acid. Arabidopsis has at least four PAL enzymes, which are all localized in the cytoplasm (Huang et al. 2010). The second pathway is dependent on ICS, which catalyzes the conversion of chorismate to isochorismate (Wildermuth et al. 2001; Strawn et al. 2007; Garcion et al. 2008). The reaction leading to SA is expected to involve an as of yet unidentified IPL enzyme (Macaulay et al. 2017). Due to the chloroplast localization of ICS in plants and the fact that transgenic Arabidopsis overexpressing the *NahG* (salicylate hydroxylase) gene in the chloroplasts failed to accumulate SA after pathogen infection or UV exposure (a stimulus for SA accumulation), this pathway

is likely to be localized in the chloroplasts (Nawrath et al. 2002; Garcion et al. 2008; Fragnière et al. 2011). The contribution of the IC pathway for SA biosynthesis differ depending on the plant species. For instance, the IC biosynthesis pathway contributes more than 90% of the SA produced during pathogenesis in *Arabidopsis*, while the PAL and IC pathways are equally important for pathogen-induced SA biosynthesis in soybean (Garcion et al. 2008; Shine et al. 2016).

The conventional methods of SA quantification in plant tissues were performed by high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS), which require time-consuming extraction steps with organic solvents (Nawrath, 1999; Verberne et al. 2000; Huang et al. 2006; Garcion et al. 2008). Given the importance of SA, salicylate responsive biosensors were designed to detect SA and SA derivatives faster and for larger scale quantifications (Huang et al. 2005; Shin, 2010). Huang et al. (2005) engineered an *Acinetobacter* biosensor originating from *Acinetobacter baylyi* sp. ADP1. This strain is able to use SA as sole the carbon source (Jones et al. 2000). It contains the *salA* operon encoding salicylate hydroxylase involved in SA degradation to catechol. Transcription of this operon is activated in the presence of SA that interacts with regulatory protein SalR. SalR belongs to the LysR-type transcriptional regulator (LTTR) family of proteins that contain two typical major domains: an N-terminal helix-turn-helix motif (residues 18-47) and a C-terminal co-factor binding domain. SalR activates the transcription of *salA* though binding to a dyadic sequence (TTCA-N₁₂-TGAT) around -190 bp upstream of the *salA* transcription start site, in response to the inducer SA (Schell, 1993; Jones et al. 2000). For the construction of the *Acinetobacter* biosensor, the *luxCDABE* operon from *Photobacterium luminescens*, encoding luciferase and enzymes involved in biosynthesis of its substrate (Winson et al. 1998a,b) was integrated into the *Acinetobacter* genome, resulting in *Acinetobacter* strain ADPWH_*lux*. This biosensor is highly sensitive to SA, methyl-SA, and acetyl-SA, and has been used to monitor SA in plant tissues (Huang et al. 2006; DeFraia et al. 2008; Ding et al. 2014).

Large scale genetic screens for mutants in *Arabidopsis* with reduced SA accumulation after pathogen infection have resulted in identification of genes *EDS5* encoding a chloroplast MATE (multidrug and toxin extrusion) transporter shown to transport SA from the chloroplast to the cytosol and the SA biosynthetic enzyme ICS1 (Nawrath and Métraux 1999; Dewdney et al. 2000; Serrano et al. 2013). For years, biologists have struggled with seeking for an enzyme with IPL activity in plants to complete the ICS branch in the SA biosynthesis pathway, but as of yet no such activity was found.

In this article, we describe the construction and characterization of a plasmid-based *E. coli* SA biosensor to screen a cDNA library of *Arabidopsis* for genes encoding the unidentified IPL. The sensing components, including the SA inducible promoter *PsaI/A*, the reporter operon *luxCDABE* and the *saI/R* gene encoding the LysR-type regulator of *saI/A*, were subcloned from *Acinetobacter* ADPWH_*lux* into a plasmid vector. As proof of principle, an expression plasmid was constructed carrying the *pmsB* gene from *P. fluorescens* encoding bacterial IPL behind the IPTG-inducible *lac* promoter, which served as a positive control for the biosensor. Since *E. coli* contains the *entC* gene encoding ICS that supports the production of isochorismate, transformation with the *pmsB* plasmid resulted in synthesis of SA, which activated the SaIR regulator, leading to expression of the *lux* genes and generation of light. This made it possible to use the *E. coli* biosensor strain to screen a cDNA expression library for cDNAs encoding proteins with IPL activity, by determining the light production of individual clones.

Results

Construction of a luminescence reporter plasmid

Our aim was to engineer a biosensor that could detect endogenous SA in *E. coli*. In our construct, the promoterless *luxCDABE* operon encoding luciferase was cloned behind the upstream promoter region of the *saI/A* operon (*PsaI/A*) to replace the original *saI/A* gene. SA-inducible expression of the resulting promoter-reporter operon is regulated by the product of the *saI/R* gene (Jones et al. 2000; Mercado-Blanco et al. 2001) (Fig. 1a). The resulting plasmid containing *saI/R* downstream of the new-

ly created *PsalA::luxCDABE* operon was named pLUX. *E. coli* XL-1 Blue MRF' transformants carrying pLUX (XL1-LUX) were able to produce luminescence due to the constitutive expression of *luxCDABE* in the presence of exogenous SA and this phenomenon could be easily captured by auto-exposure in the dark (Fig. 1b).

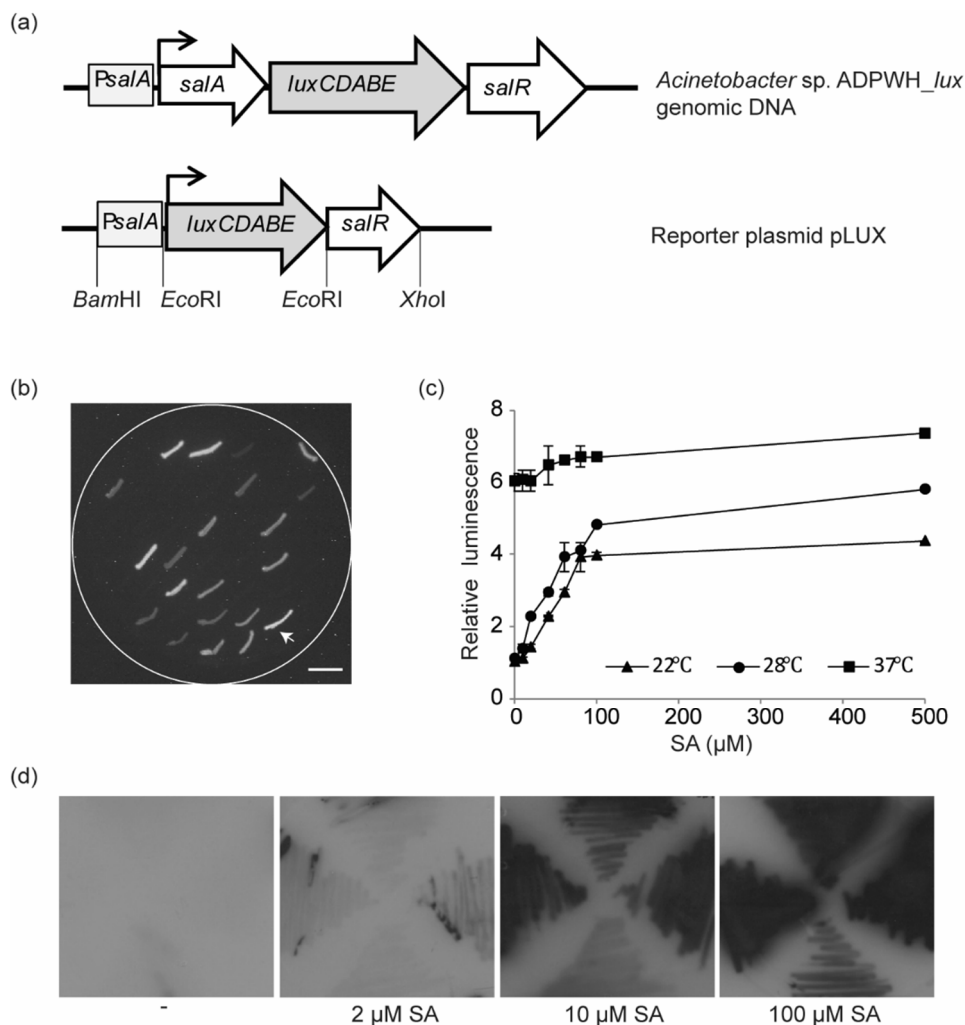


Fig. 1 *E. coli* based whole-cell biosensor XL1-LUX induction by SA. (a) Representation of chromosomally integrated *luxCDABE* in *Acinetobacter* sp. ADPWH_ *lux* (Huang et al. 2005) and pLUX reporter cassette. (b) Selection of *E. coli* XL1-LUX transformants on LC plate supplemented with SA. The *E. coli* transformant selected for our experiments is indicated by the arrow. Scale bar = 1 cm. (c) Relative luminescence values from XL1-LUX grown in liquid medium with different concentrations of SA, incubating at different temperature. Luminescence values of uninduced cells (0 μ M SA) that incubated at room temperature (22°C) were taken as 1. Error bars represent the mean $\pm$ SE (n=3). (d) Luminescence images on film of XL1-LUX streaked onto solid medium containing different concentrations of SA.

In general, XL1-LUX exhibited increased luciferase expression for concentrations of salicylate between 0 and 500 μM when the cells were grown in liquid medium at room temperature (22°C) and 28°C with 4-fold and 5-fold increased luminescence respectively, compared to uninduced cells (Fig. 1c). The results show un-induced cells (0 μM SA) had strong basal luminescence and luminescence was barely changed when induced with SA at 37°C. When grown on solid medium at room temperature, luminescence responded likewise to increasing SA concentrations (Fig. 1d). These results indicated that the XL1-LUX biosensor is capable of detecting exogenous SA.

Endogenous SA generation and luminescence monitoring

In *E. coli*, isochorismate synthase EntC transforms chorismate into isochorismate, which provides substrate for enterobactin biosynthesis (Ozenberger et al. 1989; Walsh et al. 1990). PmsB, originally from *P. fluorescens*, would convert the endogenous isochorismate into SA in *E. coli* (Mercado-Blanco et al. 2001; Chapter 3). In order to test whether XL1-LUX is able to respond to endogenous SA, a pBK-CMV (pBK) plasmid carrying the *pmsB* gene under the control of the strong isopropyl thio- β -D-galactoside (IPTG)-inducible lac promoter (pBK-pmsB), was transformed into XL1-LUX (Fig. 2). As a negative control, a pBK vector without insertion was transformed into XL1-LUX. We observed stronger luminescence in the cells containing the expressed PmsB compared to the negative control and the uninduced cells (Fig. 2). This result indicated that *E. coli* XL1-LUX is sensitive to the endogenously produced SA.

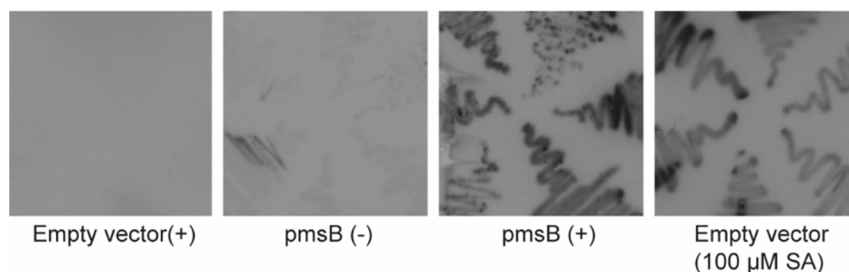


Fig. 2 *E. coli* based biosensor XL1-LUX monitoring endogenous SA. Images of luminescence of XL1-LUX containing pBK without insertion or pBK-pmsB in the absence (-) or presence of IPTG (+). Luminescence of XL1-LUX with empty vector in the presence of 100 μM SA was included as a control.

Arabidopsis cDNA library screening using XL1-LUX

Previous work showed that inoculation of turnip crinkle virus (TCV) on the resistant *Arabidopsis* ecotype Dijon (Di-17) results in the increase of SA contents, the development of a hypersensitive response (HR) and induction of *PR* gene expression (Uknes et al. 1993; Dempsey et al. 1997; Kachroo et al. 2000; Chandra-Shekara et al. 2006). This indicates that the genes involved in SA biosynthesis are active upon TCV inoculation. To seek out genes encoding IPL in *Arabidopsis* using the biosensor, a cDNA plasmid (pBK) library made from mRNA of *Arabidopsis* Di-17 leaves infected by TCV (Ausubel et al. 1987; Cooley et al. 2000) was transformed into *E. coli* XL1-LUX. Cells were selected for the presence of both the pLUX and cDNA plasmid using carbenicillin and kanamycin (Fig. 3).

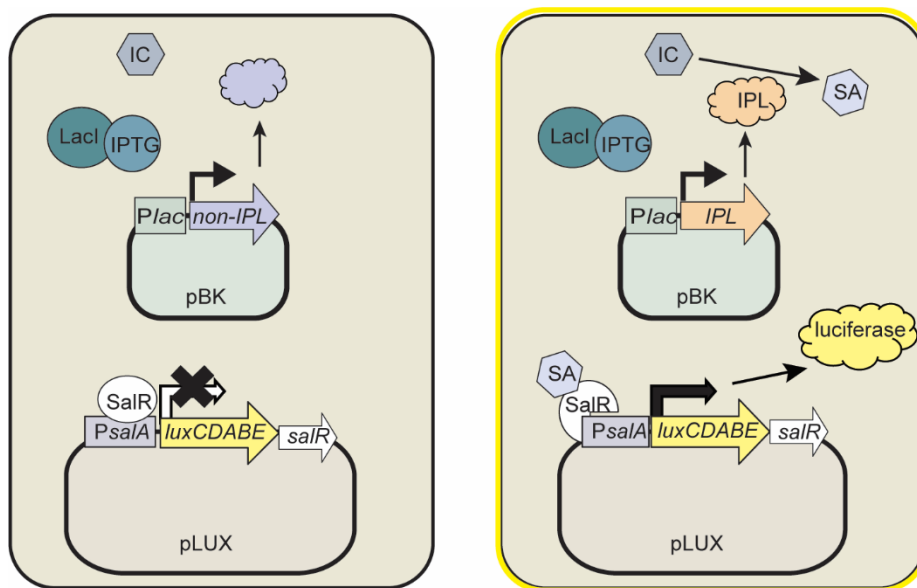


Fig. 3 Schematic representation of cDNA library screening in XL1-LUX. *E. coli* cells containing the reporter plasmid pLUX and cDNA-harboring plasmid pBK. In the presence of IPTG, cells containing a cDNA encoding an enzyme with IPL activity are able to convert endogenous isochlorismate to SA, resulting in the activation of *salR* and accumulation of luciferase and its substrates that make the cells producing light.

One and a half million *E. coli* transformants were screened, resulting in approximately 1000 colonies that were able to generate higher luminescence than the background. Those colonies were recovered and streaked onto fresh medium. From 220 colonies that were selected, the pBK plasmids were isolated and re-transformed into XL1-LUX. This

resulted in 43 clones displaying relatively strong luminescence (Fig. 4). The cDNAs from these clones were sequenced and 12 of them were in frame (Table S1).

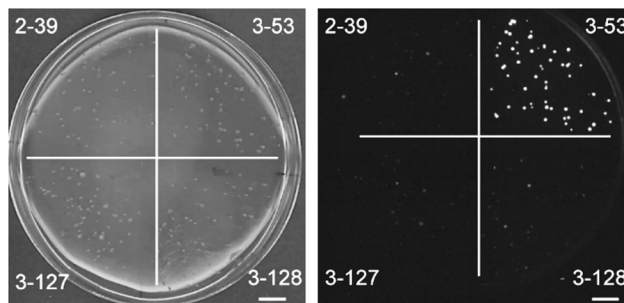


Fig. 4 Luminescence scanner images of *E. coli* colonies from the library screening. Plate with quadrants of colonies after retransformation of XL1-LUX with four different plasmids picked up in the screening, photographed in the light (left) and dark (right). Scale bar = 1 cm.

SA accumulation in *E. coli* expressing candidate cDNAs

To determine if the candidate cDNAs can direct SA accumulation in *E. coli*, we transformed the corresponding pBK plasmids into XL1-Blue MRF' and BL21 (DE3) pLysS, a strain that is optimized for high expression of cloned genes (Fig. 5). The *pmsB* gene cloned in pBK and pASK-IBA45plus served as positive controls. The positive control obtained from XL1 containing the *pmsB* gene showed slightly higher luminescence activity than the uninduced control. Obviously, the crude cell extracts of the candidate strains had no effect on luminescence, suggesting that SA was not produced or produced in such low amounts in these cells that it was not detectable by *Acinetobacter* (Fig. 5a).

We argued that *E. coli* XL1 might not produce enough isochorismate to allow sufficiently high production of SA, or that expression of the cDNAs was not sufficiently high in these cells. When crude cell extracts of these cells were incubated with chorismate, together with a crude cell extract of BL21 (DE3) pLysS transformed with an overexpression construct of the bacterial *entC* gene encoding isochorismate synthase, the SA production by the control *pmsB* extract was considerably higher. Of the candidate clones, only clone 3-53 resulted in low, but reproducible levels of SA. Sequencing revealed that the cDNA of 3-53 encodes a peroxidase named PRXR1 (ATP1a) (Fig. 5b).

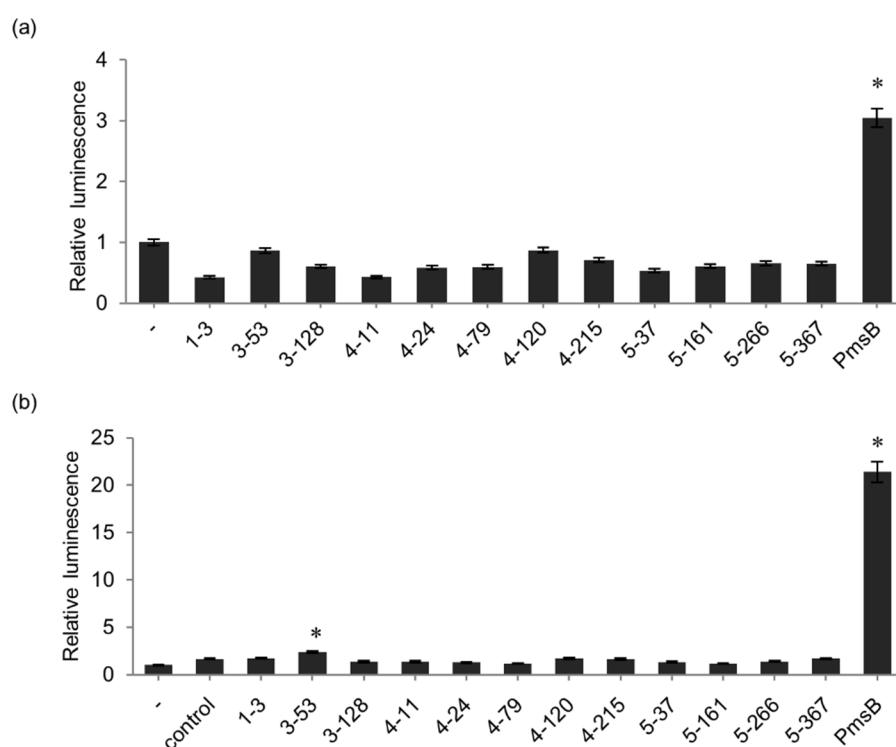


Fig. 5 Luminescence values from crude cell extracts, as measured through the *Acinetobacter* biosensor. **(a)** Luminescence values of crude cell extracts from IPTG-induced XL1-Blue MRF containing indicated pBK plasmids from the screening. “-” indicates uninduced *Acinetobacter* cells. Asterisk indicates a significant difference in comparison to the uninduced *Acinetobacter* cells ($P < 0.05$). **(b)** Luminescence values of crude cell extracts from IPTG-induced *E. coli* BL21 (DE3) pLysS transformed with the indicated plasmids, crude cell extract from *entC*-overexpressing *E. coli* and incubated for 1h with chorismate. “-” indicates non-induced *Acinetobacter* cells. “control” indicates *Acinetobacter* cells were induced by the mixture of crude cell extract from *entC*-overexpressing *E. coli* and chorismate. Asterisk indicates a significant difference in comparison to the control ($P < 0.05$). Luminescence values of uninduced *Acinetobacter* cells were taken as 1. Error bars represent the mean $\pm$ SE ($n=3$).

IPL activity of PRXR1

To establish whether PRXR1 catalyzes the conversion of isochorismate into SA, recombinant His-tagged PRXR1 protein was expressed in *E. coli* and purified by Ni-NTA agarose chromatography. The molecular subunit mass of His-PRXR1 protein was evaluated by 15% SDS-PAGE gel with coomassie brilliant blue and western blot analysis. As shown in Fig. 6a, one band was visible at the expected position of 35 ± 3 kDa. By converting luminescence values to SA concentration, the results showed that incubation of chorismate with the combination of expressed

proteins EntC and His-PRXR1 resulted in enhanced levels of SA, in a His-PRXR1-dependent manner (Fig. 6b). Because isochorismate spontaneously forms salicylate and pyruvate in the elimination reaction, basal amounts of SA were also detected with the combination of chorismate and EntC. Without EntC, incubation of PRXR1 with chorismate also resulted in a slightly higher SA content, probably because *Acinetobacter* contains ICS that could provide low amounts of isochorismate. In the absence of chorismate, PRXR1 alone or with EntC did not increase the SA amount. The results suggest that PRXR1 has IPL activity.

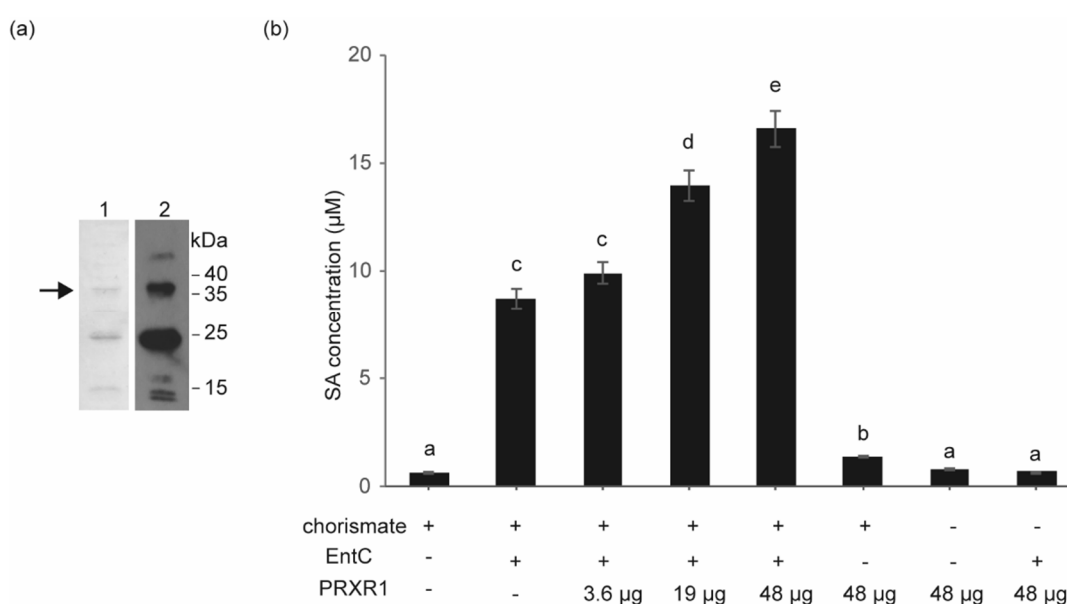


Fig. 6 SA measurements with His-tagged PRXR1. (a) His-tagged PRXR1 protein was expressed in *E. coli*, purified and electrophoresed using SDS-PAGE and either stained with Coomassie Brilliant Blue (lane 1) or visualized with Western blotting using anti-His antibodies (lane 2). The positions of molecular mass markers are indicated in kDa. The arrow indicates the position of the PRXR1 protein band. (b) Accumulation of SA *in vitro*, as measured through the *Acinetobacter* biosensor. Letters indicate significantly different groups ($P < 0.05$). Error bars represent the mean $\pm$ SE ($n=3$). This experiment was repeated three times with similar results.

Discussion

SA is an indispensable plant signaling molecule for defense against biotrophic pathogens and is associated with certain developmental processes. Two distinct pathways have been implied for its biosynthesis in plants, one involving ICS, for which extensive evidence has been obtained in *Arabidopsis*, the other mediated by PAL, which has been

described for tobacco and other plant species. Neither of these biosynthesis routes has been fully characterized. The ICS-mediated pathway in *Arabidopsis* involves the conversion of chorismate to isochorismate by the enzyme ICS. *Arabidopsis* mutants that lack an intact gene for ICS do not accumulate SA, suggesting a route similar to the biosynthesis of SA in certain prokaryotes. The bacterial pathway involves, in addition to ICS, a second enzyme, isochorismate pyruvate lyase (IPL) that converts isochorismate to SA. However, evidence for such an activity in *Arabidopsis* is still lacking.

In this article, we describe an *E. coli* biosensor that can be used to screen cDNA expression libraries for finding genes encoding proteins with IPL activity. The biosensor module was obtained by PCR from *Acinetobacter* ADPWH_*lux* genomic DNA and consisted of the SA-responsive *PsaIA* promoter in front of the *lux* operon and the constitutively expressed gene *salR* encoding the regulator protein. These modules were cloned in an *E. coli* plasmid vector, which after transformation resulted in a functional *E. coli* biosensor for detection of both exogenously supplied SA and intracellularly generated SA.

Our biosensor XL1-LUX was responsive to SA concentrations in the low μM range (above 2 μM on solid SA supplied medium), as indicated by detectable luminescence (Fig. 1). It was surprising that the optimum temperature for detecting salicylic acid was found to be lower than 28°C, which was lower than the optimum growth temperature for most of *E. coli* and *Acinetobacter* strains. Apart from being an activator of the *saIA* promoter in the presence of SA, SalR was found to be a repressor of the *saIA* operon in the absence of SA (Zhang et al. 2012). We speculate that SalR represses *saIA* gene expression by negative regulation via the binding site (TTCA-N₁₂-TGAT) in the promoter of *saIA* in *Acinetobacter* sp. ADP1 (Schell, 1993). The induction of *luxCDABE* by SalR at 37°C in the absence of SA suggests that the conformational change that turns SalR from repressor to activator is subject to temperature in *E. coli*. Compared to chromosome integration, the copy number of plasmid is much higher that might also explain leaky expression. Moreover, it is possible that in the reconstructed operon, the *PsaIA* promoter functions

sub-optimally, or that *salR* gene expression is lower in *E. coli* than in *Acinetobacter*. This would explain our finding that the expression of *luxCDABE* is activated less efficiently by SalR in *E. coli* than in *Acinetobacter*, and that *E. coli* cells show some leaky expression under non-induced conditions compared to *Acinetobacter* at 28°C (data not shown). To improve the sensitivity response to SA and decrease the leaky expression of *lux*, the intergenic region between the SalR binding site and the *luxCDABE* gene could be modified, for instance, by substitution of an *E. coli* compatible ribosome binding site, or alter the SalR binding affinity of *PsaIA* (Park et al. 2005; Blazeck and Alper, 2013; Jha et al. 2014).

The sensor was tested for detection of endogenously produced SA after transformation with a plasmid harboring the gene encoding bacterial IPL. Expression of the *lux* operon was observed after induction with IPTG, but low-level leaky expression of luciferase activity was noticeable in uninduced cells (Fig. 2). We transformed the biosensor with a cDNA plasmid library obtained from TCV-infected *Arabidopsis* to screen for luminescent clones (Fig. 3 and 4). We identified twelve clones that were in frame and able to activate *lux* gene expression in *E. coli*, and which we considered to express IPL candidates (Table S1). Subsequent analyses confirmed that at least one, PRXR1, is possibly involved in the SA biosynthesis (Fig. 5 and 6).

The IC pathway is at least partly (ICS, EDS5) localized in the chloroplast, which suggests that the hypothetical IPL could also be chloroplast localized. Previous findings by Chong et al (2014) suggested that PRXR1 interacted with an endoplasmic reticulum (ER)-localized heat-shock protein 90 (HSP90), which indicating that PRXR1 is possibly localized in the ER. PRXR1 belongs to the group of secretory class III peroxidases (EC 1.11.1.7) only found in plants. These are heme-containing glycoproteins able to oxidize various substrates, utilizing hydrogen peroxide as electron donor. The substrates include phenolic compounds, auxin, and secondary metabolites (Welinder et al. 1992; Ruiz-Duenas et al. 2001; Passardi et al. 2004). Together, these results lead to the following model. We speculate that isochorismate is

transported from the chloroplasts to the cytosol by the MATE transporter EDS5, which is an essential component in accumulation of cytoplasmic SA (Nawrath et al. 2002; Serrano et al. 2013; Yamasaki et al. 2013). Once isochorismate is in the cytosol, PRXR1 catalyzes the conversion of isochorismate to SA.

We demonstrated that only a small amount of chorismate was transformed into SA via the combination of EntC and PRXR1, suggesting the protein assay need to be optimized or PRXR1 plays only a minor role in SA biosynthesis, and other enzymes, either chloroplast or cytoplasm localized, are involved in this process as well. Another possibility relies on the fact that isochorismate spontaneously undergoes rearrangement to form isoprephenate without enzyme catalysis (DeClue et al. 2006; Luo et al. 2011). This could suggest that isoprephenate in the reaction mixture is converted to SA by PRXR1. In addition, slightly enhanced luminescence was observed in the reaction mixtures containing chorismate and EntC only, implying that a small amount of isochorismate was spontaneously converted into SA.

Materials and Methods

Reporter plasmid construction

Genomic DNA was isolated from *Acinetobacter* sp. ADPWH_ *lux* (Huang et al. 2005) with the genomic DNA purification protocol from Cold Spring Harbor (Green and Sambrook 2012). *SalA* promoter region, *salR* with promoter and *luxCDABE* were amplified from the *Acinetobacter* sp. ADPWH_ *lux* genomic DNA and cloned into pJET1.2 (Fermentas), using primers shown in Table S2. The *salA* promoter digested from pJET1.2 with *Bam*HI and *Eco*RI, introduced upstream of *salR* in pJET1.2 digested with *Bam*HI and *Eco*RI to generate plasmid pSALAR. *luxCDABE* was excised from pJET1.2 with *Eco*RI site and inserted between the *salA* promoter and *salR* region in the pSALAR plasmid, resulting in plasmid pLUX, which was transformed into *E. coli* XL-1 Blue MRF'. After transformation, plasmids were selected on carbenicillin (200 µg/mL) and 500 µM SA. The plates were photographed in the dark after auto exposure in a Gel Doc XR+ Gel Documentation System (Bio-rad).

The reporter plasmid containing the *PsalA::luxCDABE:saIR* cassette was named pLUX.

Transformation and validation of luciferase expression

Reporter plasmid pLUX was transformed into *E.coli* XL-1 Blue MRF' cells and grown on LC agar plates containing carbenicillin (200 µg/mL) at room temperature for 3 days. Individual clones were inoculated onto LC agar plates containing carbenicillin (200 µg/mL) and SA at various concentrations ranging from 0 to 100 µM, and incubated at 28°C for 2-3 days. The luminescence was detected by image scanner (ProXPRESS 2D Proteomic Imaging System, Perkin-Elmer) or X-ray film (Fuji) in the dark. Competent *E. coli* XL1-LUX cells were prepared from liquid media containing carbenicillin (200 µg/ml) and grown at room temperature until OD₆₀₀=0.4, after which the procedure followed the standard protocol (Inoue et al. 1990). For SA induction of *E. coli* XL1-LUX, 50 µL of cell cultures (OD₆₀₀=0.4) were mixed with 20 µL of various SA concentrations (0-100 µM) and 60 µL LC medium, and incubated for 1h at room temperature. The luminescence was determined immediately by using a Victor light 1420 counter (Perkin-Elmer).

Construction of a double plasmid system for sensing endogenous SA in *E. coli*

PmsB with His-tag was amplified from *P. fluorescens*, cloned into pBK-CMV (pBK) and transformed into *E. coli* XL1-LUX and selected on LC agar plates with carbenicillin (100 µg/mL) and kanamycin (25 µg/mL). Individual clones were singled out and inoculated on LC agar plates with carbenicillin (100 µg/mL), kanamycin (25 µg/mL) and 1 mM IPTG. The cells were incubated at room temperature for 3-4 days. Luminescence was detected by X-ray film in the dark room.

cDNA library screening in *E. coli*

cDNA library prepared from a mixture of RNAs from 18 days old ecotype Di-17 leaves harvested at 6, 12, 19 and 24 h after TCV infection at a ratio of 1:1:1:2 was constructed and amplified using the Stratagene Zap cDNA synthesis and cloning kit and the ZAP Express vector (Cooley et al. 2000). The lambda phage cDNA library was converted into a

kanamycin-resistant pBK-CMV plasmid library according to the instruction manual (Stratagene). The cDNA library was transformed into *E. coli* XL1-LUX and grown on LC agar plates containing carbenicillin (100 µg/mL), kanamycin (25 µg/mL) and 1 mM IPTG. The cells were incubated at room temperature for 3-4 days and luminescence was recorded using an image scanner. Luminescent clones were streaked onto fresh selected plates. Luminescence was captured after growing at room temperature for another 2-3 days. Plasmids (pBK and pLUX) were extracted from the *E. coli* strains with relatively high luminescence and transformed into *E. coli* XL1-Blue. The transformants were allowed to grow on LC plates containing carbenicillin (100 µg/mL), kanamycin (25 µg/mL) and 1 mM IPTG at 28°C for 1 day, followed by 2-3 days at room temperature. Subsequently, luminescence was captured by the image scanner.

Single plasmids were isolated from *E. coli* and sequenced. Sequenced plasmids were transformed into *E. coli* BL21 (DE3) pLysS. The cells were grown in LC medium containing kanamycin (50 µg/mL) and chloramphenicol (50 µg/mL) at 37°C until OD₆₀₀=0.6, induced with IPTG and grown overnight at 18°C. The cells were collected by centrifugation, resuspended in reaction buffer (100 mM Tris-HCl pH 7.0, 15 mM MgCl₂, 1 mM DTT, 5% glycerol) and sonicated. Crude soluble cell extracts were obtained after centrifugation. Reaction mixtures (500 µM chorismate, 10 µg EntC purified protein, 50 µL cell extract) were incubated at 30°C for 2 h. Twenty microliters supernatant from the reaction mixtures were mixed with *Acinetobacter* sp. ADPWH_*lux* cell culture (OD₆₀₀=0.4) and 50 µL LC medium, incubated at 30°C for 1 h. The luminescence was measured by the luminescence counter.

Expression and isolation of recombinant protein

The full-length coding sequence of PRXR1 was amplified from Arabidopsis cDNA and cloned into pASK-IBA45plus. Recombinant protein was expressed in *E. coli* strain BL21 (DE3) pLysS and purified with Ni-NTA Agarose (Qiagen) according to the manufacturer's protocol. Purified protein was dialyzed against dialysis buffer containing (100 mM Tris-HCl pH 7.0, 15 mM MgCl₂, 1 mM DTT, 5% glycerol) and stored at -

80°C. The subunit molecular mass of AtPRXR1 was estimated using a 15% SDS-PAGE gel. The primers used to amplify AtPRXR1 can be found in Table S2.

Determination of IPL activity

The IPL reaction was measured by coupling excess isochorismate synthase (recombinant EntC). In brief, the reaction mixture (500 µM chorismate, 16 µg EntC, 100 mM Tris-HCl pH 7.0, 15 mM MgCl₂, 1 mM DTT, 5% glycerol) in a final volume of 150 µL was incubated for 2 h at 30°C, vortexed and centrifuged. The supernatant was mixed with *Acinetobacter* sp. ADPWH_*lux* cell culture (OD₆₀₀=0.4) and 50 µL LC medium, followed by incubation at 30°C for 1 h. The luminescence was measured by the luminescence counter.

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Supporting information

Table S1. Genes in plasmids selected after screening

Plasmid number	Locus in Arabidopsis	Length (AA)	Amino acids fused to pBK	Description
1-3	AT1G09340	378	31-378	CRB: chloroplast RNA-binding protein
3-128	AT4G24770	329	136-329	RBP31: RNA-binding protein
3-53	AT4G21960	330	147-330	PRXR1: peroxidase
4-11	AT3G30390	460	300-460	Amino acid transmembrane transporter
4-120	AT1G67440	433	433	EMB1688: minichromosome maintenance protein
4-24	AT3G48500	697	329	PTAC10: plastid RNA-polymerase
4-79	AT5G65220	173	173	Ribosomal L29 family protein
4-215	AT1G67090	180	180	RBCS1A: ribulose 1,5-bisphosphate carboxylase/oxygenase
5-161	AT4G17090	548	31-548	BAM3: β -amylase
5-266	AT5G52760	126	30-120	Copper transporter
5-367	AT5G15090	274	70-274	VDAC3: Voltage-dependent anion channels
5-37	AT3G21200	312	63-306	GluTR: glutamyl-tRNA reductase

Table S2. Primers used in this study.

Primer	F(forward)/ R(reverse)	Sequence (5'-3')*
salA-promoter	F	GAGGGATCCCAAGTTATTTGAGGGGTATA
	R	GCGAATTCCTTCCTTTGAGAACTTGA
salR	F	GAGGGATCCGAATTCACGCTAAGAATTTGGCACA
	R	GAGCTCGAGTTACAAAACCTGAAATA
luxCDABE	F	GCGAATTCATGACTAAAAAAATT
	R	GAGAATTCCTCAACTATCAAACGCTT
PmsB-pBK-CMV	F	GCGAATTCACACCATCATCATCATATGCTGCAA
	R	CCTAAAACCCCT
PRXR1-pASK-IBA45plus	F	GAGGAATTCGATGGGAGGCAAAGGTGTG
	R	GAGGTCGACGATGGTTCTTGTTC

*Restriction sites are underlined.

Chapter 5

Summary

Salicylic acid is a phytohormone, which is involved in the defense responses to pathogens and abiotic stress and in the regulation of plant growth and development (Rivas-San Vicente and Plasencia, 2011). As a vital signaling molecule mediating the induction of plant innate immune responses, particularly the hypersensitive response (HR) and systemic acquired resistance (SAR), SA is produced rapidly upon the attack by biotrophic pathogens (Vlot *et al.*, 2009). Because of an elevated SA level in the cytosol, NON-EXPRESSOR OF PR GENES 1 (NPR1), the master regulator of HR and SAR, is converted to a monomeric form from an oligomer in the cytosol and translocated into the nucleus (Mou *et al.*, 2003). NPR1 directly interacts with TGA transcription factors and co-activates the expression of pathogenesis-related (PR) genes, which are widely taken as HR and SAR markers (Dempsey and Klessig, 2017).

It has been reported that SA is synthesized via two distinct biosynthesis pathways that are derived from chorismate in the chloroplasts (Dempsey, 2011). One pathway depends on phenylalanine ammonia-lyase (PAL) and involves the production of several aromatic intermediates. The second pathway is considered as a major SA contributor in *Arabidopsis* and proceeds via chloroplast localized isochorismate synthase (ICS) and an unidentified isochorismate pyruvate lyase (IPL). Subsequently, SA is transported to the cytosol by the multidrug and toxin extrusion (MATE) transporter EDS5 and triggers the SAR response (Serrano *et al.*, 2013). Plants compromised in SA accumulation exhibit reduced *PR* gene expression and enhanced susceptibility to pathogens (Wildermuth *et al.*, 2001; Nawrath *et al.*, 2002). Constitutively produced SA in plants elevates resistance to the pathogens, but interferes with regular growth and development, which results in a strongly dwarfed and infertile phenotype (Mauch *et al.*, 2001). This infers that SA biosynthesis must be tightly regulated. In **Chapter 1**, the current knowledge of the SA biosynthesis pathway, SA metabolism and the SA-mediated signaling pathway is reviewed.

Previous work has revealed that EDS5 is critical in pathogen-induced SA accumulation and is responsible for exporting SA from the chloroplasts to the cytosol (Nawrath *et al.*, 2002; Serrano *et al.*, 2013).

Studies described in **Chapter 2** aimed at discovering novel transcription factors that can activate or repress *EDS5* gene expression. Yeast One Hybrid (Y1H) and electro mobility shift assays (EMSA) showed that AtERF-1, WRKY11 and WRKY28 bound to the *EDS5* promoter. Furthermore, AtERF-1 and WRKY11 were found to bind *in vitro* to the *ICS1* and *PR1* promoter, respectively. According to protoplast transactivation assays, AtERF-1 was able to activate *EDS5* and *ICS1* gene expression, whereas WRKY11 induced *ICS1* and *PR1* expression. However, *aterf-1* mutants showed increased *PR1* expression, suggesting that AtERF-1 might positively regulate *EDS5* and *ICS1* gene expression but functions as a negative regulator of pathogen-induced SA responses. Together with the results of previously described studies from our lab, WRKY28 was found to function as a positive regulator of both the SA biosynthesis pathway and SA-mediated signaling.

The biosynthesis of SA proceeding via isochorismate remains unknown. In **Chapter 3**, several genes that might encode candidate isochorismate pyruvate lyase enzymes in Arabidopsis were co-expressed with the bacterial isochorismate synthase gene *entC* in *E. coli*. The bacterial biosensor *Acinetobacter* sp. ADPWH_*lux* was used to estimate the SA production. Arabidopsis protein PBS3 has been reported to influence SA accumulation and to be induced by exogenous SA treatment and pathogen infection (Jagadeeswaran *et al.*, 2007). Our results suggest that SA was produced when PBS3 and EntC were overexpressed simultaneously in *E. coli*. SA production was observed *in vitro* when PBS3 was present in crude *E. coli* extract, as opposed to the purified PBS3, suggesting that the enzyme needs co-factors for optimal activity.

The presumed cytosolic localization of PBS3 is inconsistent with the localization of the initial steps of the SA biosynthesis pathway described in previous studies (Fragniere *et al.*, 2011). One possibility is that PBS3, as a major candidate for IPL, is recruited to the chloroplasts when plants are attacked by biotrophic pathogens. Alternatively, isochorismate is transported or diffuses from the chloroplasts to the cytosol through the MATE transporter EDS5 and PBS3 converts isochorismate to SA in a subsequent cytosol-localized step. Isochorismate is the intermediate

product of phyloquinone (vitamin K1) metabolism and very small amounts of SA accumulate in transgenic tobacco plants expressing bacterial IPL in the cytosol when part of the overproduced isochorismate in the chloroplasts is transported or diffuses into the cytosol (Gross *et al.*, 2006; Verberne *et al.*, 2000). These findings suggest that it is unlikely that a large amount of SA is produced through the relocation of isochorismate. However, relocation of isochorismate could explain that plants overexpressing EDS5 accumulate high basal levels of SA in the absence of induction by pathogen or UV exposure. Another possibility is that *Arabidopsis* possesses more than one gene encoding IPL. PBS3 appears to play a minor role in early SA biosynthesis, supporting the assumption that a chloroplast-localized IPL enzyme is the major contributor to SA accumulation.

Therefore, to identify other proteins mediating isochorismate to SA conversion we engineered an *E.coli* SA-biosensor to screen a cDNA library of *Arabidopsis*, which is described in **Chapter 4**. The sensor, which is comprised of the SA inducible promoter *PsaIA*, the regulator *salR*, and the reporter operon *luxCDABE*, was responsive to exogenous and endogenous SA. A cDNA library from turnip crinkle virus (TCV) infected plants was used to screen for proteins that can transform endogenous isochorismate to SA. Surprisingly, PBS3 was not identified in the screening, possibly due to the elevated levels of SA observed when *E.coli* overproduced isochorismate, while overexpression of PBS3 in *E.coli* did not cause SA accumulation, as was found in **Chapter 3**. The screening resulted in the identification of PRXR1, which belongs to the peroxidase family.

Although the reaction conditions need to be further improved, PRXR1 did indeed demonstrate isochorismate pyruvate lyase activity *in vitro*. PRXR1 has been reported to interact with endoplasmic reticulum (ER)-localized heat-shock protein 90.7 (HSP90.7) (Chong *et al.*, 2015), which suggests that PRXR1 is localized in the ER, and which does not correspond to the proposed initial localization of SA. Similar to the case of PBS3, PRXR1 could accomplish SA accumulation via recruitment to the chloroplasts or after relocation of isochorismate.

As a conclusion, this thesis presents new findings related to the transcriptional regulation of SA signaling and the SA biosynthesis pathway. A model summarizing the main results presented in this thesis is proposed in Figure 1. AtERF-1, WRKY11 and WRKY28 play diverse roles in SA synthesis and the SA-mediated signaling pathway. AtERF-1 positively regulates the expression of *PR1* via binding to the GCC-box, whereas the exact role of AtERF-1 in the regulation of the expression of *EDS5* and *ICS1* remains unclear. The functions of WRKY11 await analysis of overexpression plants or knockout mutants. WRKY28 functions as a transcriptional activator in both SA biosynthesis and SA-dependent signaling pathways. ChIP-experiments and experiments with biotrophic pathogen-infected plants *in vivo* could be performed to firmly establish the roles of these transcription factors. It is surprising that two distinct proteins, PBS3 and PRXR1, that are not localized in the chloroplasts were able to elevate the SA level *in vitro*, inferring that plants might possess several IPL enzymes to accomplish SA biosynthesis via isochorismate.

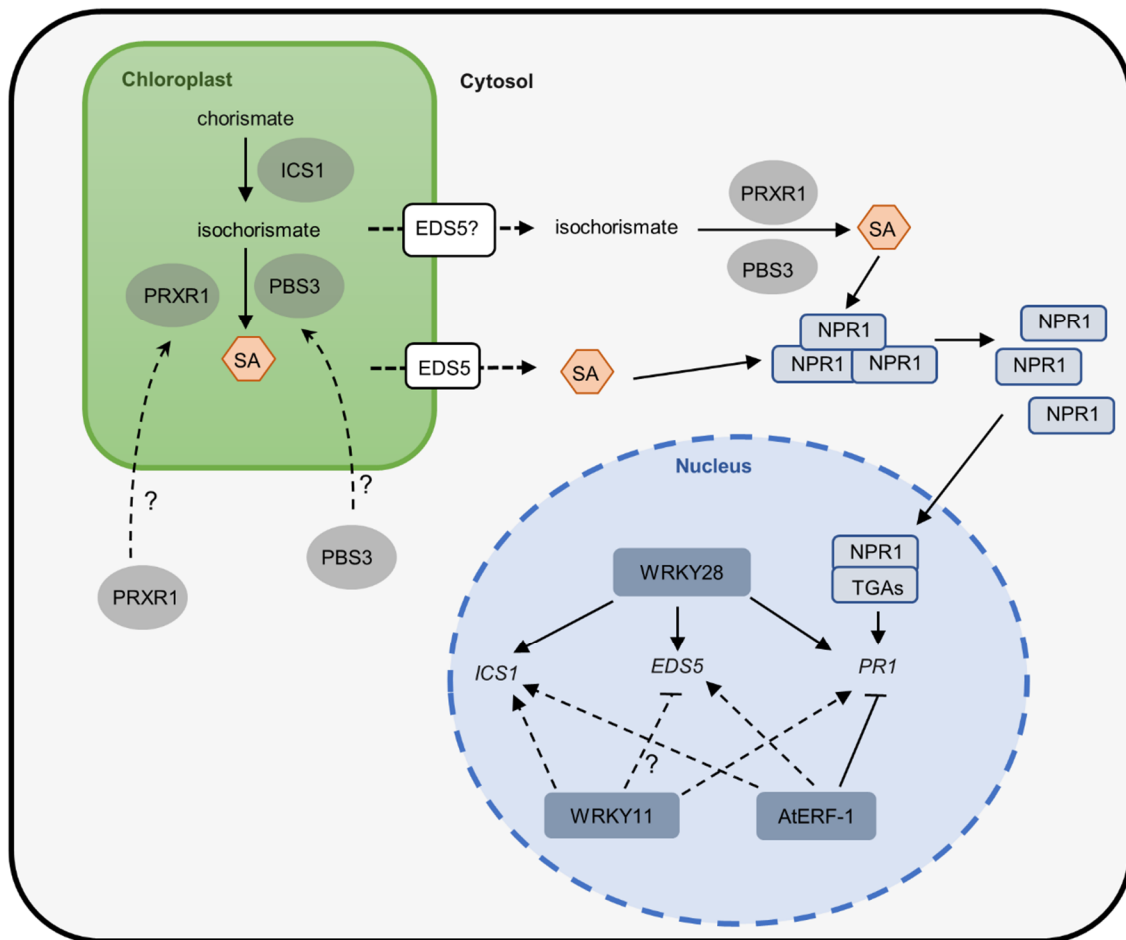


Figure 1. Model describing the transcriptional regulation of *EDS5*, *ICS1* and *PR1* and the isochorismate-dependent SA biosynthesis pathway. AtERF-1 negatively regulates *PR1* transcription and may promote the expression of *EDS5* and *ICS1* (indicated by the dashed lines). WRKY11 might contribute to *ICS1* and *PR1* expression (indicated by the dashed lines), but its function on *EDS5* expression remains unclear (indicated by the dashed line and question marker). WRKY28 is a positive regulator of *EDS5*, *ICS1* and *PR1*. ICS1 converts chorismate into the SA-precursor isochorismate. Subsequently, isochorismate may be transported through the MATE-transporter EDS5 or by another transporter to the cytosol (indicated by the dashed line and question mark) and may be converted to SA via PBS3 and PRXR1. Alternatively, PBS3 and PRXR1, which are not obvious chloroplast-targeted proteins, are re-localized into the chloroplasts (indicated by the dashed lines and question marks) and convert isochorismate into SA. SA produced in the chloroplasts is transported by EDS5 into the cytosol. The expression of *ICS1*, *EDS5* and *PBS3* is induced by exogenous SA and contributes to the positive feedback loop of SA accumulation. The accumulation of SA in the cytosol results in NPR1 monomers which migrate to the nucleus and interact with TGA transcription factors and activate transcription of *PR1*.

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SAMENVATTING

Salicylzuur (SA) is een fytohormoon, dat betrokken is bij de afweerreacties op pathogenen en abiotische stress en bij de regulatie van plantengroei en ontwikkeling (Rivas-San Vicente en Plasencia, 2011). Als een vitaal signaleringsmolecuul dat de inductie van aangeboren immuunreacties van de plant medieert, in het bijzonder de overgevoeligheidsreactie (HR) en systemisch verworven resistentie (SAR), wordt SA snel geproduceerd na infectie door biotrofe pathogenen (Vlot *et al.*, 2009). Vanwege een verhoogd SA-niveau in het cytosol, worden eiwitcomplexen van NONEXPRESSOR OF PR GENES 1 (NPR1), de hoofdregelaar van HR en SAR, omgezet in een monomere vorm in het cytosol en getransloceerd naar de kern (Mou *et al.*, 2003). NPR1 gaat een interactie aan met TGA-transcriptiefactoren en co-activeert de expressie van pathogenese-gerelateerde (PR) genen, die betrokken zijn bij HR- en fungeren als markers voor SAR (Dempsey en Klessig, 2017).

Er is gerapporteerd dat SA wordt gesynthetiseerd in de chloroplasten, via twee verschillende biosyntheseroutes die zijn afgeleid van chorismaat (Dempsey, 2011). Eén route is afhankelijk van fenylalanine ammonia-lyase (PAL) en omvat de productie van verschillende aromatische tussenproducten. De tweede route wordt beschouwd als een belangrijke SA-bijdrager in Arabidopsis en verloopt via chloroplast gelokaliseerd isochorismaatsynthase (ICS) en een niet-geïdentificeerd isochorismaatpyruvaatlyase (IPL). Vervolgens wordt SA getransporteerd naar het cytosol door de multidrug en toxine extrusie (MATE) transporter EDS5 en triggert de SAR-respons (Serrano *et al.*, 2013). Planten die aangetast zijn in de accumulatie van SA vertonen verminderde PR-genexpressie en verhoogde gevoeligheid voor pathogenen (Wildermuth *et al.*, 2001; Nawrath *et al.*, 2002). Constitutief geproduceerd SA in planten verhoogt de weerstand tegen pathogenen, maar interfereert met reguliere groei en ontwikkeling, wat resulteert in een sterk verkleind en onvruchtbaar fenotype (Mauch *et al.*, 2001). Dit betekent dat SA-biosynthese nauwkeurig moet worden gereguleerd. In **Hoofdstuk 1** wordt de huidige kennis van de SA-biosyntheseroute, het

SA-metabolisme en de SA-gemedieerde signaaltransductieroute besproken.

Eerder werk heeft onthuld dat EDS5 een kritische rol speelt in pathogeen-geïnduceerde SA-accumulatie en verantwoordelijk is voor de export van SA van de chloroplasten naar het cytosol (Nawrath *et al.*, 2002; Serrano *et al.*, 2013). Studies beschreven in **Hoofdstuk 2** zijn gericht op het ontdekken van nieuwe transcriptiefactoren die *EDS5*-genexpressie kunnen activeren of onderdrukken. Yeast One Hybrid (Y1H) assays en electro mobility shift assays (EMSA) toonden aan dat AtERF-1, WRKY11 en WRKY28 aan de *EDS5*-promoter bonden. Verder bleken AtERF-1 en WRKY11 in vitro respectievelijk te binden aan de *ICS1*- en *PR1*-promoter. Volgens protoplast-transactiverings-assays was AtERF-1 in staat om *EDS5*- en *ICS1*-genexpressie te activeren, terwijl WRKY11 *ICS1*- en *PR1*-expressie induceerde. *aterf-1*-mutanten vertoonden echter een verhoogde *PR1*-expressie, wat suggereert dat AtERF-1 de expressie van *EDS5* en *ICS1* positief zou kunnen reguleren, maar functioneert als een negatieve regulator van door pathogenen geïnduceerde SA-responsen. Samen met de resultaten van eerder beschreven studies uit ons laboratorium, bleek WRKY28 te functioneren als een positieve regulator van zowel de SA-biosynthese-route als SA-gemedieerde signalering.

De biosynthese van SA via isochorismaat is gedeeltelijk nog onbekend. In **Hoofdstuk 3** werden verschillende genen die kunnen coderen voor kandidaat isochorismaat pyruvaatlyase-enzymen in *Arabidopsis* mede tot expressie gebracht met het bacterieel isochorismaatsynthasegen *entC* in *E. coli*. De bacteriële biosensor *Acinetobacter* sp. ADPWH_*lux* werd gebruikt om de SA-productie te schatten. Van *Arabidopsis*-eiwit PBS3 is beschreven dat het SA-accumulatie beïnvloedt en dat het wordt geïnduceerd door exogene SA-behandeling en pathogene infectie (Jagadeeswaran *et al.*, 2007). Onze resultaten suggereren dat SA werd geproduceerd wanneer PBS3 en EntC gelijktijdig in *E. coli* tot overexpressie werden gebracht. SA-productie werd in vitro waargenomen wanneer PBS3 aanwezig was in ongezuiverd *E. coli*.

extract, in tegenstelling tot gezuiverd PBS3, wat suggereert dat het enzym co-factoren nodig heeft voor optimale activiteit.

De veronderstelde cytosolische lokalisatie van PBS3 komt niet overeen met de lokalisatie van de initiële stappen van de SA-biosyntheseroute in de chloroplast die in eerdere onderzoeken is beschreven (Fragniere *et al.*, 2011). Een mogelijkheid is dat PBS3, als een belangrijke kandidaat voor IPL, wordt gerekruteerd naar de chloroplasten wanneer planten worden aangevallen door biotrofe pathogenen. Een andere mogelijkheid is dat isochorismaat diffundeert of getransporteerd wordt via de MATE-transporter EDS5 van de chloroplasten naar het cytosol alwaar PBS3 isochorismaat naar SA omzet. Isochorismaat is het tussenproduct van het phyloquinon (vitamine K1)-metabolisme en zeer kleine hoeveelheden SA accumuleren in transgene tabaksplanten die het bacteriële IPL in het cytosol tot expressie brengen wanneer een deel van het in de chloroplasten overgeproduceerde isochorismaat wordt getransporteerd of diffundeert naar het cytosol (Gross *et al.*, 2006; Verberne *et al.*, 2000). Deze bevindingen suggereren dat het onwaarschijnlijk is dat een grote hoeveelheid SA wordt geproduceerd door transport van isochorismaat. Transport van isochorismaat zou echter kunnen verklaren dat planten met overexpressie van EDS5 hoge basale niveaus van SA accumuleren in afwezigheid van inductie door pathogenen of door blootstelling aan UV. Een andere mogelijkheid is dat *Arabidopsis* meer dan één gen bezit dat IPL codeert. PBS3 lijkt een ondergeschikte rol te spelen in de vroege SA-biosynthese, wat het aannemelijker maakt dat een chloroplast-gelokaliseerd IPL-enzym de belangrijkste bijdrage levert aan SA-accumulatie.

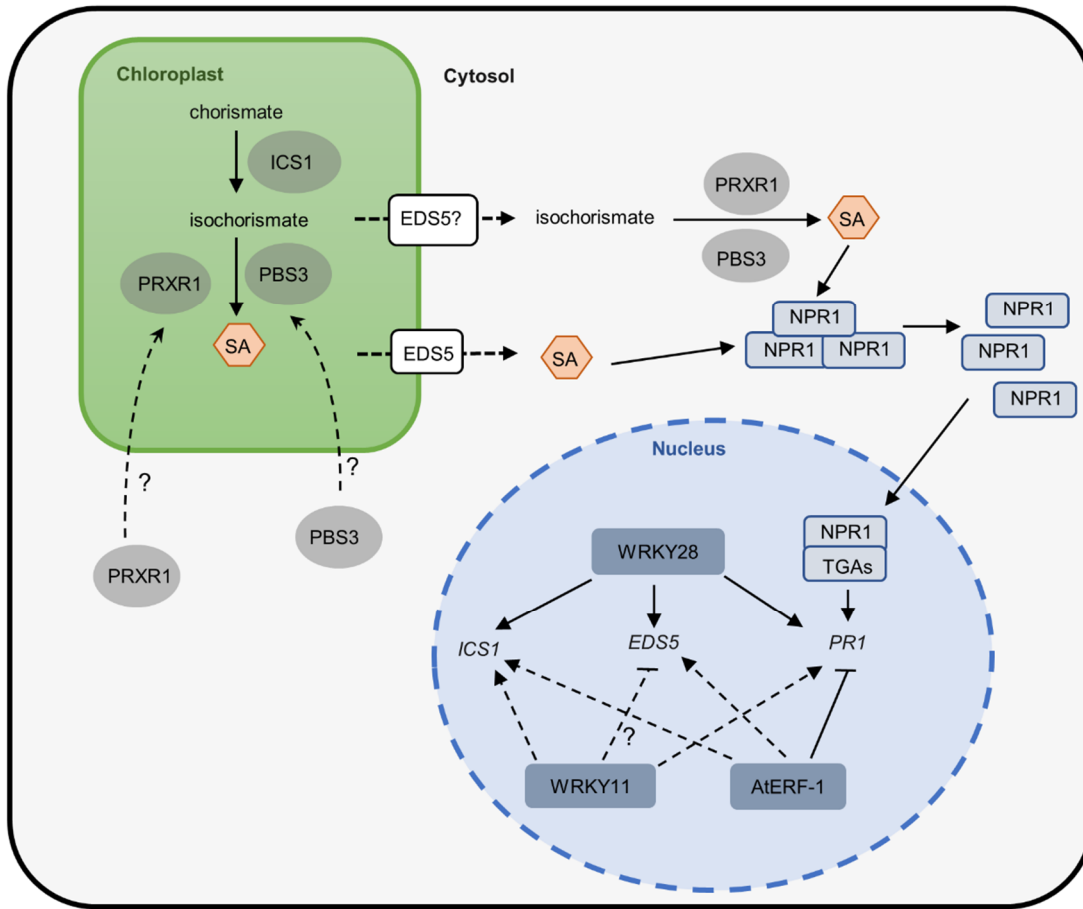
Daarom hebben we voor het identificeren van andere eiwitten die isochorismaat naar SA omzetten, een *E. coli* SA-biosensor geconstrueerd om een cDNA-bibliotheek van *Arabidopsis* te screenen. Dit wordt beschreven in **Hoofdstuk 4**. De sensor, die is samengesteld uit de SA-induceerbare promotor *PsaIA*, de regulator *SaIR*, en het reporter operon *luxCDABE*, reageerde op exogeen en endogeen SA. Een cDNA-bibliotheek van turnip crinklevirus (TCV) geïnfecteerde planten werd gebruikt om te zoeken naar eiwitten die endogeen isochorismaat

in SA om kunnen zetten. Verrassenderwijs werd PBS3 niet geïdentificeerd in deze screening. Mogelijk is dit het gevolg van de verhoogde niveaus van SA die werden waargenomen wanneer *E. coli* overmatig geproduceerd isochorismaat accumuleerde, terwijl overexpressie van PBS3 in *E. coli* geen SA-accumulatie veroorzaakte, zoals werd gevonden in **Hoofdstuk 3**. Wel resulteerde de screening in de identificatie van het eiwit PRXR1, dat tot de familie van peroxidase enzymen behoort.

Hoewel de reactieomstandigheden verder moeten worden verbeterd, toonde PRXR1 inderdaad isochorismaatpyruvaatlyase-activiteit in vitro. Van PRXR1 is gerapporteerd dat het een interactie aangaat met het endoplasmatisch reticulum (ER)-gelocaliseerde heatshock-eiwit 90.7 (HSP90.7) (Chong *et al.*, 2015), wat suggereert dat PRXR1 gelokaliseerd is in het ER, wat niet overeenkomt met de voorgestelde initiële lokalisatie van SA biosynthese in de chloroplasten. Vergelijkbaar met de hiervoor genoemde verklaringen voor PBS3 zou PRXR1 accumulatie van SA kunnen bewerkstelligen via rekrutering naar de chloroplasten of na transport van isochorismaat.

Concluderend kan gesteld worden dat dit proefschrift nieuwe bevindingen presenteert met betrekking tot de transcriptionele regulatie van SA-signalering en de SA-biosynthese-route en de enzymen die bij het laatste proces betrokken zijn. Een model met een samenvatting van de belangrijkste resultaten in dit proefschrift is weergegeven in Figuur 1. AtERF-1, WRKY11 en WRKY28 spelen verschillende rollen in SA-synthese en de SA-gemedieerde signaaltransductieroute. AtERF-1 reguleert de expressie van *PR1* positief via binding aan de GCC-box, terwijl de exacte rol van AtERF-1 in de regulatie van de expressie van *EDS5* en *ICS1* onduidelijk blijft. Voor opheldering van de functies van WRKY11 is analyse van overexpressieplanten of knockout-mutanten noodzakelijk. WRKY28 functioneert als een transcriptionele activator in zowel SA-biosynthese als SA-afhankelijke signaleringsroutes. Door middel van ChIP-experimenten en experimenten met biotrofe pathogeen geïnfecteerde planten zouden de rollen van de hier bestudeerde transcriptiefactoren beter vastgesteld kunnen worden. Het is verrassend

dat twee verschillende eiwitten, PBS3 en PRXR1, die niet gelokaliseerd zijn in de chloroplasten, in staat waren om het SA-niveau *in vitro* te verhogen, hetgeen inhoudt dat planten verschillende IPL-enzymen zouden kunnen bezitten om SA-biosynthese via isochorismaat te bewerkstelligen.



Figuur 1. Model dat de transcriptionele regulatie van *EDS5*, *ICS1* en *PR1* en de isochorismaat-afhankelijke SA-biosyntheseroute beschrijft. AtERF-1 reguleerde *PR1*-transcriptie negatief en kon de expressie van *EDS5* en *ICS1* bevorderen (aangegeven door de gestippelde lijnen). WRKY11 zou kunnen bijdragen aan *ICS1*- en *PR1*-expressie (aangegeven door de streepjeslijnen), maar de rol in expressie van *EDS5* is nog steeds onduidelijk (aangegeven door de stippellijn met vraagteken). WRKY28 is een positieve regulator of *EDS5*, *ICS1* en *PR1*. *ICS1* zet chorismaat om in de SA-precursor isochorismaat. Vervolgens zou isochorismaat kunnen worden getransporteerd door de MATE-transporter *EDS5* of door een andere transporter naar het cytosol en daar worden omgezet naar SA door PBS3 en/of PRXR1. Een andere mogelijkheid is dat PBS3 en PRXR1, die niet voor de hand liggende chloroplast eiwitten zijn, onder bepaalde omstandigheden gerelokaliseerd kunnen worden naar de chloroplasten, alwaar isochorismaat wordt omgezet in SA. SA geproduceerd in de chloroplasten wordt getransporteerd door *EDS5* in het cytosol. De expressie van *ICS1*, *EDS5* en *PBS3* wordt geïnduceerd door exogeen SA, wat bijdraagt aan een positieve feedbacklus van SA-accumulatie. De accumulatie van SA in het cytosol resulteert in NPR1-monomeren die migreren naar de kern en interacties aangaan met TGA-transcriptiefactoren en daarmee transcriptie van *PR1* activeren.

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Yingjie 洁洁

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Curriculum vitae

Yingjie Zhou was born on the 2nd of July 1986 in Chengdu, China. She started the study of Agriculture at the Sichuan Agricultural University in 2004 and obtained her bachelor's degree in 2008. She continued her Master study in plant breeding under the supervision of Prof. dr. Xiquan Zhang at the same university. After internships at the China Agricultural University on the project “Sex identification of buffalo grass” and at the International Livestock Research Institute on the project “Virus detection in forage grass” in Ethiopia, she obtained her MSc degree in July 2011. After that, she worked in a rural community as a volunteer with data collection and analysis of floating population and community library management. In March 2012, she started her PhD project, described in this thesis, in the Plant Cell Physiology group of the Institute of Biology Leiden under the supervision of Dr. Huub J. M. Linthorst and Prof. dr. Johan Memelink, supported by the China Scholarship Council. In January 2018, she continued to work as a Post-doc at Fytagoras BV in cooperation with the Leiden University-European Centre for Chinese Medicine and Natural Products.

