Study on the Mode of Action of Saccharin as a Plant Activator of Disease Resistance

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Abstract of Thesis

Plant activators, also referred to as plant resistance activators or elicitors, are compounds that lead to the improved plant protection to pathogen attacks by inducing the plant's own defense mechanisms. Unlike common toxic fungicides that directly target pathogens, resulting in drug resistance development of pathogens, side effects on non-target organisms, and polluted foods and environment, plant activators do not pose such the problems. Chemically induced resistance by plant activators can provide another useful option for practical crop protection, contributing to reduction of toxic pesticide usage. In the current study, we attempt to elucidate the molecular mechanism and efficacy of two compounds saccharin and PBZ acting as plant activators of effective defenses against some bacterial and fungal pathogens in both dicot and monocot plants.

Saccharin was previously proved to be one of the metabolites of probenazole (PBZ) in plants and acts as a plant activator. Our tests on phytotoxicity of saccharin in *Arabidopsis thaliana* and wheat plants have shown that saccharin exhibits no or moderate negative effects on the plant growth when applied at low concentrations up to 1 mM, regardless of application methods as foliar spraying or root drenching. We also confirmed that saccharin up to 10 mM had no direct antimicrobial activity on the growth of bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000) and other fungal pathogens by *in vitro* test. On the basic of these results we decide to use saccharin at 1 mM, along with PBZ at 0.1 mM as a positive control, for further analyses.

To elucidate the mechanism underlying saccharin- and PBZ-induced resistance we used the pathosystem of dicot model plant *Arabidopsis thaliana* and different bacterial and fungal pathogens. Exogenous application of saccharin and PBZ to the wild-type Arabidopsis plant triggered resistance to a hemibiotrophic bacterium *Pto* DC3000, but not to a necrotrophic bacterium *Pectobacterium carotovorum* subsp. *carotovorum*, and a hemibiotrophic fungus *Colletotrichum higginsianum*, and in the case of necrotrophic fungus *Botrytis cinerea*, they enhanced susceptibility. Analysis of gene expression profile revealed that activation of SA-regulated genes (*PR1*, *PR2*) and the concomitant suppression of JA-regulated genes (*LOX2*, *VSP2*, *PDF1.2*) by saccharin and PBZ might be the contributing event which accounted for induced resistance to *Pto* DC3000 and increased susceptibility to *Botrytis cinerea*. Consistently, analysis of Arabidopsis mutants

impaired in SA- and JA-signaling showed diminished resistance in NahG and *npr1*, but not in *jar1* plants pretreated with saccharin or PBZ. Together, these results suggest that saccharin and PBZ induce resistance in Arabidopsis against *Pto* DC3000 mainly via activation of SA-signaling leading to suppression of JA/ET- signaling and vice versa. Moreover, both saccharin and PBZ upregulated expression of other defense-related genes (*ALD1*, *PRX34*, *PAD3*, *FRK1*) and accelerated deposition of callose in Arabidopsis plants, suggesting the additive roles of multiple defense outcomes in resistance to *Pto* DC3000.

Next, we demonstrate that pretreatment of wheat seedlings with saccharin or PBZ results in a significant reduction in powdery mildew disease caused by *Blumeria graminis* f. sp. *tritici.* At 7 dpi, the less severe symptoms on saccharin-or PBZ-pretreated leaves were indicated by a reduced number of pustules approximately by 70% and 50% in the first and second leaves, respectively, as compared to those on the control leaves. In addition, the protective effect of saccharin and PBZ was maintained for at least 11 days after inoculation under high pressure of infection. Transcriptional analysis revealed expression profile of 15 defense-related genes. Indeed, saccharin and PBZ induced expression of multiple defense-related genes in wheat seedlings such as *PR* genes (*PR1.1*, *PR2*, *PR4*, *CHI3*, *CHI4*), genes associated with SA signaling and biosynthesis (*NPR1* and *PAL*), genes involved in JA biosynthesis (*LOX*, *AOS*), wheat chemically induced genes (*WCI2, WCI3*), and a transcription factor encoding gene (*WRKY72a/b*). The induced expression of those defense genes in infected/ uninfected wheat seedlings pretreated with saccharin or PBZ substantially contributed to resistance to the powdery mildew fungus *B. graminis*.

Our study on resistance induced by saccharin and PBZ in Arabidopsis and wheat plants against certain bacterial and fungal pathogens complement the current knowledge on the efficacy and the mode of action of saccharin and PBZ, which may be of interest to developers of plant defense activators and researchers working on immunity in both dicot and monocot plants. Saccharin can be expected to be used as an alternative eco-friendly protectant for reducing the use of agrochemicals in disease control by specific pathogens.

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Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations

ANOVA - analysis of variance
A. thaliana - *Arabidopsis thaliana*
bp - base pair
B. cinerea/ Bc - *Botrytis cinerea*
B. graminis/ Bg - *Blumeria graminis*
cfu/CFU - colony-forming units
Cont - control
C. higginsianum/ Ch - *Colletotrichum higginsianum*
Ct - cycle threshold
DMSO - dimethyl sulfoxide
dpi - days post-inoculation
ET - ethylene
ETI - effector triggered immunity
Fig - figure
hpi - hours post-inoculation
hpt - hours post-treatment
IR - induced resistance
ISR - induced systemic resistance
JA - jasmonic acid
KB - King's B
LB - Luria-Bertani

mins - minutes

OD - optical density

PAMPs - pathogen-associated molecular patterns

PRRs - pattern recognition receptors

PBZ - probenazole

Pcc - *Pectobacterium carotovorum* subsp. *carotovorum*

Pto DC3000/ DC3000 - *Pseudomonas syringae* pv. *tomato* DC3000

PR - pathogenesis-related

qRT-PCR - quantitative real-time polymerase chain reaction

ROS - reactive oxygen species

Saccharin - sodium saccharin dihydrate

SA - salicylic acid

secs - seconds

SD - standard deviation

SAR - systemic acquired resistance

Chapter 1

General introduction: Induced disease resistance in plants

1. Plant immune system

To survive natural invading pathogens, plants have evolved many defense mechanisms which consist of constitutive and inducible defenses. Constitutive defenses or preexisting defenses, such as physical barriers (waxy cuticles, cell walls) and antimicrobial chemicals (phytoanticipins), serve as the first line of plant protection even in the absence of pathogens (Agrios 2005). Meanwhile, inducible defenses are triggered as a consequence of specific recognition of pathogen-derived molecules by host plant receptors (Jones and Dangl 2006).

In regard to inducible immune, plants respond to pathogens using a two-branched innate immune system which is comprehensively illustrated in a zigzag model proposed by Jones and Dangl (2006). Accordingly, the first branch of immune system utilizes transmembrane pattern recognition receptors (PRRs) (e.g. kinase flagellin sensing 2) that respond to pathogen-associated molecular patterns (PAMPs) (e.g. flagellin from Gram-negative bacteria), resulting in PAMP-triggered immunity (PTI). However, successful pathogens deploy effectors which can interfere with PTI and result in effector-triggered susceptibility (ETS) or basal defense. If a given effector is recognized by NB-LRR protein encoded by most resistance (R)-gene, effector-triggered immunity (ETI) is established as the second branch of plant immune. The activation of PTI or ETI is often associated with the ROS generation, expression of defense-related genes, production of antimicrobial compounds, and hypersensitive cell death response at the infection site, resulting in plant disease resistance and restricts pathogen growth (Coll et al. 2011; Bindschedler et al. 2006; Torres 2010). So far, 100s-1000s of genes typically respond to defense induction have been revealed, together with 10 recognized classes of pathogenesis-related (PR) genes (Bektas and Eulgem 2015).

Both ETI and PTI are also known to utilize a complex network of phytohormone signaling pathways, such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Glazebrook 2005; Pieterse et al. 2012; Yi et al. 2014). It is now clear that infection with pathogens results in changes in the level of SA, JA or ET (Bari and Jones 2009). While SA- and JA-signaling acts antagonistically in most cases of defense activation, JA- and ET-signaling appears to function synergistically (Glazebrook 2005; Pieterse et al. 2012). The outcomes of antagonistic cross-talk between SA- and JA/ET-signaling often leads to the host resistance against a certain pathogen group or a distinct pathogen with a particular parasitic habit such as the biotrophs feed on living host tissue, and the necrotrophs kill host tissue and feed on the death (Bostock 2005; Glazebrook 2005). Collectively, effective defenses against biotrophic pathogens largely rely on activation of defense responses regulated by SA-dependent pathway, whereas those against necrotrophic pathogens are mainly associated with JA-mediated signaling (Glazebrook 2005; Thomma et al. 1998). Though, evidence of synergistic interactions between SA- and JA- signaling for conditioning host resistance has also been reported (Bari and Jones 2009). In addition, the lifestyles of different pathogens are not always clearly assigned as pure biotrophs or necrotrophs, particularly in the case of bacterial pathogens (Kraepiel and Barny 2016). Therefore, the positive or negative cross-talk between SA and JA/ET pathways may be regulated depending on the specific host-pathogen interaction (Glazebrook 2005).

2. Induced resistance (IR)

In addition to local responses limited to pathogen-infected tissues which are presentative for PTI, basal defense and ETI, plants can also adapt systemic immunity which includes two major types of induced resistance: systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Pieterse et al. 1998; van Wees et al. 2000). Besides the direct

activation of defenses, inducible resistance can also lead to the so-called defense priming, resulting in stronger elicitation of those defenses following pathogen attack (Walters et al. 2013). Induced resistance in the plant can be triggered by biotic and abiotic stimuli such as virulent or avirulent pathogens, usage of biocontrol agents, and application of natural or synthetic compounds (Beckers and Conrath 2007).

Systemic acquired resistance (SAR)

SAR can be initiated by local compatible or incompatible interactions or application of SAR-inducing chemicals such as SA and its analogs. This highly desirable resistance helps to protect plants from a broad-spectrum of related or unrelated pathogens in uninfected tissues and organs (Gao et al. 2014). SAR tends to be long-lasting for a period of weeks to months, but is rarely complete, with most inducing agents reducing disease by between 20 and 85% (Fu and Dong 2013; Walters et al. 2013a). In addition, resistance against biotrophs is associated with activation of SA-dependent signaling and SAR (Glazebrook 2005).

The generation of multiple signals at the local and distal sites of elicitation is involved in SAR, which can be typical, for example, an increase of SA levels and enhanced expression of several classical PR genes (Fu and Dong 2013). Some of these PR genes many of which encode PR proteins with antimicrobial activity, such as *PR1*, *PR2*, and *PR5* serve as robust markers for this systemic immune response (Bektas and Eulgem 2015). In the current concept of the plant immune system, the onset of pathogen-induced SAR is triggered upon local activation of a PTI or ETI responses (Pieterse et al. 2014). Even though the local and systemic accumulation of SA is critical for SAR induction, this hormone is proved not to serve as a general mobile signal for SAR. Nonexpressor of PR genes1 (NPR1), a transcription coactivator, is widely accepted as a

master regulator of plant defense required for induction of a large set of PR gene, local defense, and SAR. NPR1 translocates to the nucleus when SA accumulation causes changes in cellular redox potential. Wu et al. (2012) reported that NPR1 serves as a receptor for SA. However, contradictory findings suggest that SA might be perceived by the NPR1 paralogs NPR3 and NPR4, which function as SA receptors in a SA-concentration-dependent manner and as E3 ligases that mediate the degradation of NPR1 (Fu et al. 2012). Recently, Ding et al (2018) have revealed that NPR3/NPR4 serves as transcriptional corepressors for SA-responsive genes and SA inhibits their activities to promote the expression of downstream immune regulators. The study further indicated that both NPR1 and NPR3/NPR4 are bona fide SA receptors, but play opposite roles in transcriptional regulation of SA-induced defense gene expression (Ding et al. 2018).

Induced systemic resistance (ISR)

Systemic immunity can be triggered by certain strains of non-pathogenic plant growth-promoting rhizobacteria (PGPR) and fungi (PGPF) leading to induced systemic resistance (ISR) against a broad range of pathogens and insect herbivores. There is a wide variety of PGPR and PGPF including Pseudomonas, Bacillus, Trichoderma, and mycorrhiza species which can sensitize plant immune system for enhanced defense (Walters et al. 2013b; Pieterse et al. 2014).

Unlike SAR, induction of ISR is not associated with the accumulation of SA and PR proteins or PR transcripts in systemic tissues. However, both SAR and ISR require NPR1 as a common regulator of their signaling pathways. It was demonstrated that JA and ET are central players in the regulation of rhizobacteria-mediated ISR, therefore (Glazebrook, 2001). In accordance with its dependency on JA and ET signaling, ISR was

associated with activation of JA/ET-regulated genes and was shown to be effective against necrotrophic pathogens and insect herbivores (Pieterse et al. 2014).

Defense priming

Defense priming is regarded as an intrinsic adaptive part of biologically and chemically induced resistance. Indeed, priming is the plant state of preparing its defensive system for a faster and/or stronger activation of cellular defenses upon invasion, resulting in an enhanced level of resistance. Defense priming, therefore, represents a form of plant immunological memory (Conrath et al. 2002; Mauch-Mani et al. 2017). Compared to constitutively activated resistance, priming generally results in low fitness costs under pathogen pressure and appears to be a common and favorite feature of the plant's immunity system (Hulten et al. 2010; Conrath et al. 2006). It is proposed that signaling proteins and transcriptional regulators involved in expression of priming remain inactive in enemy-free conditions but provide the plant with the capacity to react with an accelerated defense response upon perception of a pathogen- or insect-derived stress signal (Pieterse et al. 2014).

Synthetic compounds of induced resistance

Plant activators, also referred to as plant resistance activators or plant resistance inducers or elicitors, are compounds that lead to improved protection to pathogen attacks by inducing the plant's own defense mechanisms (Bektas and Eulgem 2015; Alexandersson et al. 2016). Unlike common fungicides that directly target pathogens, resulting in the development of drug resistance due to repeatedly exposure, plant activators do not pose such the problem.

According to the Fungicide Resistance Action Committee (FRAC), synthetic chemicals that have been defined as host defense inducers are categorized as code P1,

such as acibenzolar-S-methyl (ASM; BTH; Bion^{R}; Actigard^{R}); code P2, such as probenazole (PBZ; Oryzemate^{R}); and code P3, such as tiadinil (TDL; V-get^{R}) and isotianil (Stout^{R}) (FRAC, 2018; Valerie Toquin et al. 2012). In addition, all of them appear to act as functional analogs of SA and are regarded as SAR inducing compounds (Bektas and Eulgem 2015). Although various synthetic compounds have been identified as plant activator, few of them have reached commercialization due to their phytotoxic effects (Noutoshi et al. 2012; Oostendorp et al. 2001). Such the various resistance-inducing compounds that have been used for studies on the plant immune system were carefully reviewed by Oostendorp et al. (2001) and Bektas and Eulgem (2015).

3. Saccharin: From an artificial sugar to a plant activator

Saccharin was accidentally discovered in 1879 by an American researcher, Constantin Fahlberg when he forgot to wash his hands after working with chemical reactions in the lab and found that his lunch bread tasted unusually sweet (Cohen 1986). Since then, saccharin has been used as a non-caloric artificial sugar to sweeten countless foodstuffs all over the world for it is approximately 300-450 times sweeter than table sugar. Saccharin is not very soluble in water, and so it is most commonly used in the form of its sodium or calcium salt (Lawrence 2003).

Saccharin is an active metabolite of probenazole

The starting point to address the new role of saccharin as an activator of plant defenses against pathogens would be traced back to the research by Uchiyama et al. (1973), who reported saccharin as one of the active metabolites of probenazole through an analysis of absorption, translocation and metabolism of probenazole in rice plant.

According to Uchiyama et al. (1973), probenazole absorbed by the plants was quickly converted to the metabolite in the following sequence: probenazole → allyl *o*-sulfamoylbenzoate → saccharin → N-*D*-glucopyranosyl saccharin. In water, probenazole decomposed rapidly to allyl *o*-sulfamoylbenzoate which was slowly hydrolyzed to give saccharin (**Fig. 1-1**). Additionally, the plants were likely to uptake an appreciable amount of allyl o-sulfamoylbenzoate, while detectable amount of probenazole in the plant was only trace. From this study results, the authors postulated that allyl *o*-sulfamoylbenzoate and saccharin play an important role in the plant defenses as principal active ingredients, which together contribute to the efficacy of probenazole in the field.

Although saccharin (in the form of sodium salt) exhibited almost the same inhibitory effect against rice blast on potted plants in the greenhouse, their efficiency was inferior to probenazole in the field. Possibly, the relative rates of the compounds through the plant membrane as influenced by the polarity of the compounds might be a contributing factor (Uchiyama et al. 1973).

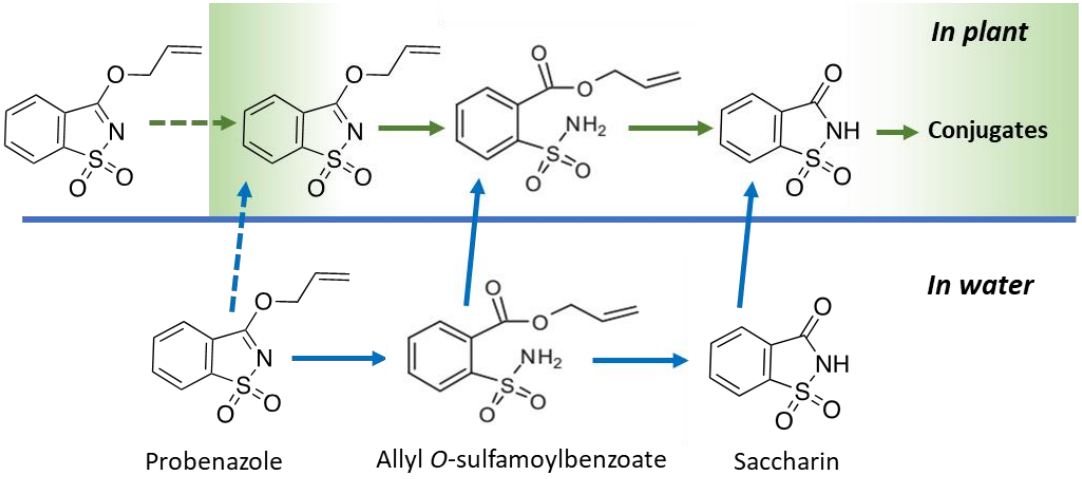

Fig. 1-1. Metabolic fate of probenazole in rice plant. Probenazole is metabolically or non-metabolically converted to allyl *o*-sulfamoylbenzoate, saccharin and N-*D*-glucopyranosyl saccharin (Uchiyama et al. 1973).

Known mode of action of saccharin in plant defenses

So far, there is ample evidence showing SAR-inducing activity of saccharin which is previously reported in both dicots and monocots, mainly against (hemi)biotrophic pathogens (Boyle and Walters, 2005, 2006; Koganezawa et al. 1998; Nakashita et al. 2002; Srivastava et al. 2011; Yoshioka et al. 2001). **Table 1-1** provides a brief overview of relevant studies on the efficacy of saccharin as a plant activator of defenses in different host-pathogen systems.

In Arabidopsis and tobacco, saccharin and PBZ were proved to activate salicylic acid (SA)-mediated signaling pathway which is accompanied by the accumulation of SA and expression of PR genes (Nakashita et al. 2002; Yoshioka et al. 2001). Unlike BTH that activate the SA-signaling at the downstream of SA accumulation, saccharin and PBZ act at the upstream of SA induction. However, these compounds induce accumulation of mRNAs from SA-marker genes (*PR1*, *PR2* and *PR5*) and they all require NPR1 as a common regulator (Lawton et al. 1996; Yoshioka et al. 2001).

Generally, the distinctive role of SA- and JA-signaling and the marker genes associated with systemic acquired resistance (SAR) is not well defined in monocots (De Vleesschauwer et al. 2013; Yoshioka et al. 2001). While saccharin and PBZ were reported to function upstream of salicylic acid (SA) in dicots, little is known about the mechanism of saccharin- and PBZ-induced resistance in monocots. Through an analysis of superoxide generation in rice seedlings, Sekizawa et al. (1987) reported that application of sodium saccharin to rice changed the compatible to incompatible combination of blast fungus races with a rice cultivar. Moreover, a comparison between the enhancing effects of saccharin and N-methylsaccharin on ROS in rice plants also suggests that the location of the biologically active group in the saccharin molecule is situated in its imide group, as methylation of the imide blocks the enhancing ability (Sekizawa et al. 1987). In

addition, Boyle and Walters (2006) showed that saccharin-induced resistance of barley to powdery mildew fungus *Blumeria graminis* might be somewhat related to the increase in peroxidase and cinnamyl alcohol dehydrogenase activities. A study by Iwai et al. (2007) also revealed that SA is involved in PBZ-induced resistance as a defense signal in adult rice leaves but not in young leaves.

4. Objectives of this study

In modern agriculture, application of chemical pesticides is one of the main strategies which has been employed to combat crop pathogens. However, this method of crop protection also remains many problems in terms of drug resistance development of pathogens, side effects on non-target organisms, and polluted foods and environment. Chemically induced resistance by plant activators can provide another useful option for practical crop protection, resulting to reduce the amount of toxic pesticides.

To explore the use of candidate compounds acting as plant activators, understanding their efficacy as well as the molecular mechanism underlying defense induction in the laboratory is essentially needed. In the current study, we examine two known compounds saccharin and PBZ in the laboratory conditions with the objectives as follows:

1. To further elucidate the mode of action and the effectiveness of saccharin and its parental compound PBZ in the model dicotyledonous plant *Arabidopsis thaliana* against bacteria *Pseudomonas syringae* pv. *tomato* DC3000 and *Pectobacterium carotovorum* subsp. c*arotovorum* and fungi *Botrytis cinerea* and *Colletotrichum higginsianum*.

2. To examine the efficacy of saccharin and PBZ in resistance induction against the powdery mildew fungus *Blumeria graminis* f. sp. *tritici* in the monocotyledonous crop *Triticum aestivum* (bread wheat) and to analyze expression profiles of wheat resistance-associated genes affected by both compounds.

Table 1-1 Review on other relevant studies on saccharin as a plant activator of defense responses

Authors et al., year	Plants	Pathogens/Elicitors	Pathogen types	Application methods	Doses	Defense responses
Sekizawa et al., 1987	Rice	*Pyricularia oryzae*	Hemi biotroph	Drenching; Foliar spotting	20-30 ppm (~ 0.1 mM)	Superoxide generation
Hiroki et al., 1998	Tobacco	*Tobacco mosaic virus*	Biotroph	Drenching	0.5 g/ pot	-
Siegrist et al., 1998	Tobacco	*Tobacco mosaic virus*	Biotroph	Drenching	3 mM (30 ml/pot)	-
	Cucumber	*Colletotrichum lagenarium*	Hemi biotroph	Drenching	1 mM (30 ml/pot)	-
	Bean	*Uromyces appendiculatus*	Biotroph	Drenching	3 mM (30 ml/pot)	-
	Parsley cell culture	Elicitor from the cell wall of fungus *Phytophthora sojae*	-	Liquid medium for cell culture	1 mM	Coumarin accumulation
Yoshioka et al., 2001	Arabidopsis	*Pto* DC3000	Hemi biotroph	Spraying	2 mM	Upregulation of *PR1*, *PR2*, *PR5*; SA production
		Peronospora parasitica	Biotroph	Spraying	0.2 mM	-
Nakashita et al., 2002	Tobacco	*Tobacco mosaic virus*	Biotroph	Spraying	2 mM	Upregulation of *PR1*, *PR2*, *PR5*; SA production
		P. syringae pv. tabaci,	Hemi biotroph	Spraying	2 mM	
		Oidium sp.	Biotroph	Drenching	2-5 mg/pot	
Yasuda et al., 2003	Arabidopsis	*Pto* DC3000	Hemi biotroph	Drenching	0.5 mg/pot	Upregulation of *PR1*, *PR2*, *PR5*; SA production
				Spraying	2 mM	
Boyle & Walters, 2005	Broad bean	*Uromyces viciae fabae*	Biotroph	Drenching	3 mM (200 ml/pot)	-
				Foliar brushing	3 mM	
Boyle & Walters, 2006	Barley	*Blumeria graminis* f. sp. *hordei*	Biotroph	Drenching	3 mM (30 ml/pot)	Increased activity of enzymes PAL, POX, CAD
				Foliar brushing	3 mM	-
Yasuda et al., 2008	Arabidopsis	*Pto* DC3000	Hemi biotroph	Drenching	1mg/pot	*PR1* upregulation; SA production
				Spraying	2 mM	
Pratibha et al., 2011	Soybean	*Phakopsora pachyrhizi*	Biotroph	Drenching,	3 mM (30 ml/pot)	-
				Foliar	3 mM	
Walter & Paterson, 2012	Barley	*Rhynchosporium commune*	Hemi biotroph	Spraying	1 mM	Transgenerational resistance
Kusajima et al., 2017	Tomato	*Pto* DC3000	Hemi biotroph	Drenching	5 mg/pot	Upregulation of *PR1*, *PR2*, *PR5*; SA production

Chapter 2

Mode of action of saccharin in Arabidopsis plant

Modified from: Antagonism between SA- and JA- signaling conditioned by saccharin in *Arabidopsis thaliana* renders resistance to a specific pathogen

Full-length article

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Abstract

Saccharin is one of the metabolites of probenazole (PBZ) in plants and acts as a plant activator. Our study of the mechanism underlying saccharin-induced systemic acquired resistance in *Arabidopsis thaliana* suggests an antagonistic interaction between salicylic acid (SA)- and jasmonic acid (JA)-signaling as revealed through gene expression analyses. In wild-type plants (Col-0) exposed to saccharin over a time course, there was a consistent increase in callose deposition and in expression of SA marker genes, *PR1* and *PR2*, which coincided with a decrease in expression of JA marker genes such as *VSP2*, *LOX2* and *PDF1.2*. Actually, pretreatment of Col-0 with saccharin or PBZ conferred resistance to *Pseudomonas syringae* pv. *tomato* DC3000, but not to *Pectobacterium carotovorum* subsp. *carotovorum*, *Botrytis cinerea,* and *Colletotrichum higginsianum*. Enhanced expression of SA- and JA-marker genes as well as the augmented deposition of callose, were evidently observed when challenged by virulent DC3000 in saccharin-pretreated plants. Consistently, pretreatment of saccharin and PBZ with SA- and JA-defective mutants showed diminished resistance in NahG and *npr1* mutant, but not in *jar1* mutant plants, suggesting that saccharin and PBZ induce resistance in Arabidopsis against *Pto* DC3000 mainly via activation of SA-signaling leading to suppression of JA/ET- signaling and *vice versa*. Collectively, an antagonism between SA- and JA-signaling conditioned by saccharin renders resistance to a specific pathogen in Arabidopsis.

Keywords: Induced resistance, Jasmonic acid (JA), Saccharin, Salicylic acid (SA), Signaling, Probenazole (PBZ).

1. Introduction

Throughout the process of co-evolution and interaction, both the plants and pathogens develop their own strategic mechanisms to overcome each other for their benefits. Plants rely on a sophisticated immune system to weaken or terminate pathogen attacks, which comprises of PAMP-triggered immunity (PTI) as the first branch of immune responses and effector-triggered immunity (ETI) as the second branch (Jones and Dangl 2006; Zipfel 2009). During PTI and ETI, a variety of immune responses are triggered including hypersensitive response (HR) (Coll et al. 2011), generation of reactive oxygen species (ROS) (Bindschedler et al. 2006; Torres 2010), production of anti-microbial phytoalexins (Schuhegger et al. 2006), deposition of callose (Ellinger and Voigt 2014) and transcriptional re-programming to activate defense-related genes (Cecchini et al. 2015). Both ETI and PTI are known to utilize common phytohormone signaling pathways including salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Glazebrook 2005; Pieterse et al. 2012; Yi et al. 2014). While SA- and JA-signaling acts antagonistically in most cases of defense activation, JA- and ET-signaling appears to function synergistically (Glazebrook 2005; Pieterse et al. 2012). The outcomes of antagonistic cross-talk between SA- and JA/ET-signaling often leads to the host resistance against a certain pathogen group or a distinct pathogen with a particular parasitic habit (e.g., biotrophs, hemibiotrophs and necrotrophs) (Bostock 2005). In general, effective defenses against biotrophic pathogens largely rely on activation of defense responses regulated by SA-dependent pathway, whereas those against necrotrophic pathogens are mainly associated with JA-mediated signaling (Glazebrook 2005; Thomma et al. 1998).

Plant immune responses can be evoked by a various source of stimuli such as pathogen attacks, application of biocontrol agents, and natural or synthetic compounds (Beckers and Conrath 2007). Although various synthetic compounds have been

discovered as plant activator, few of them have reached commercialization due to their phytotoxic effects (Noutoshi et al. 2012; Oostendorp et al. 2001). One of the most effective and commercially available plant activators is probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide; PBZ), a synthetic compound which has been used for over four decades for controlling rice blast diseases (Watanabe et al. 1977). Some of the immune responses like the oxidative burst (Iwata et al. 1980; Sekizawa et al. 1985) and increased accumulation of SA and PR proteins (Iwai et al. 2007) are evidently enhanced by PBZ. PBZ converts into saccharin metabolically in plants or physically in the aqueous medium; therefore, it is tempting to postulate that saccharin plays an important role as one of the principal active ingredients (Boyle and Walters 2005; Oostendorp et al. 2001; Uchiyama et al. 1973).

The application of saccharin changes the compatible to incompatible interaction of blast fungus races and a rice cultivar, possibly owing to an augmented superoxide generation (Sekizawa et al. 1987). Saccharin, but not probenazole activated cultured parsley cells for an enhanced elicitor-mediated furanocoumarin accumulation in response to fungal cell wall elicitors (Siegrist et al. 1998). Both PBZ and saccharin activate expression of PR genes and development of systemic acquired resistance (SAR) in tobacco and Arabidopsis plants, upstream of SA accumulation (Nakashita et al. 2002; Yasuda et al. 2008; Yoshioka et al. 2001). So far, there is ample evidence that shows the potential of saccharin to activate SAR in both mono- and dicots mostly against (hemi)biotrophic pathogens including fungi, bacteria and viruses. For example, treatment with saccharin enhanced resistance in Arabidopsis against *Peronospora parasitica*, in tobacco against *Oidium* sp. and tobacco mosaic virus (TMV) (Koganezawa et al. 1998; Nakashita et al 2002), in broad bean against *Uromyces viciae-fabae* (Boyle and Walters 2005), in barley against *Blumeria graminis* f. sp. *hordei* (Boyle and Walters 2006), and in

soybean against *Phakopsora pachyrhizi* (Srivastava et al. 2011). Interestingly, when parental barleys are treated with saccharin, the state of induced resistance is transmitted to the progeny and helps to resist against hemibiotrophic fungus *Rhynchosporium commune* (Walters and Paterson 2012).

Even though the mode of action of saccharin for the induction of plant immune responses has been characterized to some extent, it remains to be additionally elucidated. The purpose of this study is thus to further clarify the molecular mechanism of plants underlying saccharin-induced immune responses, and to point out again the cautions on the use of saccharin or similar chemical(s) for plant disease control. In this study, we investigated the effect of saccharin and its parental compound, PBZ, on immune responses of Arabidopsis plants before and during pathogen infection. We report that saccharin and PBZ induce resistance to a hemibiotrophic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000; DC3000), but not to a necrotrophic bacterium, *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), a necrotrophic fungus, *Botrytis cinerea* (*Bc*), and a hemibiotrophic fungus, *Collectotrichum higgisianum* (*Ch*). Rapid activation of SA-regulated genes and the concomitant suppression of JA-regulated genes by saccharin and PBZ may be the contributing event which accounts for the induced resistance to *Pto* DC3000 and the increased susceptibility to *Bc*. In addition, we observed a diminished resistance to *Pto* DC3000 in NahG and *npr1* mutant, but not in *jar1* mutant plants pretreated with saccharin and PBZ. Our present results together suggest that saccharin- and PBZ-treated Arabidopsis plants triggered resistance against *Pto* DC3000 probably via activation of SA-signaling, resulting in suppression of JA/ET-signaling and *vice versa*. Combined with other findings that both saccharin and PBZ upregulated expression of other defense-related genes and accelerated deposition of callose in Arabidopsis plants, we demonstrate an antagonistic interaction between SA- and JA-

signaling conditioned by saccharin renders resistance to a specific pathogen in Arabidopsis.

2. Materials and methods

Plants

Seeds of *Arabidopsis thaliana* were sown on water-swelled Jiffy-7 peat pellets (AS Jiffy Products, Oslo, Norway) and grown for 2 weeks, and the seedlings were transferred to small plastic pots containing Supermix-A soil (Sakata Seed Co., Ltd., Yokohama, Japan) mixed with vermiculite in ratio 1:1. Seedlings were grown in the growth room at 22°C, with a 10 h light/14 h dark cycle at 11.8 $W \cdot m^{-2}$ for additional 3-4 weeks before use. The mutants, salicylic acid (SA)-deficient *NahG* transgenic line (Delaney et al. 1994), *npr1-2* (Cao et al. 1997) and *jar1-1* mutant impaired in JA-signaling (Staswick and Tiryaki 2004) were used to evaluate the role of saccharin in induced resistance in Arabidopsis, with a particular focus on the antagonism between SA- and JA-mediated signaling pathways. The NahG transgenic plant and mutants *jar1* and *npr1-2* were in the *A. thaliana* Col-0 background.

Pathogens

Pseudomonas syringae pv. *tomato* DC3000*NalR* (*Pto* DC3000) was cultivated on/in King's B (KB) medium at 28°C as described previously (Ishiga and Ichinose 2016). *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) strain Pc1 were cultivated at 28°C on/in Luria-Bertani (LB) medium (Haque et al. 2015). Agar at 1.5% (w/v) was added when cultivated on solid medium. Antibiotic nalidixic acid was used for selection at a final concentration of 50 μg/ml. For use in preparation of the inoculum, bacteria were plated and grown on solid medium, then scraped off the plate. Bacteria were then

suspended and diluted in distilled water to appropriate concentrations. Fungal pathogens, *Collectotrichum higginsianum* (*Ch*) and *Botrytis cinerea* (*Bc*) were cultured on potato dextrose agar (PDA) (Becton, Dickinson and Company, USA) at 23°C in the dark (with blue light for *Bc*). Conidia of cultures grown for 10 days were suspended in distilled water for *Ch* or in 1% Maltose Sabouroud Broth for *Bc* by gentle scraping and were filtered through sterile cheesecloth to remove hyphae.

Chemicals

Sodium saccharin dihydrate (saccharin) and probenazole (PBZ) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Saccharin was dissolved in distilled water. PBZ was dissolved in dimethyl sulfoxide (DMSO) to give a stock concentration of 20 mM. The stock solution was diluted with distilled water to give a final concentration of 0.1 mM, thus containing 0.5% (v/v) DMSO. PBZ was used as a positive control that induces SA-dependent resistance throughout experiments.

Phytotoxicity *in planta* and anti-bacterial effect *in vitro* of saccharin

To assess saccharin phytotoxicity, 6-week-old Col-0 plants were treated with water (control) or saccharin solution ranging from 0.1-10 mM by foliar spraying with a hand-pump spray bottle (until dews evenly form on leaf surface) or by drenching 10 ml of test solution into soil. The plant growth (leaf size, shape, and color) after treatment were carefully observed (supplementary **Fig. S2-1**).

To assess the direct antibacterial effect of saccharin, *Pto* DC3000 was grown at 28°C on KB liquid medium with shaking to reach $OD_{600} = 1$ (or 5×10^8 colony-forming units (cfu)/ml). Ten microliters of bacterial suspension were diluted with 3 ml of fresh KB medium, then 3 µl of the diluted bacterium was placed into 3 ml of KB medium

containing water or saccharin of 0.1, 1 or 10 mM at final concentrations. The OD_{600} value was measured in a course of 5 days using Biochrom WPA Colorimeter CO7500 (Biochrom Ltd., Cambridge, UK). Similarly, bacteria were gently scraped off from plate culture, suspended in distilled water and diluted to $OD_{600} = 10^{-5}$ and 10^{-4} (5×10 cfu/ml and 5×10^2 cfu/ml). Droplets of 10 µl diluted bacterium were plated onto KB agar plate containing saccharin at indicated concentrations. The number of bacteria was determined by counting the colonies (see **Appendix 4** for further details). Three replicates were made for each treatment (supplementary **Fig. S2-2**).

Chemical treatments and inoculation bioassays

Arabidopsis plants were pretreated for 2 days with/ without either saccharin or PBZ, then challenged with bacterial or fungal pathogens on 3 fully expanded, rosette leaves per plant. After inoculation, plants were placed under high humidity in a plastic container covered with a clear lid. *Pto* DC3000 infection assay was done as described previously by Katagiri et al. (2002). Briefly, bacterial suspension ($OD_{600} = 0.0001$ or 5×10^4 cfu/ml) was infiltrated from the abaxial side into a sample leaf using a 1 ml syringe without a needle. Leaves were considered diseased when showing chlorosis and necrosis (Katagiri et al. 2002). For counting bacterial proliferation, three infected leaves were harvested from one plant among three biological replicates at days post-inoculation (dpi), and two leaf discs were obtained from one leaf using a Biopsy Punch (0.5 cm in diameter; KAI Medical, Seki City, Gifu Prefecture, Japan). A total of 6 leaf discs from one plant was combined to make one biological sample, then crushed by zirconia beads in distilled water using a Micro Smash M-100 (Tomy Seiko Co., Ltd., Tokyo, Japan). Appropriate dilutions were made and plated on a fresh KB agar plate. The number of bacteria was determined by counting colonies (see **Appendix 3** for further details).

For the *Pcc* infection assay, selected leaves were punctured with a pipette tip and inoculated with 10 µl liquid culture of *Pcc* (OD_{600} = 0.01 or 4 × 10^6 cfu/ml) at injured sites according to Hsiao et al. (2017). Photos of bacterial soft rot symptom on infected leaves were taken at 1 and 2 dpi, and lesion area was measured at 1 dpi. For *Ch* and *Bc* infection assay, 10 µl of spore suspension (2 × 10^5 spores/ml) were spotted onto the adaxial side of leaves. Infection by *Ch* was observed microscopically at 3 dpi for appressoria and infection hyphae formation. Symptoms and lesion area on leaves by *Ch* or *Bc* were assessed at 4 to 5 dpi. Lesion area caused by tested pathogens was calculated using the ImageJ software (https://imagej.net).

Analysis of gene expression by quantitative reverse transcriptase-polymerase (qRT-PCR)

For the assessment of induced gene expression over the time course, wild-type plants were treated with saccharin or PBZ for 3, 6, 12, 24, 48 and 72 hours prior to RNA extraction. The water-treated plants were used as a control. For the assessment of gene expression during bacterial infection, plants were pretreated with either saccharin or PBZ for 2 days and subsequently inoculated with *Pto* DC3000 (OD_{600} = 0.0001) for 12 and 24 hours before RNA extraction. The water-treated and uninoculated plants were used as a control.

The plant material was fixed in liquid nitrogen and then crushed using zirconia beads in a Micro Smash M-100 (Tomy Seiko Co., Ltd). RNA was extracted using a Total Plant RNA Miniprep Purification Kit (GMbiolab Co, Ltd. Taichung City, Taiwan) according to the manufacturer's protocol. Sample quality was assessed using gel electrophoresis and measured using the spectrophotometer NanoDrop 1000UV/Vis (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (0.5 µg of each sample) was

reverse-transcribed at 42°C to synthesize cDNA in a reaction mixture (10 µl) containing 10 U of RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTPs, 0.4 µg of oligo(dT)$_{12\text{-}18}$ and 2.5 U of AMV reverse transcriptase (Takara Bio, Otsu, Japan), followed by heat inactivation at 60°C for 10 min. cDNA was amplified in 10 µl of reaction volume containing 5 µl of KAPA SYBR FAST Universal qPCR Master Mix (KAPA Biosystems, Boston, MA, USA) and 100 nM of each gene-specific primer set, listed in Table 2-1.

Table 2-1 Summary information for primer sequences used for qRT-PCR

Gene	Accession	Forward (5′ > 3′)	Reverse (5′ > 3′)	Size (bp)
PR1	At2g14610	CCCACAAGATTATCTAAGGGTT	CCCTCTCGTCCCACTGCAT	77
PR2	At3g57260	CTTCAACCACACAGCTGGA	GCATTCGCTGGATGTTTTGT	113
LOX2 *	At3g45140	TTGCTCGCCAGACACTTGC	GGGATCACCATAAACGGCC	101
VSP2 *	At5g24770	GTTAGGGACCGGAGCATCAA	AACGGTCACTGAGTATGATGGGT	101
PDF1.2	At5g44420	CCATCATCACCCTTATCTTCGC	TGTCCCACTTGGCTTCTCG	101
PR3	At3g12500	GGCAAACGCTACTACGGAAG	AAGCGATCACTGCGTCGTT	130
ALD1 **	At2g13810	GTGCAAGATCCTACCTTCCCGGC	CGGTCCTTGGGGTCATAGCCAGA	160
PAD3	At3g26830	AATCTCGCCGAAATGTATGG	GCATCAGACTCCACTCGTCA	211
PRX34	At3g49120	AGTTAAGGTCGGACCCTCGT	GAGCTGCAATGGTGAGCATA	243
FRK1	At2g19190	ACGGGCATAGTTCCACAAAG	CGTCAAAAGAACGACGATGA	177
EF1-α	At5g60390	CATCATTTGGCACCCTTCTT	TGGTGACGCTGGTATGGTTA	201

** Fujimoto et al. (2015), ** Navarova et al. (2012)*

The quantitative PCR reactions were performed on a Shimadzu GVP-9600 Gene Detection System (Shimadzu, Kyoto, Japan) using the following amplification program: an initial incubation cycle of 95°C for 3 minutes, 50 cycles at 95°C for 3 seconds, 60°C for 30 seconds, 1 ending cycle at 72°C for 2 minutes and a final melt curve analysis. The resulting qRT-PCR data, cycle threshold (Ct) values, were used to calculate the relative mRNA abundance according to the ΔΔ cycle threshold method (Livak and Schmittgen 2001). The values were normalized to those of the reference gene *EF1-α* and expressed

relative to the water-treated control sample. The detailed procedure for analysis of gene expression is described in **Appendix 6**.

Callose detection by aniline blue staining

For the assessment of saccharin-induced callose deposition over the time course, Col-0 plants were treated with/ without saccharin for 3, 6, 12, 24, 48 and 72 h prior to leaf harvest for aniline staining. The water-treated plants were used as a control. For measurement of callose deposition during bacterial infection, plants were pretreated with either saccharin or PBZ for 2 days and subsequently infected with *Pto* DC3000 (OD_{600} = 0.0001) for 24 and 48 h prior to aniline staining. *In situ* detection of callose was performed as described previously by Survila et al. (2016) with minor modifications. Briefly, leaves were harvested and placed in sterile 6-well plates, then washed with acetic acid:ethanol (1:3, by vol.) at room temperature for 6 h to destain chlorophyll from the leaves, followed by 70% (v/v) ethanol for 2 hours, and finally water for 2 h twice. Leaves were stained with 0.5% (w/v) aniline blue (Nacarai Tesque, Tokyo, Japan) in 0.1 M potassium phosphate buffer (pH 9) for at least 1 hour in the dark, transferred to 50% glycerol (Sigma) and examined under Olympus IX70 inverted fluorescent microscope (Olympus, Tokyo, Japan) equipped with excitation filter 400-440 nm and emission filter 475 nm. The number of callose deposits (per 1 mm^2) was calculated using the ImageJ software (see **Appendix 5** for further details).

Light microscopy

The inoculated leaves were fixed with a mixture of ethanol and acetic acid (24:1, v/v) and decolorized with the same mixture at room temperature overnight and stained with 0.5% (w/v) cotton blue (Nacarai Tesque, Tokyo, Japan) before observation. The samples were

observed with differential interference contrast (DIC) microscopy (Olympus BX60, Olympus) as described previously (Suzuki et al. 2017).

Statistical analysis

Statistical analyses were performed using the KyPlot 5.0 statistic software (KyensLab Inc., Tokyo, Japan) with tools for Student's *t*-test for the two means, Tukey's test and Dunnett's test for the multiple comparisons of means. Statistical significance was considered when $p < 0.05$. Data are presented as mean ± standard deviation (SD). All experiments were repeated at least three times with similar results and a representative result was presented.

3. Results

Expression of defense-related genes by saccharin and PBZ

The effect of saccharin and PBZ on expression of 10 defense-related genes was analyzed in Arabidopsis wild-type plants (**Fig. 2-1**). Expression was assessed for *PR1* and *PR2*, which are markers of SA-signaling; *LOX2* and *VSP2*, which are markers of JA signaling; and *PR3* and *PDF1.2,* which are indicators of JA/ET-signaling (Seo et al. 2016). Other examined genes include *ALD1* (AGD2-Like Defense Response Protein1) which encodes an L-lysine alpha-aminotransferase functioning in the pipecolic acid biosynthetic pathway, where it catalyzes the biochemical conversion of lysine to epsilon-amino-alpha-ketocaproic acid (Navarova et al. 2012; Song et al. 2004); *PAD3* (Phytoalexin deficient 3) which encodes the cytochrome P450 enzyme 71B15 catalyzing the final step in camalexin biosynthesis (Schuhegger et al. 2006); *PRX34* (Peroxidase), which is one of key apoplastic peroxidase genes responsible for ROS generation (Bindschedler et al.

2006; Zhao et al. 2019); and *FRK1* (Flg22-induced receptor-like kinase1) which is a molecular marker for MAMP responses during PTI (Yeh et al. 2015).

Both saccharin and PBZ induced the early accumulation of transcripts of SA-signaling genes (*PR1*, *PR2*) at around 3 and 6 hours post-treatment (hpt). Expression levels of *PR1* and *PR2* consistently increased over time in plants treated with saccharin and PBZ, with peaks at 72 hpt (Fig. 2-1a, b). This result is in agreement with the previous findings by Yoshioka et al. (2001) that PBZ and saccharin activated the expression of SA-signaling genes (*PR1, PR2,* and *PR5*) in Arabidopsis within 6 h and 48 h after treatment. In contrast, expression of JA and JA/ET signaling marker genes was significantly suppressed by saccharin and PBZ within the time course in the cases of *LOX2* and *VSP2* or at the later points in time in the case of *PDF1.2* (**Fig. 2-1c-e**). *PR3* did not seem to respond to both saccharin and PBZ over the time course of treatment (**Fig. 2-1f**). In addition, expression of *ALD1, PAD3, PRX34,* and *FRK1* was significantly upregulated by saccharin and PBZ, with peaks at around 24 - 48 hpt. However, transcript levels of these 4 genes were quickly dropped or even not detected at 72 hpt (**Fig. 2-1g-j**).

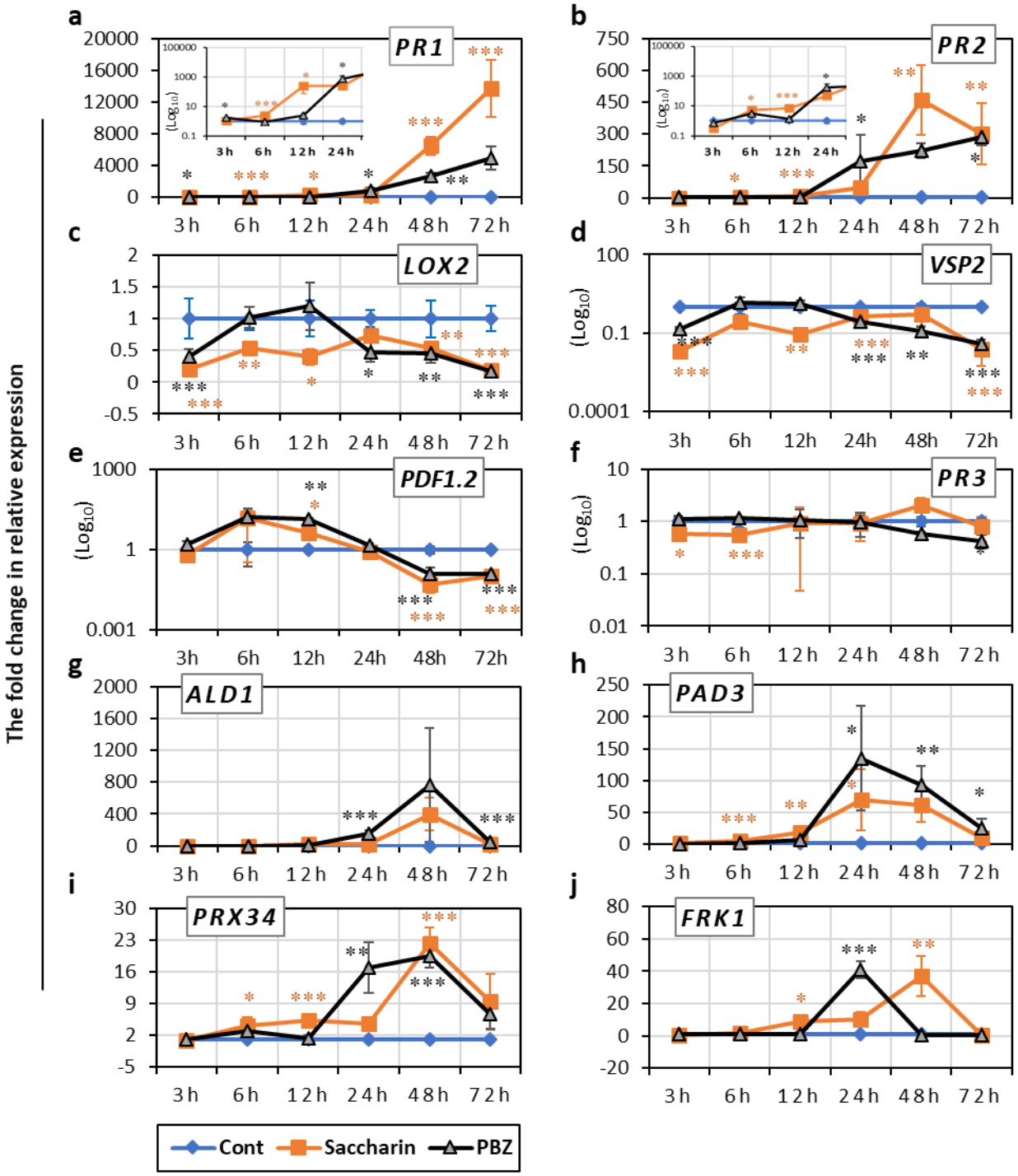

Fig. 2-1 Expression of defense-related genes after chemical treatment. Col-0 plants were sprayed with water (as a control), saccharin (1 mM) or PBZ (0.1 mM), then leaves were harvested at indicated points in time after treatment and subjected to qPCR. Transcriptional levels of SA marker genes (**a, b**), JA-marker genes (**c, d**), JA/ET marker genes (**e, f**) and other defense-related genes (**g-j**) were analyzed. The expression value of genes was normalized using *EF1-α* as an internal standard and expressed relative to average levels in the control (set at 1). Expression level

is plotted on a $\log_{10}$ scale for **a-b** (insets) and **d-f**. Data present the average ± SD from the triplicate reaction in each experiment. Asterisks indicate significant differences from the control at each time point. Dunnett's test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Saccharin and PBZ enhanced resistance to bacterium *Pto* DC3000, but not to *Pcc*

We examined whether the exogenous application of saccharin and PBZ can induce disease resistance to a hemibiotrophic bacterium, *Pto* DC3000, and a necrotrophic bacterium, *Pcc*, in Col-0 plants. As shown in **Fig. 2-2a**, saccharin and PBZ reduced the disease severity caused by *Pto* DC3000, but not by *Pcc* (**Fig. 2-2c**). Three days post-inoculation (dpi), plants pretreated with saccharin or PBZ showed less severe disease symptoms (yellowish) as compared to those pretreated with water (necrosis and water-soaked) (**Fig. 2-2a**). The less severe symptoms were accompanied by the reduced growth of *Pto* DC3000. Bacterial proliferation in leaves pretreated with saccharin and PBZ was significantly reduced at 24 hpi and 48 hpi by approximately 10% and 30%, respectively, as compared with the control (**Fig. 2-2b**). In contrast to the case of *Pto* DC3000, pretreatment of saccharin and PBZ failed to reduce disease severity caused by bacterium *Pcc.* Soft rot lesion area was slightly higher in saccharin and PBZ treatment; however, there was no significant difference when compared to the control (**Fig. 2-2c, d**).

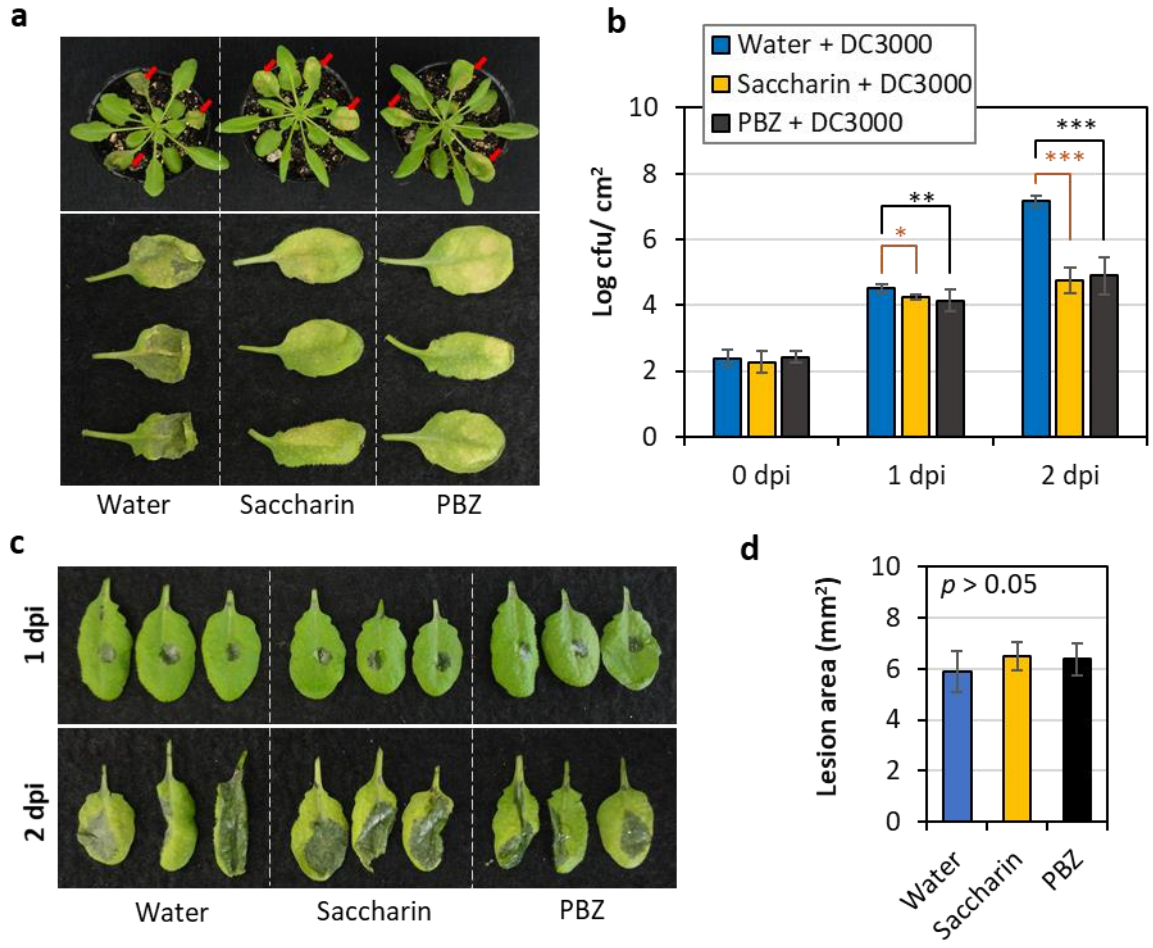

Fig. 2-2 Saccharin conferred resistance in Col-0 against DC3000, but not *Pcc*. Col-0 plants were pretreated for 2 days with water (as a control), saccharin (1 mM), or PBZ (0.1 mM), then three leaves of each plant (red arrows) were inoculated with DC3000 by infiltration (OD_{600} = 0.0001) or with *Pcc* by droplet (OD_{600} = 0.01). **a** Disease symptoms caused by DC3000 at 3 dpi. **b** Leaf discs were harvested at 0, 1 and 2 dpi and the bacterial growth was measured by counting the number of cfu. **c** Disease symptoms caused by *Pcc* at 1 and 2 dpi. **d** The lesion area (mm^2) on leaves were measured at 1 dpi using the software ImageJ. Bars present the mean values (± SD) of colony-forming units (cfu) per cm^2 or lesion area in mm^2. Asterisks indicate significant differences from the control. Dunnett's-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Saccharin and PBZ failed to protect Arabidopsis from fungal pathogens

Given that saccharin and PBZ induced resistance to *Pto* DC3000, but not to *Pcc*, we examined whether the same effect can be observed with fungal pathogens, namely *Ch*, a

hemibiotroph causing anthracnose disease, and *Bc*, a necrotroph causing gray mold. Expectedly, pretreatment with saccharin and PBZ to Col-0 plants failed to reduce disease severity caused by both fungal pathogens (**Fig. 2-3a-d**). Ahead of infection, symptoms caused by *Ch* seemed to remain similar between treatments at 5 dpi (**Fig. 2-3c, d**). On the other hand, inoculation with *Bc* caused increased susceptibility accompanied with severer symptoms and higher lesion area in the leaves pretreated with saccharin and PBZ at 4 dpi (**Fig. 2-3a, b**).

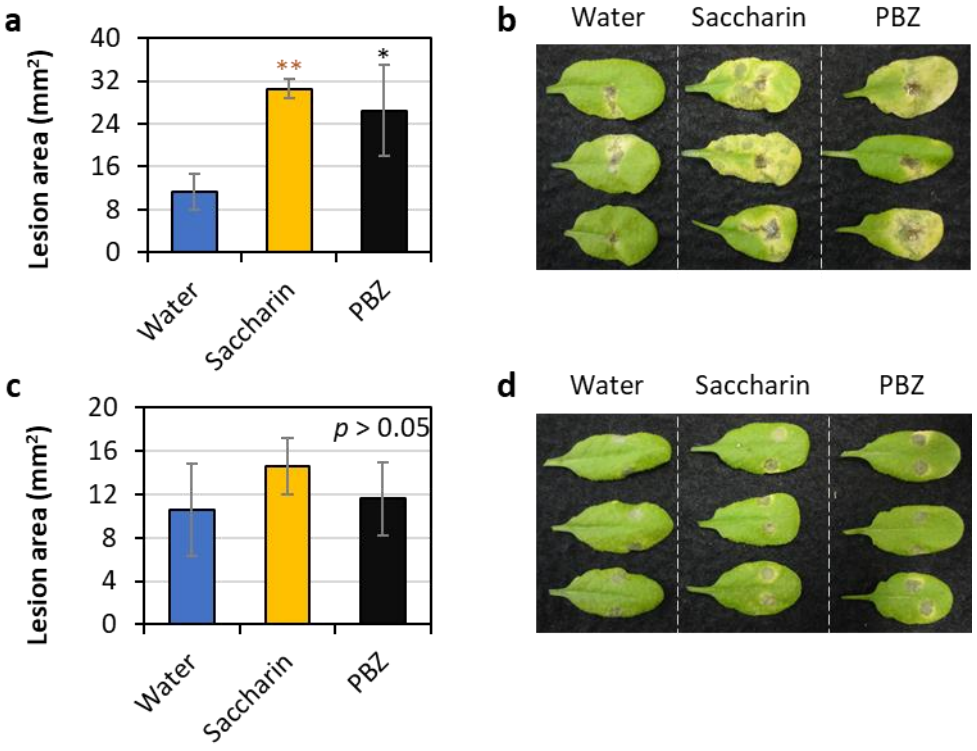

Fig. 2-3 Saccharin and PBZ failed to reduce disease severity by pathogenic fungi *Ch* and *Bc*. Col-0 plants were pretreated for 2 days with water (as a control), saccharin (1 mM) or PBZ (0.1 mM), then inoculated with *Ch* or *Bc* at concentration of 5×10^5 spores/ml **a, b** Lesion area (4 dpi) and disease symptoms (5 dpi) caused by *Bc*. **c, d** Lesion area and disease symptoms caused by *Ch* at 5 dpi. The lesion area (mm^2) on leaves was calculated by the software ImageJ. Bars present the mean values (± SD) of lesion area in mm^2. Asterisks indicate significant differences from the control. Dunnett's-test, $*p < 0.05$, $**p < 0.01$.

Expression of defense-related genes during bacterial infection

In order to gain more insight into the mechanism of SAR induced by saccharin and PBZ, we examined expression of the same set of genes during *Pto* DC3000 infection in Col-0 plants with or without two compounds (**Fig. 2-4**). Expression of SA-signaling marker genes (*PR1, PR2*) was typically induced by *Pto* DC3000 within 12 and 24 hpi in water-pretreated plants, but was more strongly enhanced in plants pretreated with saccharin or PBZ (**Fig. 2-4a, b**). Expression of JA-responsive genes (*VSP2, LOX2*) and a JA/ET-responsive gene (*PDF1.2*), on the other hand, was found to be significantly enhanced at 24 hpi by *Pto* DC3000 in plants pretreated with water, but it was suppressed in plants pretreated with saccharin or PBZ (**Fig. 2-4c-e**). Our result of activation of JA-responsive genes by *Pto* DC3000 is consistent with the finding of Brooks et al. (2005) that *Pto* DC3000 strongly induced expression of *LOX2* and *PDF1.2* at 24 hpi through secretion of phytotoxin coronatine.

Pretreatment with saccharin and PBZ to Col-0 plants also enhanced expression of other defense-related genes (**Fig. 2-4g-j**). Marked up-regulated expression was observed at 12 and 24 hpi for *ALD1* and *PRX34* and at 12 hpi for *PAD3* and *FRK1* in plants pretreated with saccharin and PBZ, suggesting the involvement of these genes in induced resistance to *Pto* DC3000. At 24 hpi, the enhanced expression of *PAD3* and *FRK1* faded or was abolished since there were no significant differences in expression between the treatments (**Fig. 2-4h, i**).

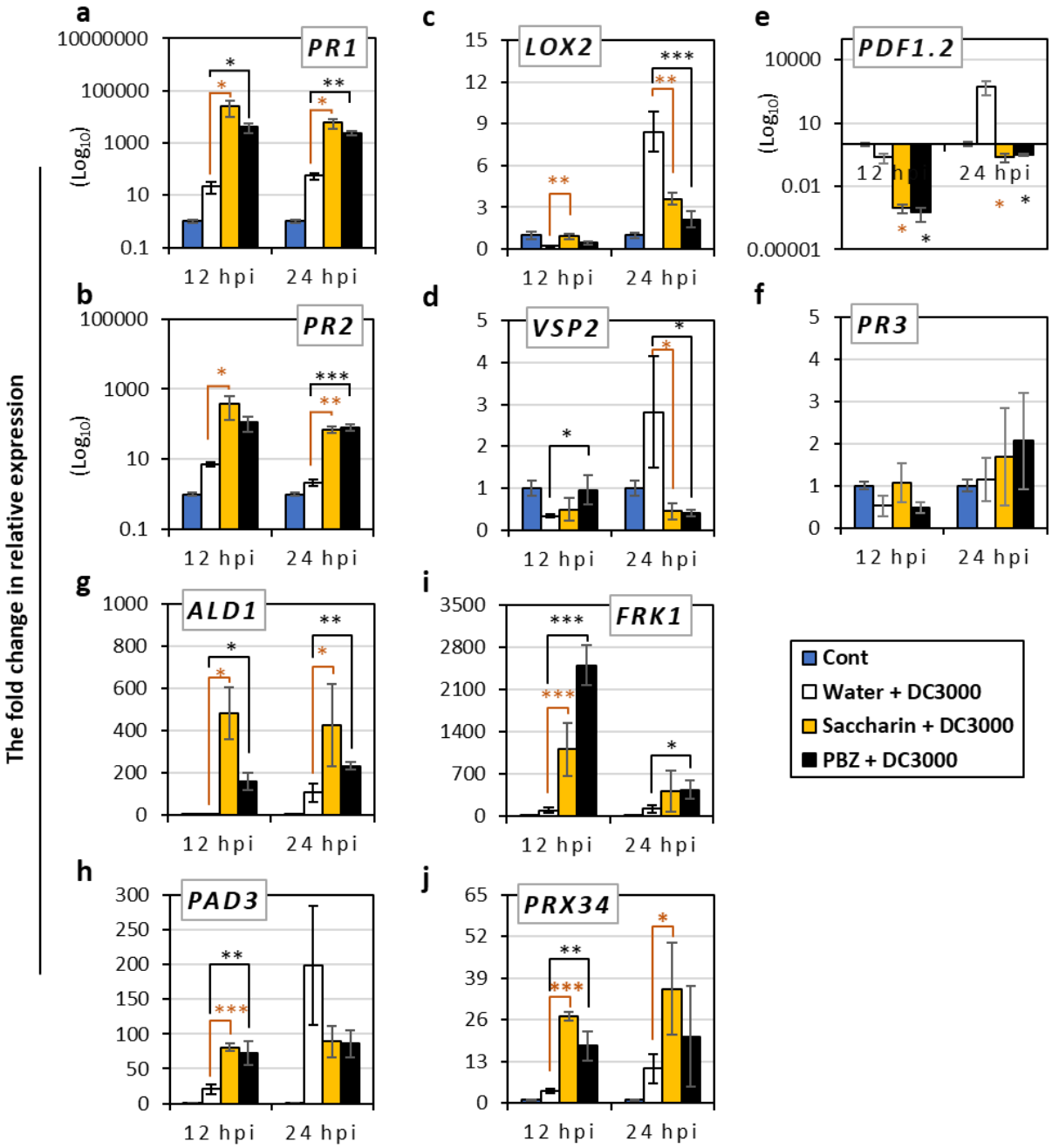

Fig. 2-4 Expression of defense-related genes during DC3000 infection. Col-0 plants were pretreated with water (as a control), saccharin (1 mM) or PBZ (0.1 mM) for 2 days, then inoculated with DC3000 by infiltration (OD_{600} = 0.0001). Leaves were harvested at 12, 24 hpi and subjected to qPCR. The water-treated and uninoculated plants were used as a control. The expression value of genes was normalized using *EF1-α* as an internal standard and expressed relative to average levels in the control (set at 1). Expression level is plotted on a $\log_{10}$ scale for *PR1*, *PR2* and *PDF1.2*. Data present the average ± SD from the triplicate reaction in each

experiment. Asterisks indicate significant differences from water-treated and inoculated plants. Student's *t*-test,*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Absence of SA compromised saccharin- and PBZ-induced resistance

To further confirm which phytohormone is involved in SAR induced by saccharin and PBZ, Arabidopsis mutants impaired in SA- or JA- signaling pathways were tested for the ability to induce resistance. Saccharin and PBZ pretreatment did not protect transgenic NahG and *npr1* plants against *Pto* DC3000, but enhanced resistance in both Col-0 and *jar1* plants (**Fig. 2-5**). In contrast, a significant reduction in bacterial proliferation at 2 dpi in Col-0 and *jar1* plants was observed when pretreated with either saccharin or PBZ as compared to the plants pretreated with water. Consistently, NahG and *npr1* plants allowed a higher number of bacterial proliferation, eventually causing severe disease symptoms (**Fig. 2-5a, b**).

Saccharin and PBZ induced callose deposition in Arabidopsis

Callose is a β-1,3-glucan cell wall polymer with some 1,6-branches which is deposited between the plasma membrane and the cell wall to act as a physical barrier to stop or slow invading pathogens (Voigt 2014; Xin and He 2013). Here, we observed cytologically that callose deposition in plants treated with saccharin and PBZ was detectable as early as 24 h after treatment and peaked at around 48-72 hpt (**Fig. 2-6a, b**). In addition, callose deposition was also increased upon infection by *Pto* DC3000 in control-treated plants, but was further augmented in plants pretreated with saccharin or PBZ (**Fig. 2-6c, d**).

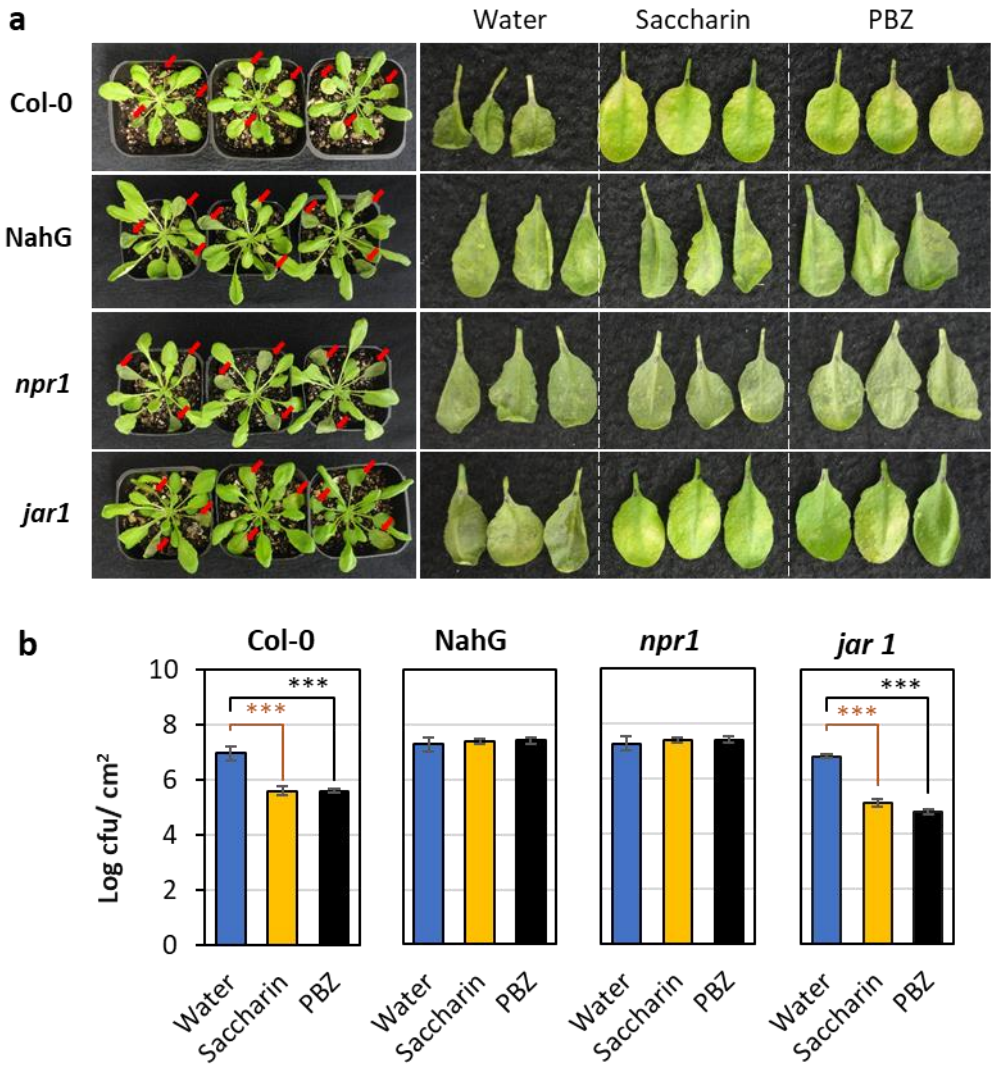

Fig. 2-5 Saccharin induced resistance in *jar1,* but not in NahG and *npr1 plants*. Plants were pretreated for 2 days with water (as a control), saccharin (1 mM) or PBZ (0.1 mM), then three leaves of each plant (red arrows) were challenged by DC3000 infiltration (OD_{600} = 0.0001). **a** Disease symptoms of representative plants were photted at 3 dpi. **b** Leaf discs were harvested at 2 dpi and assessed for bacterial growth by counting the number of colonies. Bars present the mean values (± SD) of colony-forming units (cfu) per cm^2. Asterisks indicate significant differences from the control. Dunnett's-test, ***$p < 0.001$.

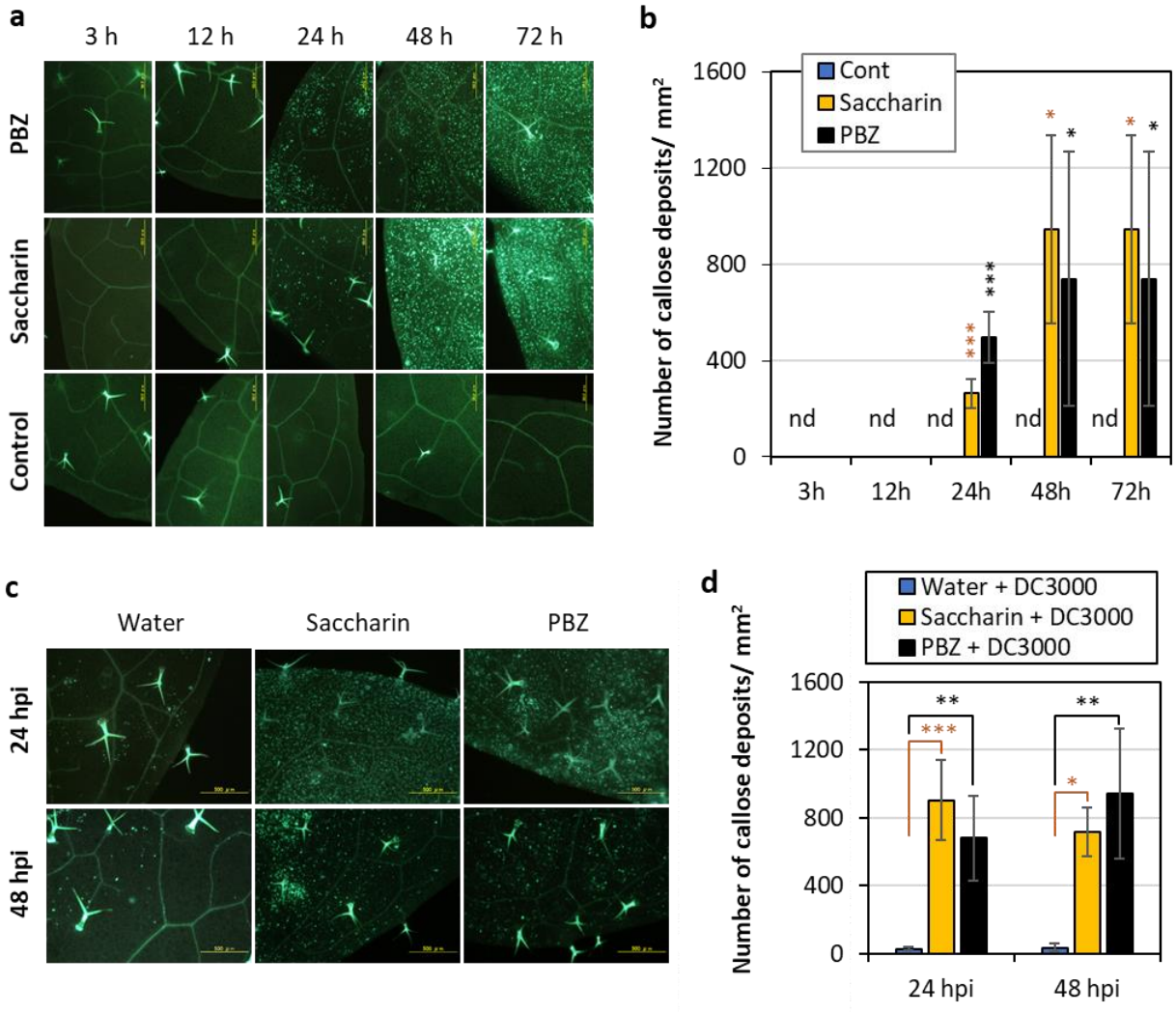

Fig. 2-6 Callose deposition induced by saccharin and PBZ. Col-0 plants were sprayed with water (as a control), saccharin (1 mM) or PBZ (0.1 mM), then leaves were harvested at indicated points in time after treatment (**a**, **b**), or 2-day-pretreated leaves were challenged by DC3000 infiltration (OD_{600} = 0.0001) and then harvested at 24 and 48 hpi (**c**, **d**). Harvested leaves were stained with aniline blue and examined for callose deposition. **a**, **c** Visualized callose deposits under a fluorescent microscope. **b**, **d** Number of callose deposits was calculated by the software ImageJ. Bars present the mean values (± SD) of the number of callose deposits per 1 mm^2. Asterisks indicate significant differences from the control at each time point. Dunnett's-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar, 500 μm. nd, not detectable.

4. Discussion

To be considered a true plant activator or elicitor in crop protection, a candidate compound(s) must fulfill the initial condition that very little or no antimicrobial activity is to be displayed either by the compound itself or by its possible metabolites (Gozzo 2003). It is also expected that weak or no phytotoxic side effects in plants are exhibited because the phytotoxicity of plant activators limits their use as agrochemicals (Bektas and Eulgem 2015). Generally, plant activators directly activate defense responses, resulting in fitness cost where plants pay the price of inhibitory growth and decreased yield. This process occurs naturally in plants and is referred to as growth-defense tradeoffs (Huot et al. 2014). Therefore, it is essential to identify optimal applied doses of plant activators for achieving a balance between the growth and defense.

Through the tests on phytotoxicity of saccharin, we have shown that saccharin exhibits no or moderate negative effects on the growth of Arabidopsis plants, irrespective of application methods when applied at low concentrations (up to 1 mM) (**Fig. S2-1**). Previous observations revealed that saccharin at 1 mM strongly enhanced coumarin accumulation in parsley cell without exhibiting phytotoxicity, but weakly did at 0.1 mM, and killed the cells at 10 mM (Siegrist et al. 1998). In addition, it was reported that 1-3 mM saccharin was effective to induce SAR in tobacco, cucumber, bean, and barley (Siegrist et al. 1998; Boyle and Walters 2005; 2006). However, 3 mM saccharin occasionally caused severe wilt in bean (Boyle and Walters 2005). We also confirmed that saccharin had no direct toxic effect on the growth of hemibiotrophic bacterium *Pto* DC3000 (**Fig. S2-2**) and other fungal pathogens *C. higginsianum*, *A. brassicicola* and *B. cinerea* (data not shown). On the basis of these results, we used saccharin at 1 mM for most experiments since saccharin at 0.1 mM could not provide consistent results of defense induction in our study (data not shown). PBZ, a well-characterized SAR-inducer,

was used at 0.1 mM based on previous studies in rice plant by Iwata et al. (1980), Sekizawa el al. (1985) and Uchiyama et al. (1973). Although saccharin is proved to be one of the active metabolites of PBZ in the plants, it is likely that saccharin requires higher concentration than PBZ when applied exogenously to perform comparable efficacy on defense induction of Arabidopsis in the present study. Since there has been no study on the rate of plant uptake of PBZ and saccharin, we accept the explanation by Uchiyama et al. 1973 that the polarity of the compounds might affect the rate of plant uptake, resulting in differential efficacy.

Previous studies have reported that saccharin induced SAR in both dicots and monocots mostly against (hemi)biotrophic pathogens (Yoshioka et al. 2001; Nakashita et al. 2002; Boyle and Walters 2005; 2006). In this study, we demonstrated that the exogenous application of saccharin and PBZ induced resistance in Arabidopsis wild-type plants to hemibiotrophic bacterium *Pto* DC3000 (**Fig. 2-2a, b**), but not to necrotrophic bacterium *Pcc*, necrotrophic fungus *Bc*, and hemibiotrophic fungus *Ch* (**Figs. 2-2c, d; 2-3a-d**). In the case of fungus *Bc*, increased susceptibility was observed in saccharin and PBZ pretreatment (**Fig. 2-3a, b**). Moreover, our study clearly indicated that saccharin and PBZ altered expression profile of multiple defense-related genes and induced callose deposition, which is presumably related to the conferred resistance (**Figs. 2-1; 2-4; 2-6**). Notably, we observed antagonism between SA- and JA-signaling conditioned by saccharin and PBZ, through contrasting expression profile of SA- and JA-signaling marker genes (**Figs. 2-1; 2-4**). Further analysis using Arabidopsis mutants further confirmed the main role of SA-signaling in SAR induced by saccharin and PBZ against *Pto* DC3000 (**Fig. 2-5**; Yoshioka et al. 2001). Collectively, these findings illustrated the nature of SA and JA antagonistic interaction in Arabidopsis.

Pathogens can manipulate the antagonistic cross-talk between SA- and JA-signaling to triumph over their hosts (Bostock 2005; Pieterse et al. 2012). It has been demonstrated that virulent *Pseudomonas syringae* stimulates JA-signaling pathway by producing coronatine (COR), a structural mimic of JA-isoleucine, thereby interfering with SA-dependent immune responses (Katagiri et al. 2002; Brooks et al. 2005; Laurie-Berry et al. 2006). Our result is well consistent with these findings in that *Pto* DC3000 alone activated expression of JA- and JA/ET- marker genes (**Fig. 2-4c-e**), suggesting the stimulation of JA signaling. Furthermore, as a hemibiotrophic pathogen, infection process of *Pto* DC3000 goes through a biotrophic stage followed by a necrotrophic one. The biotrophic stage of *Pto* DC3000 is the most aggressive phase of multiplication *in planta* (Xin and He 2013). Therefore, to impede *Pto* DC3000, plants probably develop a SA-dependent immune mechanism targeting its early and highly aggressive biotrophic stage. The results of this study showed that saccharin and PBZ activated and suppressed the expression of SA- and JA-marker genes, respectively (**Fig. 2-1a-e**). Furthermore, the effect of these compounds on the gene expression was still present during *Pto* DC3000 infection of plants pretreated for 48 hours (**Fig. 2-4a-e**). Previously, Laurie-Berry et al. (2006) reported that *PR-1* was induced by *Pto* DC3000 in wild-type Arabidopsis plants, and more strongly induced in jasmonate-insensitive1 (*jin1-1*) mutants. This is consistent with our observation of induced SA-responsive genes by *Pto* DC3000 (**Fig. 2-4a, b**). However, it is possible that *Pto* DC3000 infection did not affect the up-regulated of SA-signaling conditioned previously by pretreatment of saccharin or PBZ, but did affect the up-regulation of JA-signaling. Together, these findings further highlight the importance of suppression of JA-signaling and activation of SA-signaling in resistance to *Pto* DC3000.

Plants impaired in SA- and JA-signaling indicated that SAR-inducing effect of saccharin and PBZ to *Pto* DC3000 was maintained in *jar1*, but not in NahG and *npr1*

plants (**Fig. 2-5**). These findings are in agreement with those by Yasuda et al. (2003), who reported that SAR triggered by saccharin against *Pto* DC3000 was diminished in NahG and *npr1* plants. Furthermore, studies using different pathosystems, e.g., NahG transgenic tobacco plant with mosaic tobacco virus and trangenic and mutant Arabidopsis plants with fungus *Peronospora parasitica*, also showed similar results (Nakashita et al. 2002; Yoshioka et al. 2001). These findings, therefore, indicate that SAR induced by saccharin and PBZ against biotrophic pathogens and a specific hemibiotrophic bacterium, *Pto* DC3000, is mainly dependent on the SA-signaling pathway. This is also consistent with a previous conclusion by Delaney et al. (1994) and Yang et al. (2015) that SA-signaling pathway plays a vital role in Arabidopsis resistance to *Pto* DC3000. However, it is noteworthy that the above conclusion is drawn from single-mutant approaches whereby disruption of a specific hormone, but not disruption of its interactions, is used to explain the response outcomes. Thus, it may be more substantial to suggest that SAR induced by saccharin and PBZ against *Pto* DC3000 is given through activation of SA-signaling resulting in suppression of JA/ET- signaling in Arabidopsis plants and *vice versa*.

It has been shown that induction of SA pathway can lead to suppression of JA signaling, and as a consequence, renders infected plants resistant to biotrophs and more susceptible to necrotrophs (Bostock 2005; Glazebrook 2005). Since defense responses against necrotrophic fungus *Bc* and bacterium *Pcc* are vitally JA/ET-dependent (Norman-Setterblad et al. 2000; Thomma et al. 1998), it is not surprising in our study that saccharin and PBZ failed to protect Arabidopsis from *Pcc*, and obviously enhanced susceptibility to *Bc* (**Figs. 2-2c, d; 2-3a, b**). Conversely, saccharin-treated plants trigger resistance to all tested biotrophic pathogens that have been reported in many previous studies (Boyle and Walters 2005, 2006; Koganezawa et al. 1998; Nakashita et al. 2002; Srivastava et al 2011). In the case of hemibiotrophic fungus *Ch*, unlike hemibiotrophic bacterium *Pto*

DC3000 as discussed above, treatment with saccharin could not reduce disease susceptibility in the infected Arabidopsis (**Fig. 2-3c, d**). Microscopic examination of *Ch* infection showed a reduced number of swollen primary infection hyphae restricted inside of the living cells in saccharin-treated leaves, corresponding to the early biotrophic stage (**Fig. S2-3e, f**). However, once thin secondary infection hyphae were formed, they vigorously ramify within and between host cells and caused necrosis ahead of infection, presenting the later prominent necrotrophic stage (Narusaka et al. 2004; O'Connell et al. 2012). In addition, during *Ch* infection, pretreatment of Col-0 plants with saccharin and PBZ enhanced expression of SA-signaling genes and suppressed that of JA-signaling marker genes (**Fig. S2-3a-d**). These results suggest that in the early biotrophic stage of *Ch* infection, the increasing induction of SA-signaling substantially contribute to the reduced infection. However, induction of SA-signaling might subsequently facilitate colonization when *Ch* switches into the aggressive necrotrophic stage. Our observation can be further explained by the finding of Narusaka et al. (2004) that defense reaction against *Ch* depends primarily on JA/ET signaling pathways. Additionaly, Fujioka et al. (2015) have reported that a volatile compound, limonene, triggered resistance to *Ch* in Arabidopsis by activating expression of *PDF1.2,* but not *PR1*.

Resistance to a specific pathogen has been linked to a number of defense responses including transcriptional re-programming to activate defense-related genes. We found that saccharin and PBZ directly activated transcript accumulation of defense-related genes *ALD1, PAD3, PRX34,* and *FRK1* which function in generation of multiple related amino acid-derived molecules, camalexin biosynthesis, ROS generation, and MAMPs responses, respectively (**Figs. 2-1g-j; 2-4g-j**). These results raise the prospect of multi contribution of different immune responses evoked by saccharin and PBZ in resistance to *Pto* DC3000. Noteworthy is the case of a gene *ALD1* which is considered to belong to a

group of the so-called type II regulators of SA. ALD1 affects the accumulation of SA, but it is not directly implicated in SA synthesis (Lu et al. 2009; Navarova et al. 2012). A study by Cecchini et al. (2015) has highlighted that ALD1 regulates basal immune components and early inducible defense responses in Arabidopsis. Considering that saccharin and PBZ markedly upregulated expression of *ALD1* as well as SA-signaling marker genes (**Figs. 2-1g; 2-4g**), it is more likely that ALD1 plays a role in SAR induced by saccharin and PBZ against *Pto* DC3000.

Formation of physical barriers is a common immune response to pathogen attack including callose deposition to fortify cell walls at the infection site (Xin and He 2013). In this work, we showed that saccharin and PBZ directly induced the formation of callose in Col-0 plants, and this immune output was still sustained when challenged with *Pto* DC3000 (**Fig. 2-6**). Although it remains unclear as to what extent the facilitated formation of callose by saccharin and PBZ directly contributes to resistance against *Pto* DC3000, we cannot exclude a possibility that callose may play the additive role in complex combinations of different defense responses.

In summary, we showed that application of saccharin and PBZ to the wild-type Arabidopsis plant triggered resistance to hemibiotrophic bacterium *Pto* DC3000. The contribution of saccharin and PBZ to the induced resistance might be attributed to the alteration of expression pattern of defense-related genes, especially that of SA- and JA-responsive genes. Our results suggest that saccharin and PBZ induce resistance against *Pto* DC3000 probably via activation of SA- signaling resulting in suppression of JA/ET-signaling and *vice versa*. However, given that saccharin and PBZ enhanced susceptibility to necrotrophic fungus *Bc*, it also raises a question about the extent to which saccharin can be applied in crops to induce effective defense against (hemi)biotrophic pathogens in balance with susceptibility to necrotrophic pathogens. Saccharin is used as a food additive

and can be manufactured industrially at a very low cost. In addition, since the adverse effect on plants and microorganisms is extremely low or almost negligible, saccharin can be expected to be used as an alternative eco-friendly protectant for reducing the use of agrochemicals in disease control by specific pathogens.

5. Supplementary information

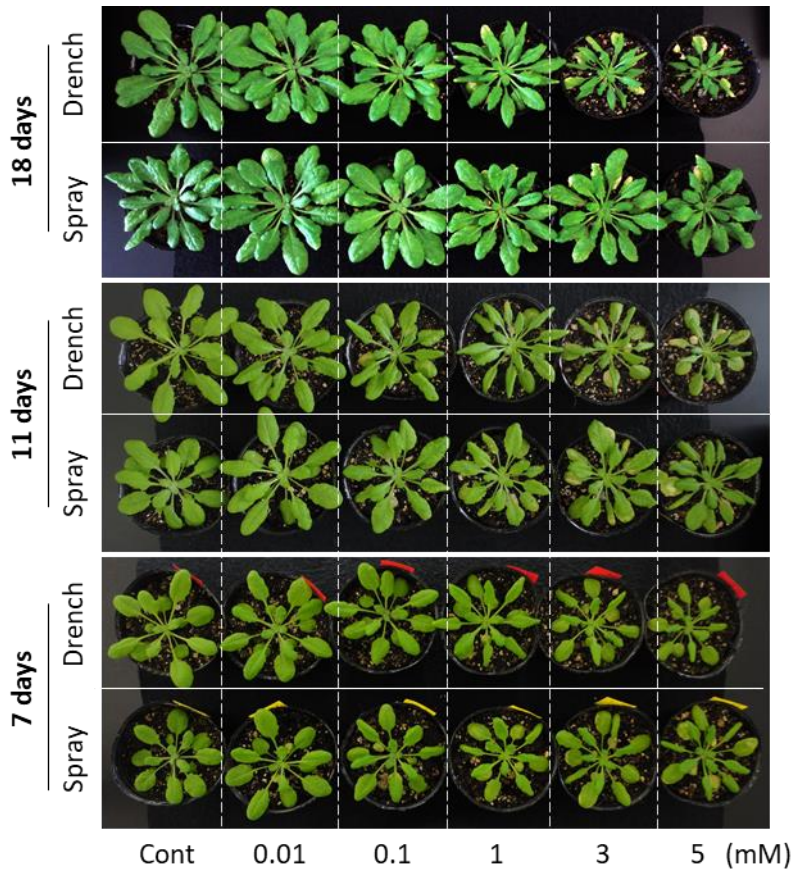

Fig. S2-1 Effect of saccharin on the growth of Arabidopsis plants. Col-0 plants were treated with water (as a control) or saccharin ranging from 0.01 to 5 mM by spraying until dews evenly formed on the leaf surface or drenching 10 ml of each solution into soil. Plant growth was observed for leaf size, shape and color after 7, 11 and 18 days of treatment. This experiment was repeated twice with similar results.

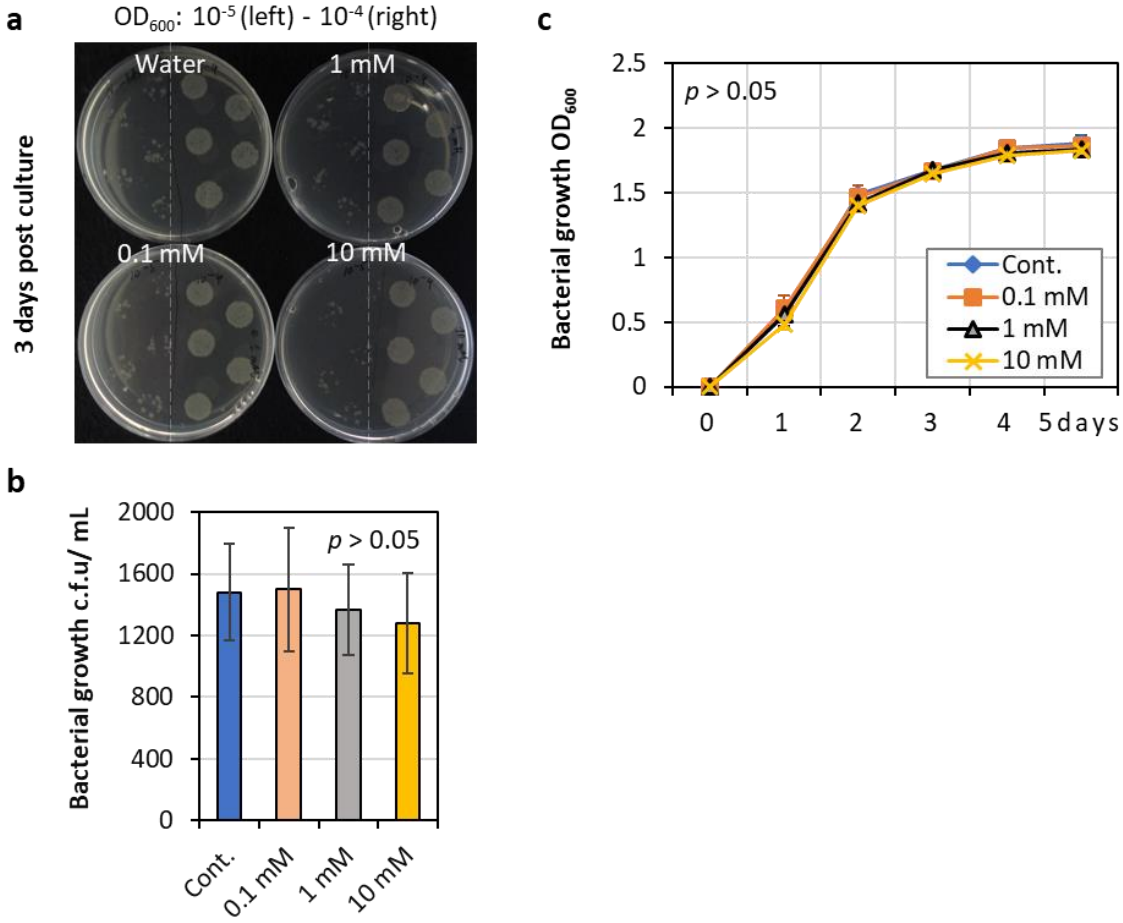

Fig. S2-2 Effect of saccharin on the growth of *Pto* DC3000. Liquid and solid KB media were amended with water (control) or saccharin at concentrations of 0.1, 1 or 10 mM. **a** 10 µl of bacteria suspension were placed onto KB agar petri disk at the left side for $OD_{600} = 10^{-5}$ and the right side for $OD_{600} = 10^{-4}$. **b** The number of viable bacteria was determined by counting the colonies at 3 days after culture. **c** Bacterium was grown in test tubes with shaking at 28°C. The OD_{600} was recorded in a course of 5 days. Data present the mean ± SD from the three replicates in each experiment. Turkey's test, $p > 0.05$.

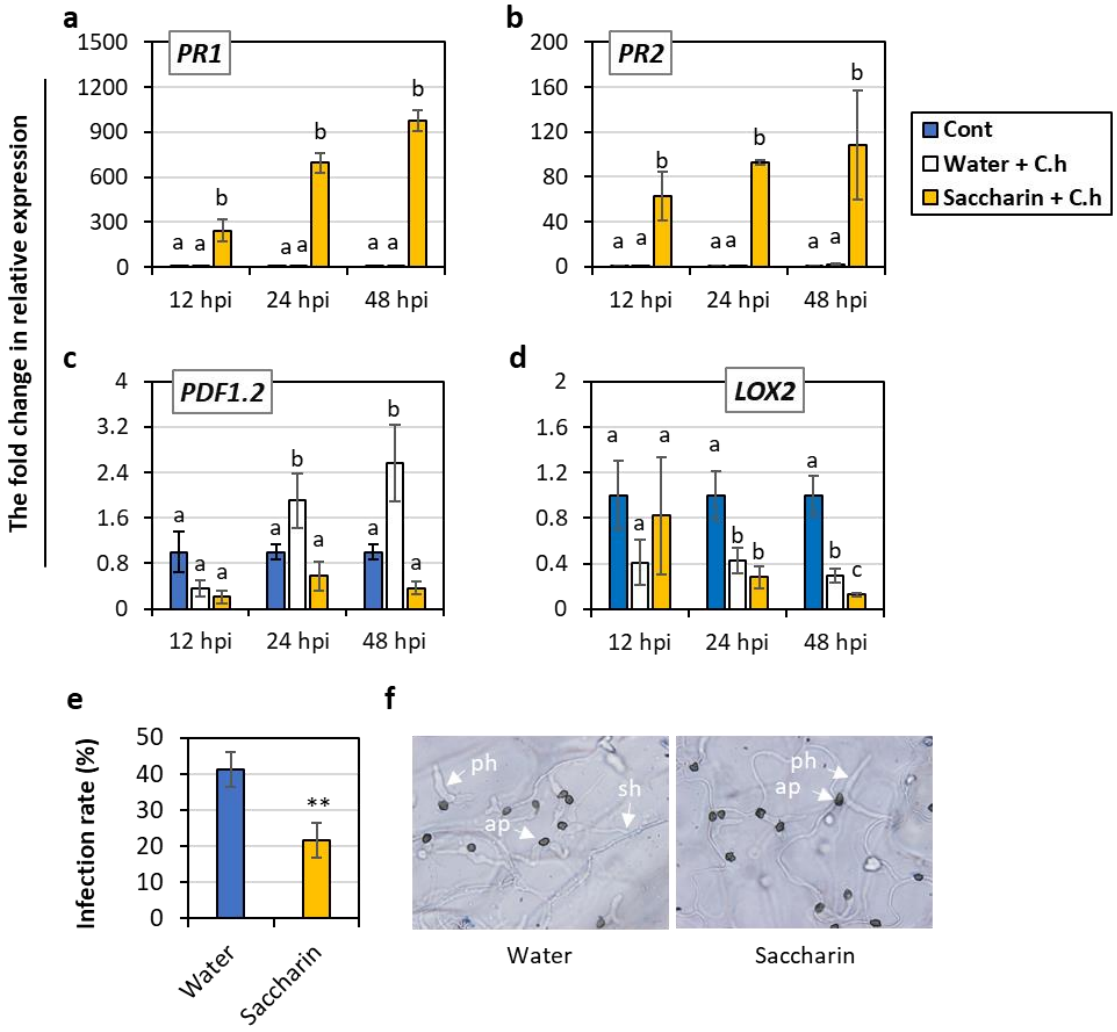

Fig. S2-3 Effect of saccharin on expression of SA- and JA/ET-responsive genes and penetration during infection by *Ch*. Col-0 plants were pretreated with water or saccharin (1 mM) for 2 days, then inoculated with *Ch* (5×10^4 spores/ml). **a** to **d** Leaves were harvested at 12, 24 and 48 hpi and subjected to qPCR. The water-treated and uninoculated plants were used as the control. The expression value of genes was normalized using *EF1-α* as an internal standard and expressed relative to average levels in the control. Data present the average ± SD from the triplicate reaction in each experiment. Letters indicate significant differences between treatments at each time point post-inoculation. Tukey's test, $p < 0.05$. **e** Penetration efficiency (%) at 3 dpi was calculated as a percentage of the number of conidia with appressoria forming primary infection hyphae per total number of conidia with appressoria. **f** Micrographs showing large intracellular primary hyphae 3 dpi. ap, appressorium; ph, primary hypha, sh, secondary hypha. Bars present the mean values (± SD) of infection rate (%). Asterisks indicate significant difference from the water treatment. Student's *t*-test, $^{**}p < 0.01$.

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Chapter 3

Mode of action of saccharin in wheat

Modified from: The plant activator saccharin induces resistance to wheat powdery mildew through activation of multiple defense-related genes

Short communication

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Abstract

Saccharin and its parental compound probenazole (PBZ) are the plant activators of effective defense responses to (hemi)biotrophic pathogens. Here, we demonstrate that pretreatment of wheat seedlings with saccharin or PBZ results in a significant reduction in powdery mildew disease caused by *Blumeria graminis* f. sp. *tritici.* Transcriptional analysis revealed expression profile of 15 defense-related genes including *PR* genes, *WCI* genes, *LOX*, *AOS*, *NPR1*, *PAL* and *WRKY* genes in wheat seedlings exposed to either saccharin or PBZ. Moreover, the saccharin- and PBZ-enhanced expression of those genes during fungal infection further proved a close correlation with increased resistance in wheat.

Keywords: *Blumeria graminis* f. sp. *tritici*, Induced resistance, Plant activator, Probenazole (PBZ), Saccharin, Wheat.

1. Introduction

Saccharin is commonly known as a non-caloric artificial sweetener which has a long history of 140 years since its accidental discovery in 1879 (Lawrence 2003). The starting point to address the new role of saccharin as an activator of plant defenses against pathogens can be traced back to the research by Uchiyama et al. (1973), who reported saccharin as one of the active metabolites of probenazole (PBZ), a well-known plant activator (**Fig. 3-1**). Comparatively, saccharin is obviously safer and cheaper than its parental compound PBZ, as it is widely used to sweeten countless foodstuffs all over the world (Bassoli and Merlini 2003; Yamaguchi 1982). Ample evidence showing the systemic acquired resistance (SAR)-inducing activity of saccharin is previously reported in both dicots and monocots, mainly against (hemi)biotrophic pathogens (Boyle and Walters, 2005, 2006; Koganezawa et al. 1998; Nakashita et al. 2002; Srivastava et al. 2011; Yoshioka et al. 2001). In Arabidopsis and tobacco, saccharin and PBZ were proved to activate salicylic acid (SA)-mediated signaling pathway which is accompanied by the accumulation of SA and expression of *PR* genes (Nakashita et al. 2002; Yoshioka et al. 2001).

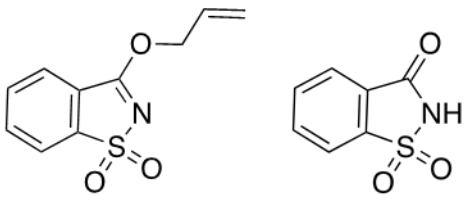

Fig. 3-1 Chemical structures of saccharin and probenazole (PBZ). Probenazole is non-metabolically or metabolically changed to saccharin in the plant (Uchiyama et al. 1973). Two compounds resemble salicylic acid (SA) in that they are composed of a cyclohexane ring, and are commonly regarded as functional analogs of SA (Bektas and Eulgem 2015).

Exogenous application of chemical plant activators, such as SA and its functional analogs benzothiadiazole (BTH) and PBZ, activates various defense responses which are tightly associated with extensive transcriptional-reprogramming regulated by a complex network of phytohormone signaling pathways (Pieterse et al. 2012; Bektas and Eulgem 2015). About 100s - 1000s of genes typically respond to defense induction have been revealed, together with 10 recognized classes of pathogenesis-related (PR) genes (Bektas and Eulgem 2015). In dicots, antagonistic interaction between SA- and JA-signaling appears to act predominantly in most cases of defense activation, whereas synergistic interaction mainly occurs between JA- and ET-signaling (Glazebrook 2005). In contrast, the distinctive role of SA- and JA-signaling and the marker genes associated with systemic acquired resistance (SAR) in monocots is not well defined (De Vleesschauwer et al. 2013; Yoshioka et al. 2001). For example, rice resistance to the hemibiotrophic fungus *Magnaporthe oryzae,* accompanied by accumulation of SA and PR proteins increased in response to SA and PBZ in adult plants but not in young plants (Iwai et al. 2007). On the other hand, exogenously applied JA or PBZ, but not SA, improved resistance against rice blast in young rice plants (Mei et al. 2006). In wheat, resistance to the biotrophic fungus *Blumeria graminis* f. sp. *tritici* (hereafter *B. graminis*), conferred by application of SA and its analogs BTH and INA (Gorlach et al. 1996). However, both SA and BTH did not turn on expression of *PR* genes such as *PR1.1* and *PR1.2*, which are commonly used as SA-marker genes and always respond to SA and its analogs in dicots (Gorlach et al. 1996; Molina et al. 1999). Instead, activation of wheat chemically-induced (WCI) genes showed a close correlation with BTH-induced resistance to *B. graminis* (Gorlach et al. 1996). Ding et al. (2011) and Sahu et al. (2016) recently reported for wheat that resistance to the hemibiotrophic fungi, *Fusarium graminearum* (hereafter *F. graminearum*) and *Bipolaris sorokiniana*, closely linked with increased expression of

both SA- and JA-related genes. However, knowledge on saccharin-altered expression of defense genes in monocots is so far elusive. Here, this study thus aims to analyze expression profiles of resistance-associated genes in wheat seedlings exposed to saccharin and PBZ. Significant reduction of wheat powdery mildew caused by *B. graminis* f. sp. *tritici* is also reported following pretreatment with saccharin and PBZ.

2. Materials and methods

A susceptible variety of wheat, *Triticum aestivum* cv. Nourin No. 61 and the powdery mildew fungus *B. graminis* f. sp. *tritici* races t2 were used (**Fig. S3-3**). The fungus was maintained on the same wheat cultivar by weekly transfer to fresh seedlings. Seeds were placed on wet filter paper to germinate and three of those that germinated were selected and grown in 200 cm^3 pots containing sterile soil mixed with vermiculite at 1:2 ratio. Seedlings were then transferred in controlled growth chamber held at 22°C, 16 h light/8 h dark cycle at 11.8 Wm^2 until when they were ready for analysis after 10 days.

Inoculation with the powdery mildew fungus was carried out by exposing seedlings to heavily infected wheat plants in the chamber. Sodium saccharin dihydrate (saccharin) and PBZ acquired from Wako Pure Chemical Industries (Osaka, Japan) were dissolved in distilled water and dimethyl sulfoxide (DMSO), respectively. In this experiment, PBZ was used as a positive control that induces resistance throughout experiments (Iwai et al. 2007; Mei et al. 2006). For foliar application, all test solutions were amended with 0.01% (v/v) Silwet L-77 (PhytoTech Labs, Shawnee, USA) as a surfactant. For analyses of defense-related genes, the first leaves of tested seedlings were used to quantify the expression levels as previously described by Fujioka et al. (2015). Statistical analyses of the qRT-PCR data were performed in Kyplot 5.0 (KyensLab Inc., Tokyo, Japan). An analysis of variance (ANOVA) was carried out, followed by the Tukey's multiple

comparison tests. The genes analyzed in this experiment are listed in **Table 3-1**. Microscopic specimens to observe fungal structures and associated host response were essentially prepared according to the trypan blue staining protocol described by Wang et al. (2016) with minor modifications.

Table 3-1 Summary information for wheat defense genes and primer sequences used for qPCR

Gene	Accession	Description		Sequence (5' > 3')	Size (bp)	References
RNase L inhibitor-like	Ta2776	68 kDa protein HP68 (a house keeping gene)	F	CGATTCAGAGCAGCGTATTGTTG	242	Wang et al. 2016
			R	AGTTGGTCGGGTCTCTTCTAAATG		
PR1.1	AJ007348	PR-1 (basic), pathogenesis-related protein 1	F	CTGGAGCACGAAGCTGCAG	76	Molina et al. 1999
			R	CGAGTGCTGGAGCTTGCAGT		
PR2	Y18212	Beta-1,3-endoglucanase	F	CTCGACATCGGTAACGACCAG	119	Desmond et al. 2006
			R	GCGGCGATGTACTTGATGTTC		
PR3; *CHI1*	AB029934	Chitinase 1	F	AGAGATAAGCAAGGCCACGTC	116	Desmond et al. 2006
			R	GGTTGCTCACCAGGTCCTTC		
PR4a; *PR4*	AJ006098	Wheatwin 1-2	F	CGAGGATCGTGGACCAGTG	128	Desmond et al. 2006
			R	GTCGACGAACTGGTAGTTGACG		
TaPERO; *PR9*	X56011	Wheat peroxidase	F	GAGATTCCACAGATGCAAACGAG	102	Desmond et al. 2006
			R	GGAGGCCCTTGTTTCTGAATG		
WCI2	U32428	Wheat chemically induced gene, lipoxygenase	F	TAGGAACTGGAACTTCACCGAGC	113	Gorlach et al. 1996
			R	GGTAGTCCTTGATGTGCAGCGAC		
WCI3	U32429	Wheat chemically induced gene, sulfur-rich/thionin-like protein	F	AAAGTTGGTCTTGCCACTGACTG	107	Gorlach et al. 1996
			R	TCGACAAAGCACTTCTGGATTTC		
TaNPR1	AX049430	Wheat nonexpressor of pathogenesis-related gene 1	F	TGAGGGAAGTCGATCTGAATGAG	92	Liu et al. 2016
			R	GCCCAGTTCCACTGTTTTCACT		
CHI3	AB029936	Class 1b neutral chitinase	F	GACCTCCTTGGCGTCAGCTA	95	Liu et al. 2016
			R	TGCATGTCTTCTCGCATCATATAGTC		
CHI4	AF112966	Class 4 acidic chitinase	F	AACGTCGACCCAGGGAACA	91	Liu et al. 2016
			R	AGCAGTAGGAGCATCGCTAGAAAG		
TaWRKY72a/b	CN009320	Transcription factor gene (TF)	F	ACAACTTCCCCAGGAGCTACTACC	108	Desmond et al. 2008
			R	CCTCGTATGTCGTTACCACCACA		
TaWRKY78	HM013818	Transcription factor gene (TF)	F	GATGCAATCCATGGCTTCGA	91	Desmond et al. 2008
			R	CATGCGGCCAGCAGAGTTT		
TaAOS	AY196004	*Triticum aestivum* allene oxide synthase	F	TCCCGAGAGCGCTGTTTAAA	98	Liu et al. 2016
			R	GACGATTGACGGCTGCTATGA		
LOX	BT008992.1	Lipoxygenase	F	TGTTGATAGACTGGTGCTGTG	85	Liu et al. 2016
			R	TGAGGATTAACGCTTAGGATCG		
TaPAL	AK448430.1	Phenylalanine ammonia-lyase	F	CGTCAAGAGCTGTGTGAAGATGG	158	Liu et al. 2016
			R	GGTAGTTGGAGCTGCAAGGGTC		

3. Results and discussions

First, we evaluated whether saccharin exhibited phytotoxic side effects on the growth of wheat seedlings since phytotoxicity of plant activators limits their use as agrochemicals. As shown in **Fig. S3-1**, saccharin was found to show no negative effects on wheat height and fresh weight, irrespective of application methods when applied at low concentrations up to 1 mM. However, saccharin at higher concentrations (3-5 mM) exhibited necrosis on the tip of the first leaves (**Fig. S3-1**). Pretreatment of saccharin or PBZ for 2 days by drenching induced wheat resistance to powdery mildew fungus *B. graminis* (**Fig. 3-2**). Differences were observed at 2 days post-inoculation (dpi) manifested by the failure of the number of branched appressoria to penetrate host cells, where also the area of dead cells at infection sites were higher on saccharin- or PBZ- pretreated leaves (**Fig. 3-2b**, *right*). These observations indicated the possibility of hypersensitive reaction which could culminate to cell death. At 7 dpi, the less severe symptoms on saccharin-pretreated leaves were indicated by a reduced number of pustules approximately by 70% and 50% in the first and second leaves, respectively, as compared to those on the control leaves (**Fig. 3-2a**). PBZ showed similar efficiency with saccharin. In addition, the protective effect of saccharin and PBZ was maintained for at least 11 days after inoculation under high pressure of infection (**Fig. 3-2b**, *left*). Foliar application of saccharin also reduced the infection of powdery mildew on wheat seedlings (**Fig. S3-2**).

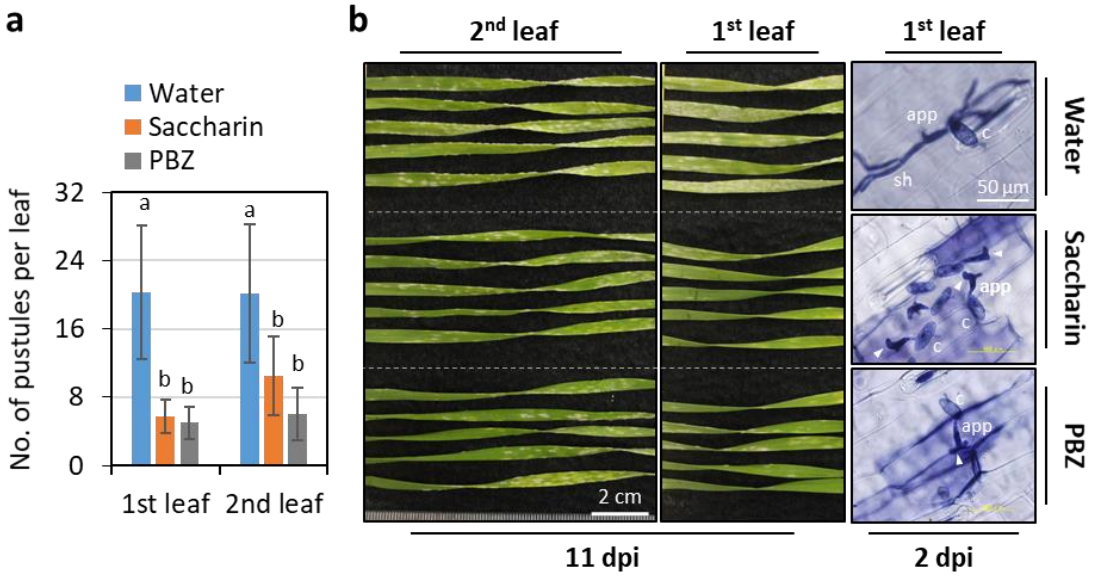

Fig. 3-2 Saccharin and PBZ reduced disease symptom caused by *Blumeria graminis*. Two days before inoculation with powdery mildew fungus, each pot containing 3 ten-day-old seedlings were drenched with 10 ml of water as a control, saccharin (1 mM) or PBZ (0.1 mM). After inoculation, the 1st and 2nd leaves were cut off for counting the number of pustules on the adaxial side at 7 days after inoculation (dpi) (**a**) and taking the photos of symptoms at 11 dpi (**b**, *left*). Bars present the mean values (± SD) of at least 20 leaves. Different letters indicate significant differences between groups using Tukey's test analysis ($p < 0.05$). For microscopic observation of fungal growth and cell death, 3-cm-long segments of infected leaves were stained with trypan blue at 2 dpi (**b**, *right*). The fungal structures such as conidia (c), appressoria (app) and epiphytic secondary hyphae (sh) and dead cells were stained blue. The white arrow points to the branched tips of appressoria as they failed to make penetration into host cells. The experiment was repeated three times with similar results and a representative result was presented.

We further examined whether saccharin or PBZ treatment of wheat seedlings could affect the expression profile of defense-related genes. A set of 20 genes involved in wheat defense responses was initially selected and assessed for their transcript levels in 10-day-old wheat seedlings considering 4-time points, 3, 12, 48 and 72 h post-treatment (hpt) with saccharin and PBZ. A total of 15 genes showed consistent expression including *PR* genes (*PR1.1*, *PR2*, *PR4*, *CHI3*, *CHI4*, *PR3* and *PR9*), wheat chemically-induced genes (*WCI2*, *WCI3*), JA biosynthesis-related genes (*LOX*, *AOS*), SA signaling and biosynthesis-associated genes (*NPR1*, *PAL*), and transcription factor-encoding genes

(*WRKY72a/b*, *WRKY78*) (**Fig. 3-3**). To investigate possible synergistic action of saccharin and PBZ on gene expression during fungal infection, wheat seedlings were pretreated with saccharin and PBZ by drenching for 2 days, followed by *B. graminis* inoculation, and expression levels of the 15 selected genes were analyzed at 2 and 3 days post-inoculation (dpi) (**Fig. 3-4**).

As shown in **Fig. 3-3a-e**, expression of pathogenesis-related genes, *PR1.1*, *PR2*, *PR4*, and 2 chitinase-encoding genes (*CHI3*, *CHI4*) were downregulated by saccharin and PBZ at an early time point 3 hpt, but apparently up-regulated at 12 hpt and 48 hpt as compared to the control. At 72 hpi, highly induced transcripts of *RR1.1* and *CHI4* were detectable in saccharin- and PBZ-treatments, but not those of *PR2*, *PR4* and *CHI3*. Previous studies have reported that those 5 *PR* genes did not respond to SA, INA and BTH, but to JA in wheat (Desmond et al. 2006; Lu et al. 2006; Molina et al. 1999; Yu and Muehlbauer 2001). *PR3* (chitinase 1) and *PR9* (wheat peroxidase) are the only two of total 7 tested *PR* genes, which showed downregulated expression or did not respond to saccharin and PBZ within the time course (**Fig. 3-3m, n**). During infection, expression of 6 *PR* genes (except for *PR4*) was obviously induced by *B. graminis* as compared to the control (Fig. 4a-e, m, n) even though statistical analysis did not give significance in the cases of *PR1.1*, *PR2* and *CHI3*. In the infected seedlings pretreated with saccharin or PBZ, expression of *PR1.1*, *PR2*, *PR4*, *CHI3* and *CHI4* was significantly enhanced as compared to those pretreated with water (**Fig. 4a-e**). For *PR3* and *PR9*, their expression during infection was suppressed by saccharin and PBZ, that was consistent with the results of time-course treatment (**Fig. 3-4m, n**). Since INA, BTH, PBZ and saccharin are regarded as SA analogs, the fact that only saccharin and PBZ directly activate expression of genes *PR1.1*, *PR2*, *PR4*, *CHI3* and *CHI4* suggests differential responsiveness of these *PR* genes to different chemical activators in wheat.

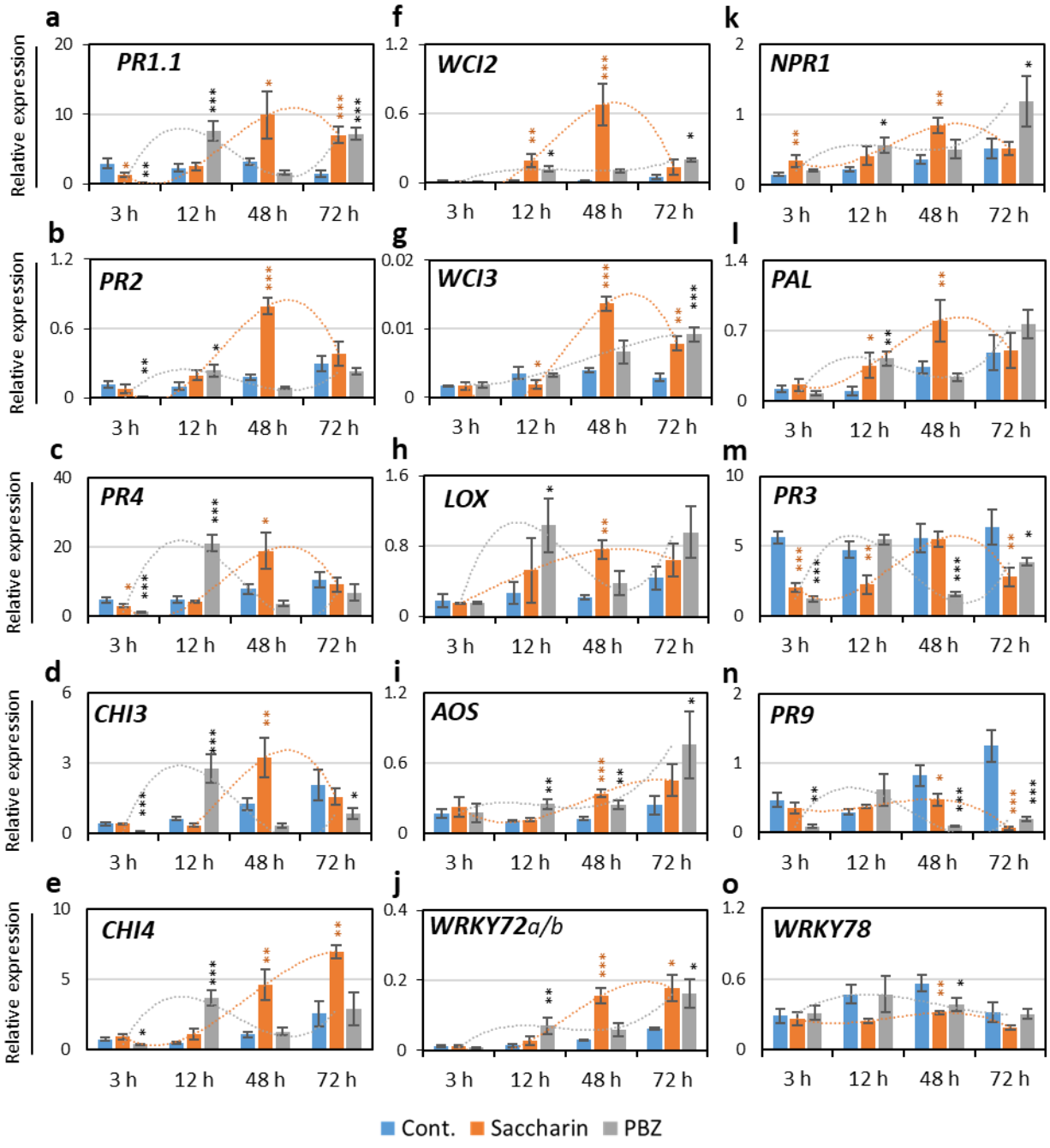

Fig. 3-3 Expression profiling of wheat defense-related genes by saccharin and PBZ. Ten-day-old seedlings were drenched with 10 ml of water (as a control; Cont.), saccharin (1 mM) or PBZ (0.1 mM), then the first leaves were harvested at indicated points in the time course of 72 h after treatment and subjected to qPCR. The expression value of genes was normalized using the gene RNase L inhibitor-like (Ta2776) as an internal standard. Data present the average ± standard deviation (SD) from three independent plants. The orange and grey trend lines present the expression levels of saccharin and PBZ treatment, respectively. Asterisks indicate significant

differences between treatments at each time point as revealed by Tukey's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The experiment was repeated twice with similar results and a representative result was presented.

TaNPR1 (Non-expressor of pathogenesis-related) is a key downstream regulator of SA-mediated signaling, and is involved in the cross-talk between SA- and JA-dependent pathways (De Vleesschauwer et al. 2013; Pieterse et al. 2012). TaPAL (phenylalanine ammonia-lyase) is a key enzyme for SA synthesis in the phenylpropanoid pathway, which occurs predominantly in monocots beside the isochorismate pathway (Pieterse et al. 2012). Increased expression of *NPR1* and *PAL* were supposed to relate to wheat resistance to rust fungus *Puccinia graminis* f. sp. *tritici* and *F. graminearum* (Ding et al. 2011; Li et al. 2001). In our study, *TaNPR1* was up-regulated by saccharin at 3 hpt and PBZ at 12 hpt. Induced expression of *TaNPR1* was maintained until 72 hpt in PBZ treatment, and 48 hpt in saccharin treatment (**Fig. 3-3k**). Similarly, *PAL* showed increased levels of the transcript at 12 and 48 hpt (**Fig. 3-3l**). During fungal infection, *PAL* was down-regulated by *B. graminis* while *NPR1* showed no response. But their expression was activated to higher levels in the presence of PBZ at 3 dpi (**Fig. 3-4k, l**). Collectively, the upregulation of *NPR1*, *PAL*, and some *PR* genes probably infers the positive effect of saccharin and PBZ on SA-mediated signaling in wheat.

LOX and *AOS* are both involved in JA biosynthesis and regarded as good markers for JA-mediated signaling pathway in both dicots and monocots (Kouzai et al. 2016; Liu et al. 2016; Seo et al. 2016). As shown in **Fig. 3-3h, i**, expression of *LOX* and *AOS* was induced by saccharin at 48 hpt, and PBZ at 12 hpt (for *LOX*, *AOS*) and 48-72 hpt (for *AOS*). When infected with the powdery mildew fungus, both genes were not responsive to *B. graminis* infection in the seedlings pretreated with water. However, they markedly responded to PBZ in the infected seedlings (**Fig. 3-4h, i**). Pretreatment with saccharin

enhanced expression of *AOS* at 2 dpi (**Fig. 3-4i**). These results indicate that the JA-mediated signaling is probably activated by saccharin and PBZ.

Both saccharin and PBZ clearly activated expression of the 2 chemically induced genes, *WCI2* (12-48 hpt) and *WCI3* (48-72 hpt), of which expression of *WCI2* preceded that of *WCI3* (**Fig. 3-3f, g**). During infection with *B. graminis*, the up-regulated expression of *WCI2* was maintained at 2 and 3 dpi in the seedlings pretreated with saccharin and PBZ, while that of *WCI3* was observed at 2 dpi in saccharin pretreatment (**Fig. 3-4f, g**). In addition, expression of *WCI3* appeared to be down-regulated by *B. graminis* in the water-pretreatment (**Fig. 3-4g**). These results are consistent with previous studies that reported 5 chemically induced genes (*WCI1-5*) were highly activated in wheat plants by BTH, SA and INA (Gorlach et al. 1996; Yu and Muehlbauer 2001), and by JA also (Liu et al. 2016). In the case of BTH, the induction of *WCIs* was tightly correlated with induced resistance to *B. graminis* (Gorlach et al. 1996). Previous findings have shown that *WCI* genes were not induced by infection with *B. graminis* and *F. graminearum*, at least until 12 dpi in the case of *B. graminis* (Gorlach et al. 1996; Yu and Muehlbauer 2001). Furthermore, *WCI2* was identified to encode a lipoxygenase which is involved in JA synthesis (Gorlach et al. 1996), and it was regulated by both JA (Liu et al. 2016) and SA (or SA analogs) (**Figs. 3-3f; 3-4f**; Gorlach et al. 1996; Yu and Muehlbauer 2001). These findings strongly imply that the JA- and SA-dependent signaling pathways may be cooperative in resistance of wheat seedlings. This idea might be in line with Tamaoki et al. (2013) who proposed, for rice, that both JA and SA activate a common defense system which plays an important role in pathogen defense responses through comparative transcriptome analysis of BTH- and JA-treated rice.

WRKY transcription factors are a large family of regulatory proteins which play an important role in plant immune (Pieterse et al. 2012). While some of WRKYs are reported

to be involved in SA signaling in Arabidopsis, some show strong responsiveness to JA in rice and wheat (Desmond et al. 2008; Liu et al. 2016; Tamaoki et al. 2013). In this study, the two WRKY genes *TaWRKY72a/b* and *TaWRKY78* previously reported as JA-responsive genes (Liu et al. 2016) were analyzed for their response to saccharin and PBZ. *WRKY72a/b* expression was strongly activated by saccharin and PBZ at 12 hpt, and this induced expression was still observed at 72 hpt (**Fig. 3-3j**). Also, *WRKY72a/b* was directly induced by *B. graminis*, significantly at 2 dpi, but its expression was highly enhanced in saccharin and PBZ treatments (**Fig. 3-4j**). In contrast, down-regulated expression of *WRKY78* was seen in wheat exposed to either saccharin or PBZ at 48 hpt (Fig. 3o) and in *B. graminis* infected wheat at 3 dpi (**Fig. 3-4o**). The contrasting expression of *WRKY72a/b* and *WRKY78* in response to *B. graminis* under chemical elicitors demonstrated the positive and negative roles of WRKYs acting in a complex defense response network in wheat seedling (**Figs. 3-3j, o; 3-4j, o**; Pandey and Somssich 2009).

In summary, expression profiling seems to provide a starting point to unveil the mechanism underlying saccharin- and PBZ-induced resistance in wheat seedlings to the powdery mildew fungus *B. graminis*. Indeed, saccharin and PBZ induced expression of multiple defense-related genes in wheat seedlings such as *PR* genes (*PR1.1*, *PR2*, *PR4*, *CHI3*, *CHI4*), genes associated with SA signaling and biosynthesis (*NPR1* and *PAL*), genes involved in JA biosynthesis (*LOX*, *AOS*), wheat chemically induced genes (*WCI2, WCI3*), and a transcription factor encoding gene (*WRKY72a/b*). The enhanced expression of those defense genes in wheat exposed to saccharin or PBZ substantially contributed to resistance to the powdery mildew fungus *B. graminis*.

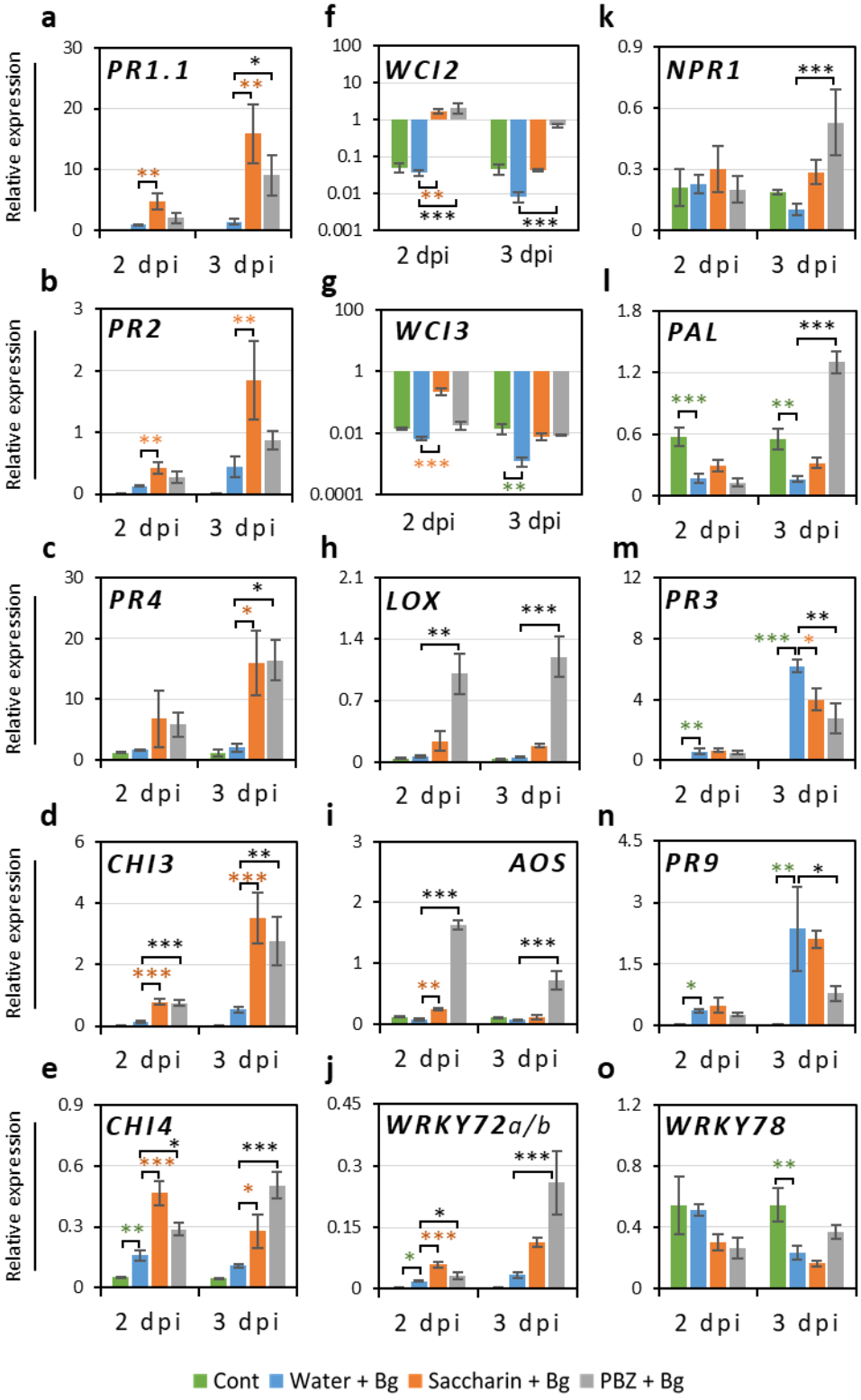

Fig. 3-4 Change in gene expression of wheat in response to inoculation with *Blumeria graminis*. Conidia of *B. graminis* were inoculated onto leaves of ten-day-old seedlings that had been pretreated by drenching for 2 days with 10 ml of water (water + Bg), saccharin (1 mM; saccharin

+ Bg) or PBZ (0.1 mM; PBZ + Bg). The first leaves were harvested at 2 and 3 days post-inoculation (dpi) and subjected to qPCR. The water-treated and uninoculated plants were used as control (Cont.). The expression value of genes was normalized using the gene RNase L inhibitor-like (Ta2776) as an internal standard. The expression is plotted on a $\log_{10}$ scale for genes *WCI2* and *WCI3*. Data present the average ± SD from three independent plants. Asterisks indicate significant differences between treatments at each time point as revealed by Tukey's test (*p < 0.05, **p < 0.01, ***p < 0.001). The experiment was repeated twice with similar results and a representative result was presented.

4. Supplementary information

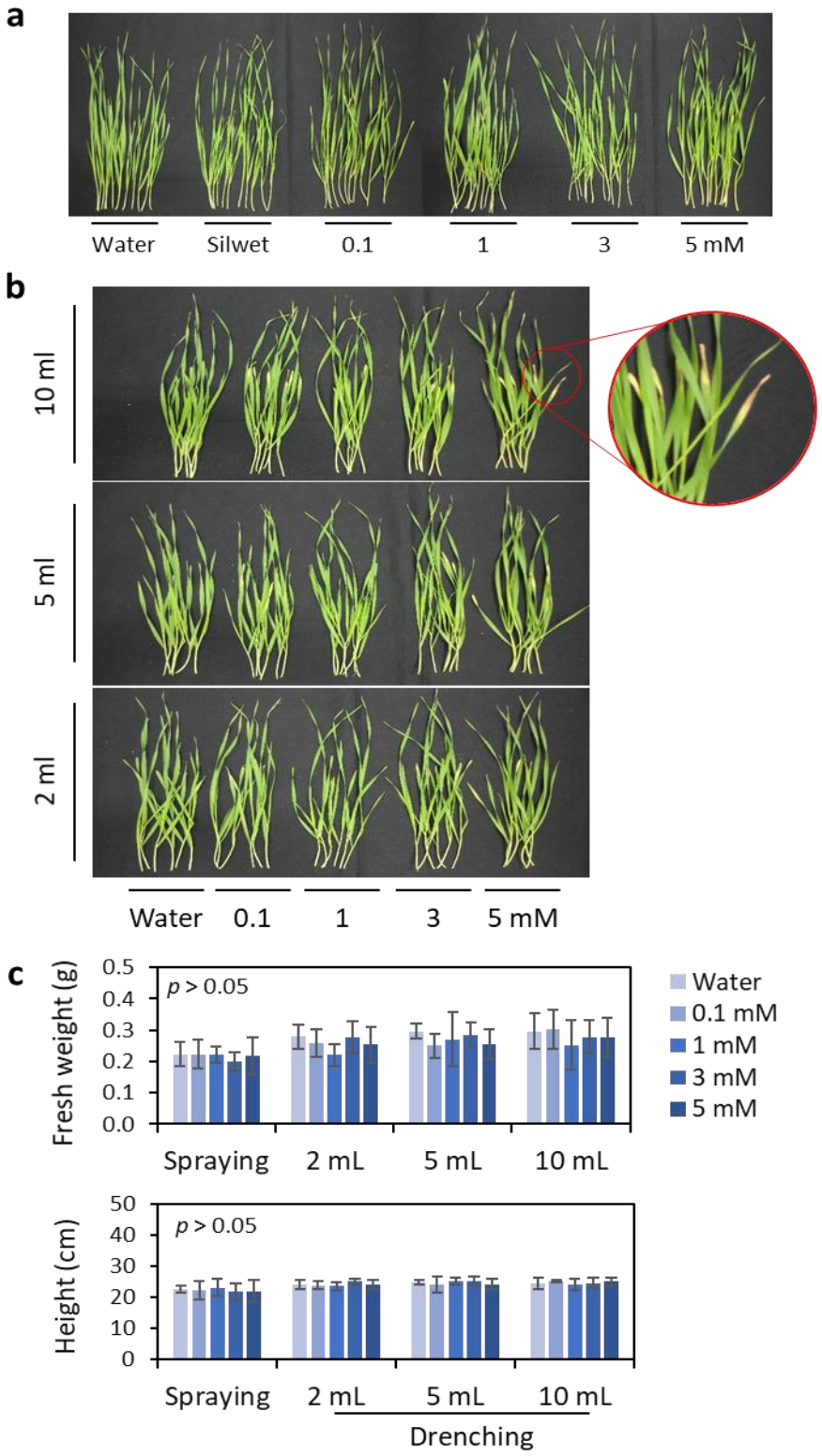

Fig. S3-1 Effect of saccharin doses and application methods on the growth of wheat seedlings. Ten-day-old seedlings of susceptible wheat (*Triticum aestivum* cv. Nourin No. 61) were used for

all experiments. Seedlings were treated with water (as a control) or saccharin at various concentrations (0, 0.1, 1, 3 and 5 mM) by spraying solutions amended with 0.01% (v/v) Silwet L-77 as a surfactant (**a**) or by drenching 2, 5 and 10 ml of each solution into pots (**b**). Wheat seedlings (aerial parts) were harvested for growth assessment after 10 days of treatment. No significant differences in plant height and fresh weight were observed between doses and application methods (**c**) (Tukey's test, $p > 0.05$). Data present the average ± SD of plant height in cm and fresh weight in g per seedling from twenty wheat seedlings. However, necrosis appeared on the tip of the first leaves as shown in the zoomed circle picture, when applied saccharin at high doses (3-5 mM and 10 ml). The experiment was repeated twice with similar results and a representative result was presented.

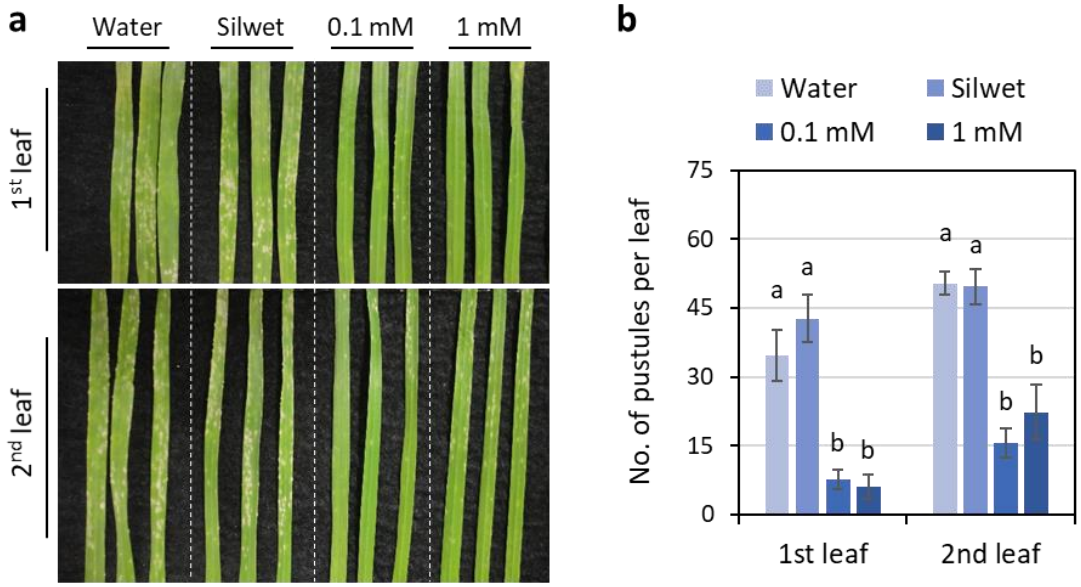

Fig. S3-2 Foliar application of saccharin reduced disease development of *B. graminis*. Ten-day-old wheat seedlings were sprayed with saccharin at different concentrations (0, 0.1 and 1 mM) amended with 0.01% (v/v) Silwet L-77. After 2 days of treatment, the seedlings were inoculated with *B. graminis* by putting them into an inoculation chamber containing heavily infected wheat leaves. The number of pustules on the adaxial side of leaves was counted (**b**) and photos of symptoms were taken at 7 days post inoculation (**a**). Bars present the mean values (± SD) of 15-20 leaves. Different letters indicate significant differences between groups using Tukey's test analysis ($p < 0.05$). The experiment was repeated twice with similar results and a representative result was presented.

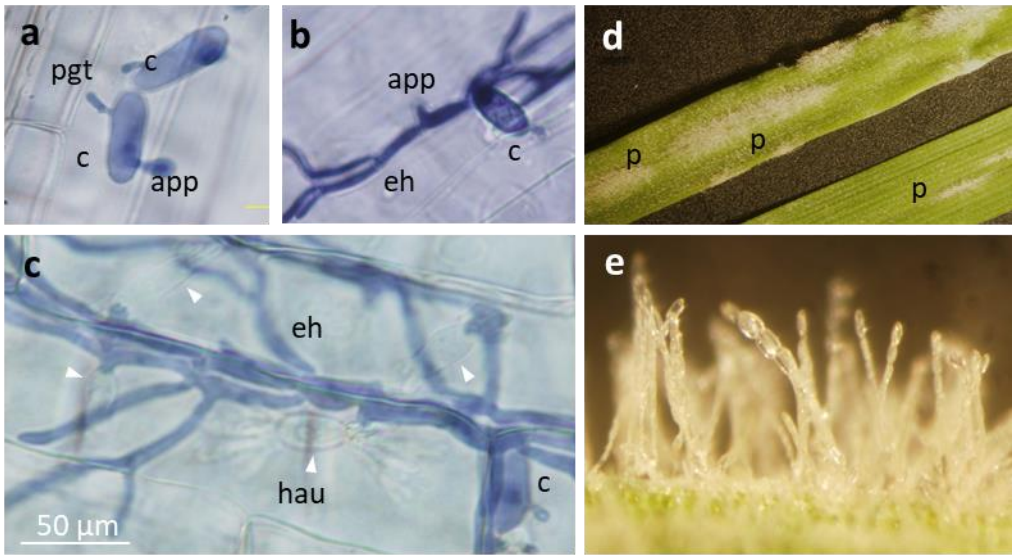

Fig. S3-3 The development of powdery mildew fungus *B. graminis* on wheat seedlings. Infection of *B. graminis* in wheat seedlings started with the landing of conidia (c) from heavily infected wheat leaves as a source of inoculum on the surface of test leaves. Within an hour after inoculation, conidium germinates a short primary germ tube (pgt) to anchor and sense the host leaf surface (**a**). Then conidium produces a secondary germ tube and differentiates appressorium (app), from which a peg penetrates through the host cell wall and develops a finger-like haustorium (hau) for taking nutrients from the host (**b, c**). Within 3 -5 days after inoculation, pustules (p) which consist of masses of epiphytic hyphae (eh) and conidia trains appear and quickly develop over leaf surface, giving this fungus the powdery-like appearance and the new source of inoculum (**d, e**). Three-cm-long segments from infected leaves at various points in time were stained with trypan blue and examined under a standard light microscope (**a, b, c**). The white arrow points to the finger-like haustoria forming inside the host cells (**c**). Photos of pustules and trains of conidia forming on leaf surface at 5 dpi were taken under a stereo microscope with a camera (**d, e**).

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Chapter 4

General discussions and conclusions

1. Phytotoxicity and antimicrobial activity of saccharin

Since plant activators directly activate defense responses which carry the fitness cost, requiring allocation of limited resources away from growth and reproduction as a consequence of the growth-defense tradeoffs (Bostock 2005; Huot et al. 2014). To be considered as plant activators, candidate compounds, beside the defense-inducing capacity, should exhibit no or little phytotoxicity and weak antimicrobial activities as well (Gozzo 2003; Bektas and Eulgem 2015).

Our tests on phytotoxicity of saccharin in *Arabidopsis thaliana* and wheat plants have shown that saccharin exhibits no or moderate negative effects on the plant growth when applied at low concentrations up to 1 mM, regardless of foliar spraying or root drenching (**Figs. S2-1; S3-1**). Basically, phytotoxicity caused by high doses of saccharin can be viewed by the yellow turning and curl leaves on Arabidopsis and the necrosis in the tip of the first and second leaves on wheat. Previous observations reported the toxic effect of saccharin at high concentration (3-10 mM) in parley cells and beans (Siegrist et al. 1998; Boyle and Walters 2005).

We also confirmed that saccharin up to 10 mM had no direct toxic effect on the growth of the hemibiotrophic bacterium *P. syringae* DC3000 (**Fig. S2-2**) and other fungal pathogens by *in vitro* test. No antifungal test was performed *in vitro* for the powdery mildew fungus *B. graminis* f.sp. *tritici* because it is a strictly obligate biotroph. However, an *in vivo* test done by Boyle and Walters (2006), applying 3 mM saccharin to first leaves of barley 2 h prior to inoculation with conidia of *B. graminis* f.sp. *hordei*, showed no significant effect on conidial germination or formation of appressoria. Together, we assume that saccharin generally had no direct antimicrobial activities on fungal and bacterial pathogens.

Since most of the studies showed saccharin at 1-3 mM could effectively induce defenses without exhibiting severe phytotoxicity, we decide to use saccharin at 1 mM, along with PBZ at 0.1 mM as a positive control, for further analyses.

2. Saccharin and PBZ induce Arabidopsis resistance to *P. syringae* DC3000 via conditioning an antagonism between SA- and JA-signaling

Previously, saccharin, and presumably PBZ, were proved to SA-mediated signaling pathway which was accompanied by the accumulation of SA and expression of *PR* genes in Arabidopsis (Yoshioka et al. 2001). However, the molecular mechanism of plants underlying saccharin-induced immune responses needs to be further elucidated.

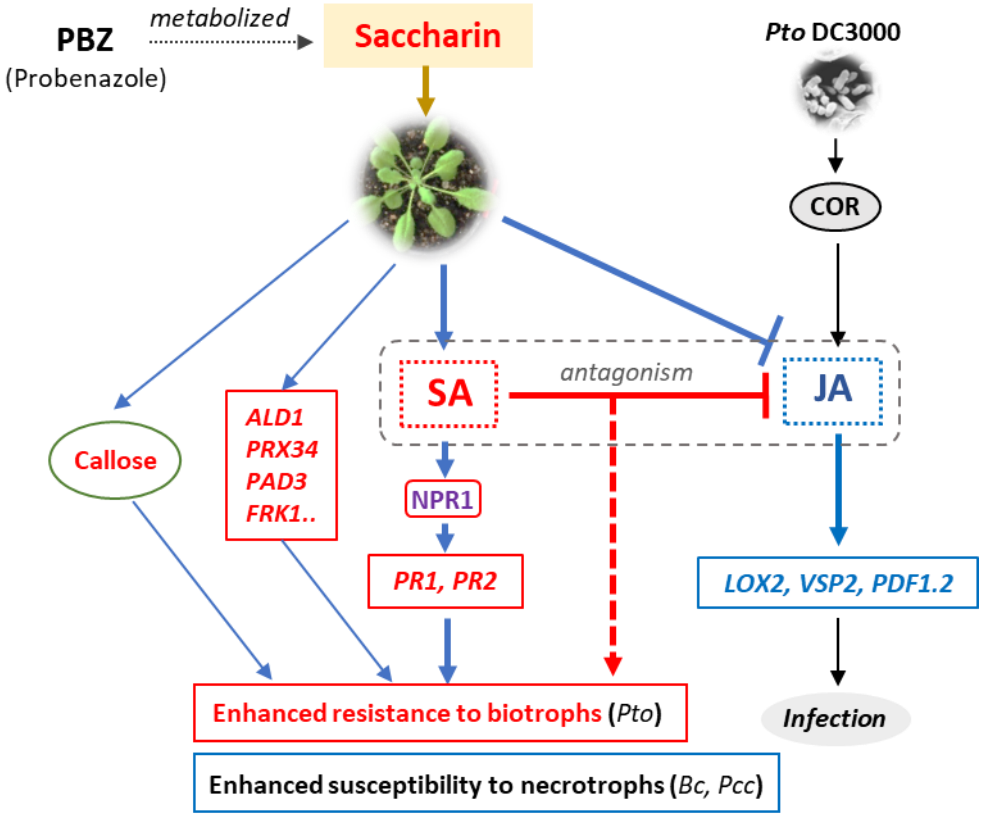

Fig. 4-1 Graphical abstract depicts the mode of action of saccharin in the induction of defenses in the model plant *Arabidopsis thaliana*.

In the current study, we show that exogenous application of saccharin and PBZ to the wild-type Arabidopsis plant triggered resistance to a hemibiotrophic bacterium *Pto* DC3000, but not to a necrotrophic bacterium *Pcc*, a hemibiotrophic fungus C*h*, and in the case of necrotrophic fungus *Bc*, they enhanced susceptibility (**Figs. 2-2; 2-3**). It is widely accepted that SA- and JA- signaling antagonistically interact, resulting in resistance or susceptibility to a specific group of pathogens as the final outcomes of this cross-talk (Glazebrook 2005; Pieterse et al. 2014). For example, resistance to biotrophs is associated with activation of SA-signaling while that to necrotrophs is related to activation of JA-signaling, and in reverse. Our analysis of gene expression profile reveal that activation of SA-regulated genes (*PR1*, *PR2*) and the concomitant suppression of JA-regulated genes (*LOS2*, *VSP2*, *PDF1.2*) by saccharin and PBZ might be the contributing event which accounted for the induced resistance to *Pto* DC3000 and the increased susceptibility to *Bc* (**Figs. 2-1; 2-4**). Our result is also consistent with previous findings that virulent *Pseudomonas syringae* produces coronatine, a structural mimic of JA-isoleucine, thereby stimulates JA-signaling pathway accompanied by the activation of JA-marker genes (Katagiri et al. 2002; Brooks et al. 2005; Laurie-Berry et al. 2006). In addition, Delaney et al. (1994) and Yang et al. (2015) reported that SA-signaling pathway plays a vital role in Arabidopsis resistance to *Pto* DC3000. Using mutant plants for inoculation test, we reconfirm and complement the known knowledge in that resistance conferred by saccharin and PBZ against *Pto* DC3000 is given via activation of SA-signaling, possibly resulting in suppression of JA/ET-signaling and *vice versa* (**Fig. 2-5**). Moreover, both saccharin and PBZ upregulated expression of other defense-related genes (*ALD1*, *PRX34*, *PAD3* and *FRK1*) and accelerated deposition of callose in Arabidopsis plants **(Figs. 2-1; 2-4; 2-6),** suggesting the additive roles of these defense outcomes in the conferred

resistance to *Pto* DC3000. **Fig. 4-1** graphically illustrated the mode of action of saccharin and PBZ in the induction of defenses in the model plant *Arabidopsis thaliana*.

Additionally, the cautions on the use of saccharin and PBZ or similar chemicals for plant disease control are also pointed out in this study. Since saccharin and PBZ enhanced susceptibility to necrotrophic fungus *Bc*, it also essential to have more investigation on the extent to which saccharin and PBZ can be practically applied in crops to induce effective defense against (hemi)biotrophic pathogens in balance with susceptibility to necrotrophic pathogens.

3. Wheat resistance to *Blumeria graminis* triggered by saccharin and PBZ is associated with the activation of multiple defense-related genes

In contrast to the situation in dicots, the distinctive role of phytohormone signaling and the marker genes associated with SAR in monocots is not well defined (De Vleesschauwer et al. 2013; Yoshioka et al. 2001). Furthermore, knowledge on saccharin-altered expression of defense genes in monocots is so far elusive.

In this study, we demonstrate that pretreatment of wheat seedlings with saccharin or PBZ results in a significant reduction in powdery mildew disease caused by a strictly biotrophic fungus *Blumeria graminis*, a causal agent of powdery mildew disease (**Figs. 3-1; S3-2**). Transcriptional analysis revealed expression profile of 15 defense-related genes in wheat seedlings exposed to either saccharin or PBZ. Indeed, saccharin and PBZ induced expression of multiple defense-related genes in wheat seedlings such as *PR* genes (*PR1.1*, *PR2*, *PR4*, *CHI3*, *CHI4*), genes associated with SA signaling and biosynthesis (*NPR1* and *PAL*), genes involved in JA biosynthesis (*LOX*, *AOS*), wheat chemically induced genes (*WCI2, WCI3*), and a transcription factor encoding gene (*WRKY72a/b*) (**Figs. 3-2; 3-4**). The enhanced expression of those defense genes in wheat seedlings

treated with saccharin or PBZ is closely correlated to the conferred resistance to the powdery mildew fungus *B. graminis*.

Our results complement the current knowledge on responsive genes for saccharin- and PBZ-induced resistance in wheat plants, which may be of interest to developers of plant defense activators and researchers working on immunity in wheat and other monocot plants.

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Appendix 1

Medium recipes for of pathogen culture

1. PDA

PDA	7.8 g
Distilled water	200 ml
Total volume:	200 ml

Notes:

- PDA (Potato Dextrose Agarose) is used for hemibiotrophic and necrotrophic fungal culture such as *C. higginsianum* and *B.cinerea* in this study.
- Mix ingredients and autoclave and cool to 50 - 55°C before pouring into sterile Petri discs (10 ml for each).
- For making a medium in a test tube, mixed ingredients are distributed into test tubes (10 ml for each), then autoclaved. Test tubes are arranged in a rack so that they can be put in slanting position at the same time for solidification after autoclave.

2. V8

Agarose	3 g
$CaCO_3$	0.6 g
Tomato juice	40 ml
Distilled water	160 ml
Total volume:	200 ml

Notes:

- V8 is used for fungal cultures such as *A.brassicicola* and *M. pinodes.*
- The calcium carbonate is reported to prevent the acidity of the juice from hydrolyzing the agar.
- Procedure for making V8 is the same as described for PDA.

3. King's B (KB)

Distilled water	up to 1000 ml
Protease peptone	20 mg
K_2HPO_4	1.5 g
$MgSO_2.7H_2O$	0.4 g
Glycerol	10 ml
Agarose (for solid medium)	15 g
Total volume:	1000 ml

Notes:

- King's B is used for bacterial culture such as *P. syringae* pv. *tomato* DC3000.
- For solid medium, mix ingredients, autoclave, and cool to 50 - 55°C before amending with an antibiotic solution and pouring into Petri discs (10 ml for each).

- For the liquid medium of KB (no agarose added), mixed ingredients are distributed into test tubes (3 ml for each) before autoclaving, then cool down. The antibiotic solution will be added when used.
- Nalidixic acid (Nal) is usually amended in the medium for the culture of *P. syringae* at a final concentration of 0.05 mg/ml.

Preparation of 10 ml of Nalidixic solution 50 mg/ml

Distilled water	8 ml
NaOH 5M (5N)	2 ml
Nalidixic acid sodium salt	0.5 g
Total volume:	10 ml

- Nalidixic solution 50 mg/ml is prepared by dissolving Nalidixic acid sodium salt in buffer NaOH 1M whose stock solution is available as 5M (5N).
- Since antibiotic is very sensitive to high temperature, the autoclave cannot be applied to a sterile solution. Instead, the filter is used to sterilize solution by pushing solution through a 50 ml syringe fitted with a 0.22 µm filter.
- Divide into 1 ml portions into 1.5 ml Eppendorf tubes and store at -20°C.

4. Luria-Bertani (LB)

Distilled water	up to 1000 ml
Bactotrytone	10 g
Yeast extract	5 g
NaCl	5 g
Agarose (for solid medium)	15 g
Total volume:	1000 ml

Notes:

- LB medium is used for bacterial culture such as *P. carotovorum* subsp. *carotovorum*.
- Procedure for making LB is similar to that for making King's B.

5. Maltose Sabouroud Bloth

Distilled water	up to 1000 ml
Casein	10 g
Maltose	40 g
Total volume:	1000 ml

Notes:

- Maltose Sabouraud Broth is used for the cultivation of molds and yeasts.
- In this study, 0.01% Sabouraud Maltose Broth is used to dilute spore suspension of *B. cinerea* for Arabidopsis infection.

Appendix 2

Staining of fungal infected leaves with trypan blue

This staining method is used to reveal hyphal structures and dead plant cells in plant tissues. Fungal structures and dead plant cell are stained blue.

1. Solution to be prepared:

Bleaching solution (to remove chloroform)

Lactic acid	4 ml
Ethanol	96 ml
Total volume:	100 ml

Staining solution (stock): The working solution is prepared by diluting the stock solution with ethanol (96%; 1:2 v/v)

Phenol	10 g
Glycerol	10 ml
Lactic acid	10 ml
Water	10 ml
Trypan blue	0.02 g
Total volume:	30 ml

Distaining solution (Chloral hydrate)

Chloral hydrate	1 kg
Water	400 ml
Total volume:	400 ml

2. Staining procedure:

- Infected leaves (3 leaves) are placed in 6-well-plates containing bleaching solution (2-3 ml/well) for 1-2 days at the shaker.
- Discard the bleaching solution and add staining solution. Sample is left overnight in the staining solution.
- The next day, distain by replacing the staining solution with chloral hydrate solution. If necessary, replace distaining solution several times.
- The samples can be kept in the chloral hydrate solution for several months.
- Observe the samples under a microscope.

Staining procedure by boiling:

- Infected leaves or tissues are transferred into a plastic test tube with a lid and covered with diluted trypan blue solution.
- The tube (lid slightly unscrewed) is placed in a heated water bath and the staining solution is boiled for one minute.
- The tissue is left overnight in the staining solution.

- The next day, distain by replacing the staining solution with chloral hydrate solution. If necessary replace distaining solution several times. The samples can be kept in the chloral hydrate solution for several months.

3. Microscopic observation and data evaluation:

- Place the sample onto a microscope slide and cover it by a cover glass.
- Sample is observed with a microscope (Olympus BX61).
- View sample under a microscope at 10x, 20x, 40x or 50x magnification for the fungal structures at inner- or outer cellulars such as hyphae, conidia, appressoria and infection hyphae.
- The rate of infection was calculated by a formula as below:

Rate of appressorium (%) = Number of spores forming infection hyphae x 100 / Number of spores forming appressorium.

Penetration rate (%) = Number of spores forming infection hyphae x 100 / Number of spores forming appressorium.

Reference

http://resources.rothamsted.ac.uk/sites/default/files/groups/bioimaging_dev/Staining%20of%20infected%20Arabidopsis%20leaves%20with%20trypan%20blue.pdf.

Appendix 3

How to indicate the concentration of fungal suspension and bacterial culture

1. Counting fungal spore with a hemocytometer

Before inoculation of plants with fungal pathogens, for example, *C. higginsianum* and *B. cinerea*, spore/conidial suspension from fungal culture is prepared to obtain optimal concentrations, normally 2 or 5 x 10^5 spores/ml. This process can be done by using a hemacytometer which is a specialized microscope slide used to count cells, organelles, etc.

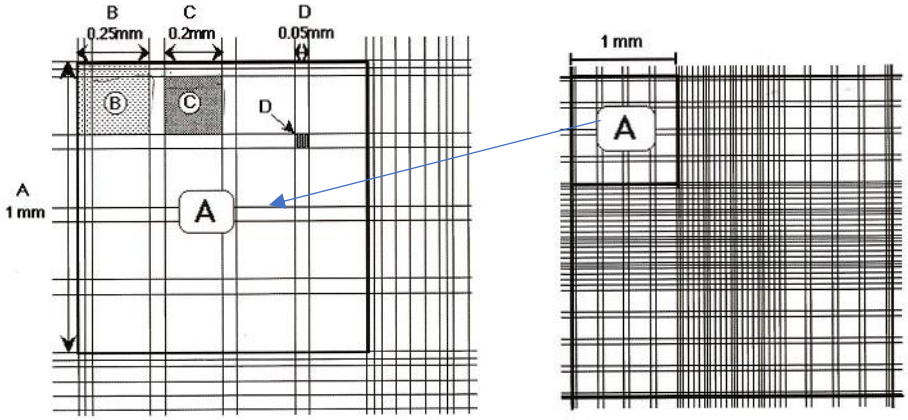

Fig. A3-1 Grid on a hemacytometer (Turker-Turk A117, Japan)

- Clean glass hemocytometer and coverslip with alcohol before use and affix coverslip to the hemocytometer.
- Take 10 µl of spore suspension by micropipette and transfer to one of the chambers of the hemocytometer by carefully touching the coverslip at its edge with the pipette tip.
- View the spores under a standard microscope at 10x magnification (Olympus). Focus the microscope on one of the 4 outer A squares in the grid.
- Count number of spores in the A squares. Each A square consisting of 16 smaller squares (indicated as B zone) has sides 1mm x 1 mm. The volume over the A square is 1 mm x 1mm x 0.1 mm = 0.1 mm^3, which is equivalent to 10^{-4} ml (**Fig. A3-1**).
- The total number of spores per 1 ml will be determined using the following calculations:

  Number of spores/ ml = Average number of spores count per A square x 10^4
- Dilute the spore solution to desired concentration (spores/ml) using the formula:

  C1 x V1= C2 x V2.

  C1, V1: original concentration and volume

  C2, V2: desired concentration and volume

2. Estimation of bacterial concentration in culture and infected leaf sample

2.1. Estimation of bacterial concentration in liquid medium by OD value

To estimate the concentration of bacterial cell cultured *in vitro* in liquid medium, the OD_{600} value, which indicates the absorbance, or optical density, of a sample measured at a wavelength of 600 nm, was indicated by using Biochrom WPA Colorimeter CO7500 (Biochrom Ltd., Cambridge, UK). The OD value can be used to identify the optimal concentration of bacteria for inoculation test or to estimate the bacterial growth in the presence of saccharin in liquid medium (see Appendix 9).

2.2. Estimation of bacterial concentration by counting the number of CFU

CFU (colony-forming unit) is a unit used to estimate the number of viable bacteria in culture or bacterial proliferation in infected leaf samples. In the case of leaf sample, leaf discs were made from the infected leaf, then crushed by zirconia beads as described in the method of Chapter 2. Appropriate dilutions of bacterial culture or solution of crashed leaf discs were made and plated on a fresh KB agar plate. The number of bacteria was determined by counting colonies appear on the agar plate. Serial dilutions (log dilutions) are used to decrease a bacterial concentration with ratio 1:10 (tenfold dilution) to a required concentration which is easier to count the number of CFU when plated to an agar plate. The CFU/ml of original solution or CFU/cm^2 of leaf area can be calculated using the below formulas.

CFU/ml = (number of colonies x dilution factor)/ volume of plate culture

CFU/cm^2 = (number of colonies x dilution factor) / (volume of plate culture x total area of leaf discs

Notes: - Dilution factor indicates how much the original sample is diluted.

- CFU/ml or CFU/cm^2 can be converted into log_{10} value for presented data.

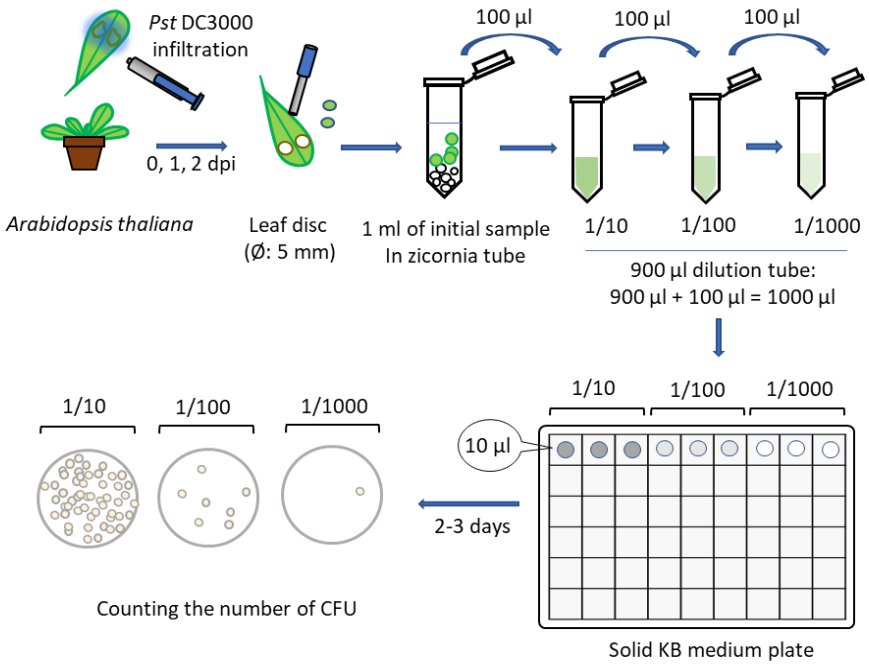

Fig. A3-2 Experiment design for estimation of bacterial concentration in the infected leaves

Appendix 4

Examination of saccharin toxicity on the growth of fungal and bacterial pathogens

1. Examination of direct antimicrobial effect of saccharin on fungi using onion epidermis

- Purchase fresh onion bulbs (6 ~ 8 cm in diameter) from a grocery store.
- Remove the dry, papery outer layers and the oldest, outermost fleshy layers.
- Excise onion into 4 sections and use the inner fleshy layers for taking whole-cell epidermis from the adaxial (inner or concave) surface of the onion layers.
- Cut some shadow lines on the adaxial surface by a razor blade, that makes many square pieces of the epidermis (1 x 1 cm).
- Peel off each piece of the epidermis by tweezers and put it into a bottle containing ethanol 100% to kill the cells.
- Incubate epidermal cells in ethanol overnight, then wash them with distilled water 2-3 times to ensure ethanol is removed completely from the cells.
- Place the onion epidermis onto the drop of water on the slide with hydrophobic side facing upward.
- Drop 5 µl of saccharin solution and 5 µl of spore suspension onto the center of the epidermis to test the effect of saccharin on the growth of fungus in terms of germination of spore, appressorial and infection hyphal formation.
- At indicated points in times after fungal inoculation, drop a small amount of cotton blue or trypan blue solution into where spore suspension was placed to stain fungal structures.
- After staining for 15 - 20 min, place the coverslip on top of the stained onion epidermis and view the samples under the microscope.

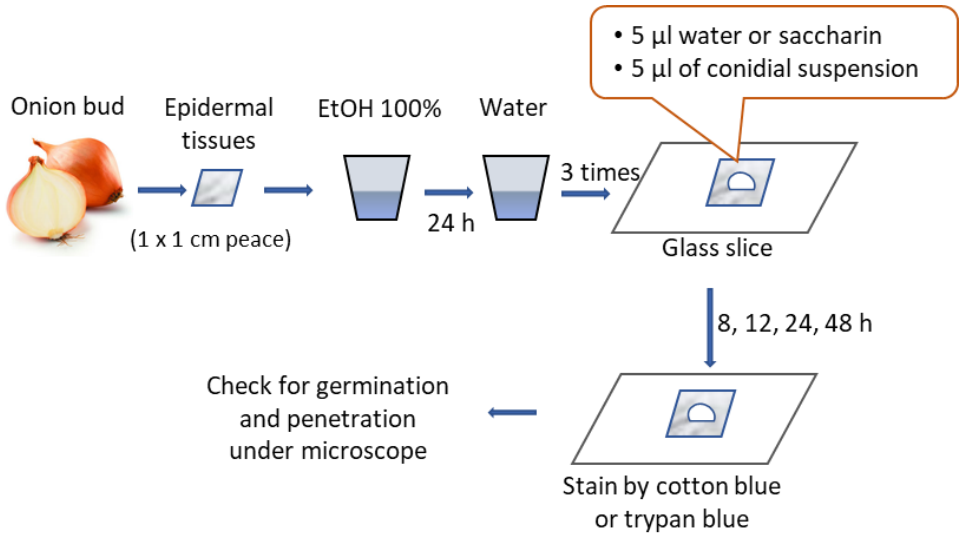

Fig. A4-1 Experiment design for examination of effects of saccharin on fungal growth using onion epidermis

2. Test of the direct effect of saccharin on *Pto* DC3000 growth

- Prepare KB solid and liquid media amended with saccharin at various concentrations in Petri disks and test tubes, respectively, as described in Appendix 7.
- For the test in liquid medium, Pto DC3000 is grown at 28°C on liquid KB with shaking to reach OD600 = 1 (or 5 × 108 colony-forming units (CFU)/ml). Ten microliters of bacterial suspension were diluted with 3 ml of fresh KB medium, then 3 µl of the diluted bacterium was placed into 3 ml of KB medium in a test tube containing water or saccharin. To estimate the concentration of bacterial cell in liquid medium, the OD600 value, which indicates the absorbance, or optical density, of a sample measured at a wavelength of 600 nm, was recorded in a course of 5 days using Biochrom WPA Colorimeter CO7500 (Biochrom Ltd., Cambridge, UK).
- For the test in solid medium, *Pto* DC3000 was grown at 28°C on liquid KB in Petri disk for 2 - 3 days, then bacteria were gently scraped off from plate culture, suspended in distilled water. A set of serial dilutions is made and droplets of 10 µl diluted bacterium were plated onto KB agar plate amended with saccharin. The number of viable bacteria was determined by counting the colonies at 2 ~ 3 days after culture (see **Fig. A3-2** for how to make a serial dilution of bacteria and calculate the number of viable bacteria).

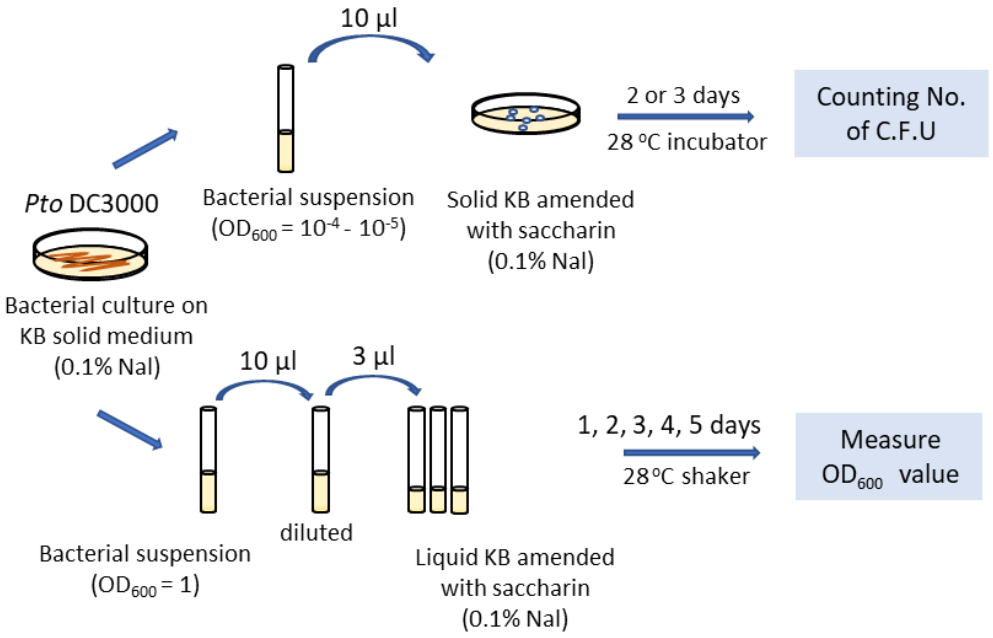

Fig. A4-2 Experiment design for *in vitro* examination of effects of saccharin on bacterial growth

Appendix 5

Callose detection by staining with aniline blue

In situ detection of callose in leaf sample was performed as described in Survila et al. (2016) with minor modifications.

1. Solution to be prepared:

Bleaching solution (to remove chloroform)

Acetic acid (:1)	20 ml
Ethanol (:3)	60 ml
Total volume:	80 ml

Phosphate buffer (pH 9):

Distilled water	100 ml
K_2HPO_4	2.6 g
Total volume:	100 ml

Staining solution (Aniline blue 5 mg/ml, or 0.5%):

Phosphate buffer	100 ml
Aniline blue	500 mg
Total volume:	100 ml

Maintaining solution (Glycerol 50%)

Glycerol	50 ml
Distilled water	50 ml
Total volume:	100 ml

2. Staining procedure:

- Leaf sample is placed in sterile 6 - well plates with bleaching solution consisting of acetic acid and ethanol (1:3 v/v) for 6 hours for Arabidopsis leaf or overnight for wheat leaf at a shaker.
- Replace the bleaching solution with ethanol 50% (v/v) for 2 hours.
- Replace ethanol 70% with distilled water for 2 hours with 2 changes.
- Leaves were stained with a phosphate buffer (pH 9) containing 5 mg/ml aniline blue (Sigma) for 1-3 hours in the dark.
- Transfer sample to 50% glycerol (Sigma).
- View callose spots under fluorescence microscopy (Olympus IX70) with UV lamp (excitation filter 400 - 440 nm; emission filter 475 nm) and take pictures for further callose measurement by a camera (Olympus DP70).

3. Analysis of callose pictures

For the calculation of number of callose deposits (in 1 mm^2) or percentage of callose area (per picture), the following Fiji tools (https://imagej.net) were used:

- Set a scale according to the scale bar on the picture (usually 50 or 500 µm) at ‘Set scale’: enter 50 or 500 into the 'Known distance' and change the 'Unit of length' to µm, check 'Global'; then draw a new line and confirm that the measurement scale is correct.
- Select ‘8 bite’ for the picture at ‘Image type’: to convert a scanned color image of the leaf to grayscale. Callose dots are in white color.
- ‘Make Binary’: white dots of callose become black, the background becomes white.
- ‘Analyze Particles’: Data window gives an area of callose in µm or percentage.

Reference

Survila, M., Davidsson, P. R., Pennanen, V., Kariola, T., and Broberg, M. (2016). Peroxidase-Generated Apoplastic ROS Impair Cuticle Integrity and Contribute to DAMP-Elicited Defenses. 7, 1–16

Appendix 6

Analysis of gene expression

1. RNA extraction using Plant Total RNA Purification Kit

The protocol described below is used for leaf samples of Arabidopsis and wheat plants with minor modifications from the manufacturer's protocol.

- Quickly weigh no more than 100 mg of fresh tissue (about 20 leaf discs 5 mm in diameter for Arabidopsis and 10 leaf discs for wheat plant) and place samples into a 2 ml-tube containing 1 g of zirconia beads. The samples were fixed by liquid nitrogen and stored at -80oC until extraction.
- Add 450 µl of Plant RNA Lysis A/2-ME Solution A into a 2 ml-tube containing zirconia bead and frozen sample and homogenize at 3,000 rpm for 90 seconds in a shaker (Micro Smash M-100).
- Incubate at 60°C for 3 min. Centrifuge the tube at top speed (12,000~14,000 xg) for 5 min.
- Transfer the supernatant into an RNA Spin Filter (with yellow ring) inserted in a clean 2 ml Collection Tube. Spin at top speed for 5 min.

  ** Most tissue clumps and cell debris can be removed by Spin Filter, but a small amount will pass through the filter and form a pellet in the collection tube. Do not disturb the pellet.*
- Transfer the flow-through from the collection tube into a new 1.5 ml microcentrifuge tube, avoiding disrupting and pipetting the pellets. 5. Add 0.5 volumes (about 225 µl) of 100% Ethanol to the lysate, and mix well by pipetting.

  ** Do not centrifuge after Ethanol is added.*
- Load the lysate/ethanol mixture (<700 µl) into the RNA Spin Column inserted in a 2 ml collection tube, spin at top speed for 1 min, and discard the flow-through.

  ** If the volume of lysate/ethanol mixture is greater than 700 µl, apply any remaining lysate/ethanol mixture into the RNA spin column and repeat centrifugation step as above.*
- Place the RNA spin column to the original collection tube and add 500 µl of RNA Wash Solution I, spin at top speed for 1 min and discard the flow-through.
- DNase I digestion. For each isolation, premix 80 µl DNase I Incubation Buffer with 2 µl DNase I in a microcentrifuge tube (mix by flicking or inverting the tube, do not vortex!). Add 82 µl of the solution to the center of RNA spin column membrane and incubate it at room temperature for 15 min.

  ** If processing multiple samples at a time, prepare a fresh master mix of DNase I solution before use; do not store a premix of DNase I solution.*
- Add 500 µl of RNA Wash Solution I to the RNA Spin Column, spin at top speed for 1 min and then discard the flow-through.

- Place the RNA Spin Column into the original collection tube and add 600 µl of RNA Wash Solution II, spin at top speed for 1 min and then discard the flow-through. Repeat this step once more.
- Place the RNA Spin Column onto the original collection tube and spin at top speed for 3 min to remove any residual ethanol from RNA Wash Solution II used in the previous step. Transfer the RNA spin column in a clean 1.5 ml microcentrifuge tube.

  ** If centrifugation speed is lower than 12,000 xg or residual ethanol from RNA Wash Solution II must be removed completely, incubate the RNA spin column at 60 ~ 65°C in a drying oven for 5 min to evaporate all of the remaining ethanol.*
- Add 30~50 µl Nuclease-free water into the center of the RNA Spin Column membrane and let stand for 1 min. Centrifuge at top speed for 1 min.
- Estimate the RNA quantity and concentration using 2 µl of an aliquot by a spectrophotometer (Nano Drop D-1000). Adjust the RNA concentration to 150 ng/µl. The RNA sample can be stored at -80°C.
- Check the RNA quality using gel electrophoresis.

2. Check the RNA quality using a gel electrophoresis

- Make a 1,2% agarose gel for 12 wells.

Agarose	0.48 g
TAE buffer	40 ml
Total volume:	40 ml

- Prepare RNA loading sample by using 2 x RNA loading buffer without Ethidium bromide (Wako). Incubate the mixture of RNA and loading buffer at 70°C for 10 min, then place on ice.

Total RNA	4 µl
Loading buffer	4 µl
Total volume:	8 µl

- Place the gel into the electrophoresis sink containing 300 ml of TAE, then load all of 8 µl loading sample into each well. Run the electrophoresis system Mupid-2plus, 100V, in 30 min (do not open the cover during the processing time).
- Dye the gel by 0.01% Ethidium bromide solution and keep in the dark on a constant shaker for 20 mins.

Ethidium bromide	20 µl
TAE (or distilled water)	200 ml
Total volume:	8 µl

- Place the stained gel into the camera system (BioRad) under UV light and assess the quality of RNA by picture.

3. cDNA synthesis reaction

- Place 0.5 µg of total RNA (4 µl) and Oligo(dT)12-18 primer (0.5 µl) in a 0.2 ml-tube in a final volume of 5 µl, and incubated at 70℃ for 10 min, then place on ice.

Total RNA (500 ng):	4 µl
Oligo(dT)12-18 primer (1 µg/ µl):	0.5 µl (0.5 µg)
Nuclease-free water:	0.5 µl
Total volume:	5 µl

- Prepare a 10 µl reaction buffer by adding the following mixture. Spin down briefly.

Denatured RNA	5 µl
5X Reverse transcription buffer	2 µl
Ribonuclease inhibitor (40 U/ul; TaKaRa 2313A)	0.25 µl (10 U)
dNTP (10 mM each)	1 µl (1 mM)
AMV Reverse transcriptase (5 U/ul; TaKaRa 2630A)	0.1 µl (0.5 U)
Nuclease-free water	1.65 µl
Total volume:	10 µl

- Incubate the reaction mixture at 42℃ for 60 min.
- Heat the sample at 95℃ for 5 min, then incubated at 4℃ for 5 min.
- Dilute the first-strand cDNA synthesis reaction with TE buffer or nuclease-free water (add to 90 µl). Alternatively, store the first-strand cDNA at -20℃ until use.

4. Design PCR primers and check specificity

4.1. Design PCR primers

- Online software: Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) is used for primer design.
- Forward and reverse primer pairs for qPCR should amplify unique target sequences between 70-150 bp long, that are areas of low secondary structure (GC content < 60%). The primers themselves should follow the guidelines for good primer design.
- Since the multiple primers will be used in a single qPCR reaction, they should have similar annealing/temperatures.
- Once the primers arrive, it is recommended to make a 100 µM (100 pmol) stock which then dilutes to make a working stock (10 µM ~ 10 pmol). The 100 µM stock is kept at -20° C and opened only to create new working stocks.

4.2. Check primer specificity

- Check primer specificity by "BLAST" (nucleotide) the proposed primer sequence to ensure specificity for the target (amplified) gene.
- Check the specificity of the primers by doing a PCR reaction first and running the products on a gel.

PCR reaction:

cDNA reaction	2.0 µl
Nuclease-free water	9.5 µl
Upstream primer	0.5 µl
Downstream primer	0.5 µl
GoTaq master mix (2X)	12.5 µl
Total volume:	25 µl

Note: Since all genes checked in this study are defense-related genes which are supposed to be chemically inducible, cDNA should be taken from samples treated with activators such as saccharin or PBZ.

PCR program:

Temperature	Time	Cycles
95°C	5 mins	1
95°C	15 secs	30
62°C	30 secs	
72°C	2 mins	
72°C	2 mins	1
4°C	∞	1

Running electrophoresis:

- Make a 1,2% agarose gel for 12 wells (0.48 g of agarose + 40 ml TAE buffer)
- Place the gel into the electrophoresis sink containing 300 ml of TAE buffer, then load 8 µl of PCR reaction into each well. Do not forget to load 5 µl of the ladder (100 bp) into a well.
- Run the electrophoresis system Mupid-2plus, 100V, in 30 min (do not open the cover while running).
- Dye the gel by SYBR Green I solution (20 µl of SYBR Green I plus 200 ml TAE buffer) and keep in the dark on a constant shaker for 30 mins.
- Load the gel into the camera system (BioRad) under UV light and check the quality of RNA by picture.

5. qPCR reaction

Two µl of cDNA was amplified in 10 µl of reaction volume containing 5 µl of KAPA SYBR Fast qPCR master mix (2x) (KAPA Biosystem) and 10 pmol/ml of both forward and reverse gene-specific primers. The quantitative PCR reactions were performed on a Gene detection system GVP-9600 (Shimazu) using the following amplification program: an initial activation cycle of 95 °C for 3 minutes, 50 cycles at 95 °C for 3 seconds, 60 °C for 30 seconds, 1 ending cycle at 72 °C for 2 minutes, and a final melt curve analysis.

cDNA reaction	2 µl
Nuclease-free water	2.6 µl
Upstream primer	0.2 µl
Downstream primer	0.2 µl
KAPA SYBR	5 µl
Total volume:	10 µl

6. Data interpretation

Data from qPCR are obtained as a number of threshold cycle (Ct) at which detectable signal is achieved. Lower Ct means larger amount of starting template/material (cDNA of target amplified genes) in sample, and in reverse.

There are two methods for qPCR data analysis which are absolute quantification (using absolute curve) and relative quantification. In this study, later method is used to compare levels or changes in gene expression in the test samples with the control (also referred to as calibrator samples). Two approaches used in relative quantification are normalization against a unit mass which requires accurate quantification of starting material (not be used in this study) and normalization against a reference gene which follows the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The Ct value of a target gene in the test sample is normalized to that of the reference gene (*EF1-α* for Arabidopsis and RNase L inhibitor-like for wheat plants) and expressed relative to that in the control sample.

The $2^{-\Delta\Delta CT}$ method with 3 steps:

1. ΔCt = Ct (target) – Ct (reference)
2. ΔΔCt = ΔCt (test sample) - ΔCt (control sample)
3. Calculate expression ratio or fold difference by $2^{-\Delta\Delta Ct}$

Or follow Livak method with 2 steps as below:

1. Normalize gene expression for each sample (control or test)
   Relative expression = $2^{\Delta Ct\ (reference)\ -\ \Delta Ct\ (target)}$
2. Calculate expression ratio = Relative expression (control or test)/ Relative expression (control).

Finally, statistic data are reported in the form of $2^{-\Delta\Delta Ct}$ (expression ratio) with variation among replicate reactions. The control is reported as 1 without any error or with derivation of the $2^{-\Delta\Delta Ct}$ equation calculated as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859)).

Reference

Applied Biosystems (1997) User Bulletin # 2 ABI P RISM 7700 Sequence Detection System SUBJECT: Relative Quantitation of Gene Expression. p.35.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408