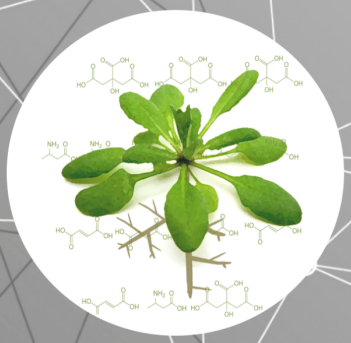
https://doi.org/10.35662/unine-thesis-2712

A dissertation submitted to the University of Neuchâtel for the degree of Doctor in Science by

Andrea Balmer

BABA and Co.- Deciphering the chemical vocabulary of priming



Thesis supervisor

Dr. Brigitte Mauch-Mani

Thesis committee

Dr. Brigitte Mauch-Mani, University of Neuchâtel, Switzerland

Dr. Victor Flors, Universitat Jaume I, Castellon, Spain

Dr. Gaétan Glauser, University of Neuchâtel, Switzerland

Dr. Daniel Croll, University of Neuchâtel, Switzerland

Defense the 4th of October of 2018

University of Neuchâtel

UNIVERSITÉ DE NEUCHÂTEL

Faculté des Sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel – Suisse
Tél : + 41 (0)32 718 21 00
E-mail : secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Andrea Balmer

Titre:

"BABA and Co. – Deciphering the chemical vocabulary of priming"

sur le rapport des membres du jury composé comme suit:

- Prof. associée, Brigitte Mauch-Mani, directrice de thèse, Université de Neuchâtel, Suisse
- Prof. assistant Daniel Croll, Université de Neuchâtel, Suisse
- Prof. Victor Flors, Universidad Jaume I, Castellon, Espagne
- Dr Gaétan Glauser, Université de Neuchâtel, Suisse

Neuchâtel, le 23 octobre 2018

Le Doyen, Prof. P. Felber

P. Fel

Keywords

β-aminobutyric acid (BABA), TCA, carboxylic acids, induced resistance, primary metabolism, priming, priming signals, abiotic stress, biotic stress.

Abstract

The defense system of a plant can be primed to respond faster and stronger to stress, resulting in an augmented stress resistance and/or tolerance. Priming can be triggered by biotic and abiotic stimuli, as well as by chemicals such as β-aminobutyric acid (BABA), a nonprotein amino acid which has been recently described to be naturally synthesized by plants. The effective induction of numerous defense mechanisms upon priming by BABA can involve a vast redistribution of the metabolic flux within both primary and secondary metabolisms. The aim of my thesis was to enhance our understanding of the priming phenomenon using the model plant Arabidopsis, the chemical priming agents synthetic BABA and tricarboxylic acids (TCAs) and natural BABA as case studies. Following a general introduction into plant defense and priming, in a first part I elaborate the capacity of some TCAs as priming agents. In a second part, I highlight the kinetics and proteomic signature triggered by the treatment with synthetic BABA in Arabidopsis and in a third part finally, I focus on the behavior of natural (endogenous) BABA from a physiological point of view.

Altogether I observed that application of the TCAs citrate and fumarate, two major components of the TCA cycle, was inducing priming in Arabidopsis against the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 through complex signaling pathways characterized by alterations in phytohormone balances and camalexine accumulation. Furthermore, in line with the described fast uptake of synthetic BABA by roots and its swift translocation into leaves, I observed a rapid and massive alteration in tissue- and time-point- specific proteomic patterns of various pathways including cellular respiration, salicylic acid signaling, carbon/nitrogen balance and stress signaling. In Arabidopsis, BABA levels were not only found to be elevated after several biotic and abiotic stresses but as well as during senescence. Intriguingly, endogenous BABA did not show a systemic accumulation in plant tissues, neither from shoot to root, nor leaf to leaf.

This thesis uncovers new aspects and sheds light on the puzzle of the plant priming phenomenon, highlighting as well a promising new role of TCAs as a priming agents that could be further tested and exploited in terms of potential and application for crop protection.

Mots clés

Acide β-Aminobutyrique (BABA), TCA, acides carboxyliques, résistance induite, métabolisme primaire, priming, signaux de défense, stress abiotiques, stress biotiques.

Résumé

Le système de défense d'une plante peut être stimulé à réagir plus rapidement et plus fortement au stress, ce qui entraîne une résistance et / ou une tolérance accrues. L'amorçage ou "priming" peut être déclenché par des stimuli biotiques et abiotiques, ainsi que par des produits chimiques tels que l'acide **β**-aminobutyrique (BABA), un acide aminé non protéique récemment décrit comme étant naturellement synthétisé par les plantes. L'induction efficace de nombreux mécanismes de défense lors du priming induit par BABA peut impliquer une vaste redistribution du flux métabolique des métabolismes primaires et secondaires. L'objectif de cette thèse était d'améliorer notre compréhension du phénomène du priming en utilisant la plante modèle Arabidopsis, les inducteurs du priming BABA synthétique et acides tricarboxyliques (TCA) ainsi que le BABA naturel. Après une introduction générale sur la défense de la plante et le priming, la capacité de certains TCAs en tant qu'agents de priming est analysée. Ensuite je présente la cinétique et la signature protéomique déclenchées par le traitement au BABA synthétique chez Arabidopsis pour finalement mettre l'accent sur le comportement du BABA naturel (endogène) d'un point de vue physiologique.

J'ai observé que l'application de citrate et de fumarate, deux composants majeurs du cycle du TCA, induisait une résistante chez Arabidopsis contre le pathogène bactérien *Pseudomonas syringae* pv. tomato DC3000 par des voies de signalisation complexes et par des altérations au niveau des phytohormones et de la camalexine. En outre, conformément à l'absorption rapide décrite de BABA synthétique par les racines et à sa translocation rapide dans les feuilles, j'ai observé une altération protéique rapide et massive au niveau des tissues et dans le temps de diverses voies, dont la respiration cellulaire, la signalisation de l'acide salicylique, et la signalisation de stress. Chez Arabidopsis, les taux de BABA ont non seulement été induits après différents stress biotiques et abiotiques, mais aussi pendant la sénescence. Curieusement, le BABA endogène n'est pas accumulé de façons systémiques dans les tissus végétaux, ni de la pousse à la racine, ni de feuille en feuille.

Cette thèse met en évidence de nouveaux aspects et contribue à l'éclaircissement du phénomène du priming, soulignant également le nouveau rôle prometteur des TCAs en tant qu'agents de

priming pouvant être davantage testés et exploités en termes de potentiel et d'application dans la protection des cultures.

Table of contents

Chapter I- **General Introduction**

The 'prime-ome': towards a holistic approach to priming

Adapted from:
Balmer, A., Pastor, V., Gamir, J., Flors, V., & Mauch-Mani, B. (2015). The 'prime-ome': towards a holistic approach to priming. Trends in plant science, 20(7), 443-452.

Keywords
Priming, induced resistance, transcriptomics, proteomics, metabolomics, biotic stress

The 'prime-ome': towards a holistic approach to priming

Introduction

Plants possess complex strategies to survive and adapt to possible damage resulting from natural interactions with many different types of biotic and abiotic stress such a localized pathogen attack, high temperatures, salinity or chemical treatments. In addition to the constitutively expressed barriers and upon recognition of stress, plants have developed a number of inducible defense mechanisms. Depending on the nature of damage and in the case of a pathogen attack, specific signal transduction pathways are induced, leading to the expression of local acquired resistance (LAR) or systemic acquired resistance (SAR), a set of local and systemic defense responses. Local contact with the pathogen or stress can also level up the defensive state of a plant before a secondary challenging stress. This phenomenon called priming is an induced state whereby basal and further layers of defense are potentiated to react more rapidly and more efficiently to a given stress. Synthetic as well as biological compounds can activate priming. Primed plants can respond faster and stronger to stress, and multi-facetted pathways - each specific for the type of encountered challenge, are orchestrating priming. This flexibility of priming makes it difficult to pinpoint an exact mechanism: the same phenotypic observation might be the consequence of unrelated underlying events. Recently, details of the molecular aspects of establishing a primed state and its transfer to the plant offspring have come to light. Technical advances for detection and quantification of small molecules spanning the fields of transcriptomics, proteomics, and metabolomics, together with adequate bioinformatics tools, will soon allow us to take a holistic and systems approach towards plant defense. In the following chapter, current knowledge and state of the art knowledge of "omics" during plant priming are reviewed and discussed.

Inducible plant immunity

During evolution plants have developed various defense strategies to counter possible stressful situations triggered by changing environmental conditions or through their contact with numerous pests and pathogenic microorganisms that are rapidly evolving to evade host defenses. One strategy first in line is mediated by inducible defenses associated with local acquired resistance (LAR) and systemic acquired resistance (SAR) (Durrant and Dong, 2004), both defense mechanisms are based on the systemic immunization of adjacent cells. Following penetration of the plant cell wall by a pathogen, the plant perceives Microbial Associated Molecular Patterns (MAMPs), leading to the activation of pattern-triggered immunity (PTI) as a basal defense response (Couto and Zipfel, 2016).The cardinal example of such a plant response to MAMPs is the perception of flagellin, a protein subunit of bacterial flagella (Zipfel et al. 2004). However, pathogens have developed a variety of means to suppress PTI by interfering with recognition of MAMPs through the secretion of effector molecules into the plant cell. But once pathogens acquired the capacity to suppress primary defenses, plants developed a more specific mechanism called effector triggered immunity (ETI, Jones and Dangl, 2006) to detect pathogen effectors; this mechanism involves resistance (*R*) gene dependent resistance (Chisholm et al. 2006).

The recognition between plants and microorganisms mediated by *R* genes and by the perception of MAMPs leads to the activation of a series of cellular signals, resulting in the establishment of a complex network of defenses, such as the initiation of localized cell death, known as hypersensitive response (HR) (Birch et al. 2018). Similarly, the production of signaling molecules includes hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (Et) (Narusaka et al. 2004). These hormones are rapidly induced in response to elicitors and play a key role in the expression of defense genes, leading amongst others to the accumulation of phytoalexins and protease inhibitors. The entire signaling process culminates in the expression of defense genes driven by transcription factors (Birkenbihl et al. 2017). Another defense system in plants is based on the potentiation of their immune system by using their 'priming memory'.

Priming has been defined as an induced state whereby a plant reacts more rapidly and or more efficiently to a stress (Conrath et al. 2009). Priming events can occur as a result of interindividual or interspecies communication, such as induced resistance mediated by rhizobacteria, mycorrhizal fungi, or virulent or avirulent pathogens or by natural or axenic compounds (Conrath et al. 2015;

Mauch-Mani et al. 2017; Fernández-Crespo et al. 2017). Plants 'remember' such events and, depending on the type of primary stimulus (initial trigger for priming) and the pathosystem involved (target of priming), primed plants can deploy a diverse set of defense mechanisms. Recently, it has become apparent that the specific defense mechanisms also depend strongly on the priming state and priming has been divided into a 'priming phase', a 'post- challenge primed state', and a 'transgenerational primed state' (Gamir et al. 2014; Pastor et al. 2014; Balmer et al. 2018) (**Figure 1**).

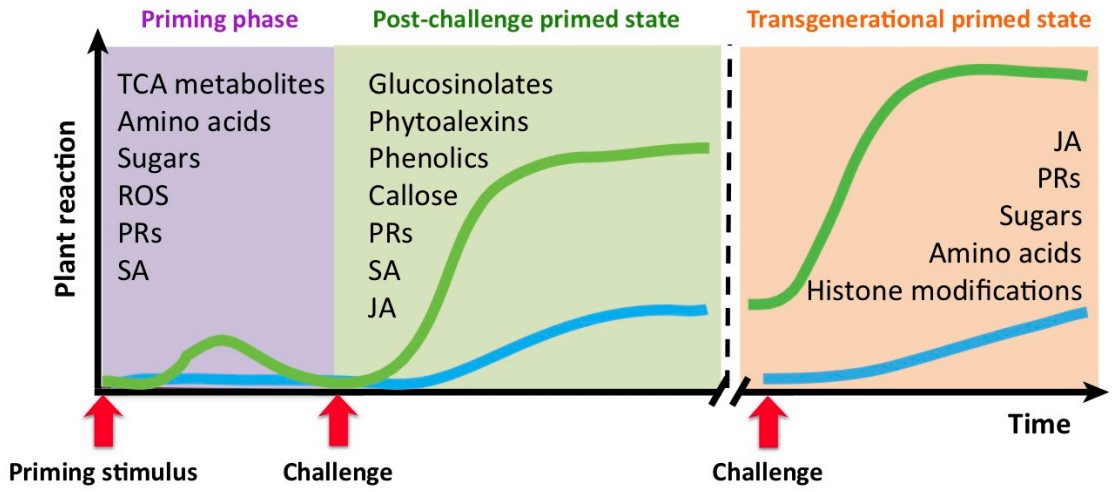


Figure 1. The various states in priming. The priming state is triggered by a priming stimulus and lasts until the plant is exposed to a challenging stress. During the priming stage, the levels of various primary and secondary metabolites, enzymes, hormones, and other molecules are slightly altered, putting the plant in a standby state. On challenge with a stress, the plant enters the post-challenge primed state during which the appropriate reactions to combat the given stressor are induced rapidly. The transgenerational primed state is found in plants generated from seeds stemming from primed parental plants that have a priming memory and are thus able to react more rapidly and more adequately when challenged by a stressor. The green line represents the reaction level and speed of plants that have been primed and the blue line shows the reaction level of plants that have not been primed.

The elucidation of priming phenomena usually focuses on a genetic, proteomic, metabolomic, or physiological approach. Consequently, it deals with only part of the entire priming event. In mammalian cells, recent reports show that mRNA regulation explains only about 40% of the changes in protein abundance (Schwanhausser et al. 2011); thus, focusing on one of the omics might lead to misinterpretation of physiological responses. In potato (*Solanum tuberosum*) plants primed by β-aminobutyric acid (BABA), the low overlap between transcript regulation and apoplast protein abundance after infection confirms the importance of combining several approaches (Bengtsson et al. 2014). Metabolites, as the final product of most cellular metabolic pathways, act

as regulatory components of biological information and thus allow a more complete picture of the physiological state of a plant (Pastor et al. 2014). Although priming is an integral part of both ISR (induced systemic resistance) and SAR (systemic acquired resistance), only priming has no or minimal negative impact on the host plants' energy status. Primed plants deploy their defense repertoire only during pathogen attack and not in a constitutive manner. Induction of direct defenses is often associated with high physiological costs that can minimise the benefits of the enhanced protection. By contrast, primed plants show almost equal protection levels but suffer from considerably lower fitness costs (Van Hulten et al. 2006; Wang et al. 2015). This makes priming-based approaches valuable for crop protection strategies.

Priming induced by BABA

Besides biotic and abiotic stress, various synthetic compounds such as the non-protein amino acid β-aminobutyric acid (BABA) are capable of inducing resistance and priming in treated plants. BABA has been shown to protect a range of plant species including Arabidopsis against different virulent pathogens, insect herbivores, nematodes and abiotic stress by potentiating specific plant resistance mechanisms (reviewed by Balmer et al. 2015).

The mechanisms of priming induced by BABA as described in Arabidopsis imply multiple defense responses such a faster induction of reactive oxygen species (ROS), callose accumulation, induction of metabolites or production of plant defense hormones, like SA, JA or abscisic acid (ABA) (Baccelli and Mauch-Mani 2016). However, it seems intricate to assign a clearly defined signaling pattern to a specific stage of priming. The choice and the effectiveness of the augmented defense reactions during the post-challenge stages depends strongly on multiple factors such as the type of plant/challenger as well as the developmental stage or environmental inputs (Balmer et al. 2015). As a biological example, BABA primed Arabidopsis develop resistance against the biotrophic pathogens *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* pv. tomato based on SA-dependent defense mechanisms. Interestingly, a similar mechanism was also described for the necrotrophic fungus *Botrytis cinerea* in which an augmented expression of the SA-inducible pathogen-related gene *PR-1* was detected (Zimmerli et al. 2000, 2001). BABA also primes for a faster and stronger deposition of callose-rich papillae under the appressoria of pathogenic fungi and oomycetes (Nishiruma et al. 2003; Ton and Mauch-Mani 2004). In contrast, it was shown that

SA-dependent resistance does not contribute to the level of BABA-triggered priming against the necrotrophic fungi *Alternaria brassicicola*, and that a functional ABA signaling pathway was required. In other pathosystems, like tomato, tobacco and potato plants primed by BABA and infected by *Phytophthora infestans*, the role of SA, ROS and callose deposition has also been documented (Sharma et al. 2012; Siegrist et al. 2000; Eschen-Lippold et al. 2010; Meller et al. 2018).

Omics in research on priming for defenses against pathogens and pests: prime-omics

In recent years the availability of improved analytical techniques revealed that plant priming can be separated into distinct stages (Gamir et al. 2014). The priming phase, starting immediately after perception of the priming stimulus and ending with the exposure to a challenge, was hitherto generally described as uneventful and strong up- or down-regulation of gene activity was observed only in the post-challenge primed phase. Recently, the effect of priming was recognized to be active in the progeny of a primed plant; that is, at the transgenerational level (Slaughter et al. 2012; Rasmann et al. 2012; Mandal et al. 2012; Luna et al. 2012). **Table 1 A,B,C** gives an overview of recently available information about priming against biotic stressors including bacteria, oomycetes, fungi, nematodes, and arthropods and the following sections give details of the underlying mechanisms.

Prime-omics in defenses against bacteria

a. Events in the priming phase

This phase usually involves changes in primary metabolites such as sugars, amino acids, and tricarboxylic acid derivatives (Pastor et al. 2014). Although priming with BABA and with avirulent bacteria leads to the same resistance phenotype, their impact on the plant's metabolome partially differs (Pastor et al. 2014). Interestingly, conjugated forms of plant hormones accumulating during the priming state support the idea that they can be rapidly hydrolyzed to their active forms to respond faster against a pathogenic invasion (Pastor et al. 2014; Pastor et al. 2013). Using a qRT-PCR approach to analyze selected marker genes, *Trichoderma asperellum* was described to prime *Arabidopsis thaliana* defense against virulent *Pseudomonas syringae* (*Pst*) without causing major

changes in gene expression during the priming phase. Only a few genes – most of them related to ethylene (Et)/jasmonic acid (JA) signaling – were significantly affected in leaves of plants primed by *T. asperellum*. However, compared with non-primed plants, *T. asperellum*-inoculated plants that suffered subsequent infection by *Pst* showed major differences in defense gene expression patterns (Brotman et al. 2012). At the metabolomic level, various amino acid precursors of plant defense metabolites (Coruzzi et al. 2000) have been shown to accumulate to a higher level following priming with *T. asperellum*, but also with chemicals such as pipecolic acid (Brotman et al. 2012; Vogel-Adghough et al. 2013).Most efforts in characterizing the changes occurring in primed plants on bacterial infection have concentrated on targeted approaches based on expression profiling of key plant defense genes involved in SA, JA, and Et defense signaling, accumulation of mitogen-activated protein kinases (MAPKs), and production of ROS. Proteomic and metabolomic mechanisms remain poorly understood.

b. Events in the post-challenge priming state

SA plays an important role in regulating the primed state during defense against bacteria (Ton et al. 2005). For instance, tobacco (*Nicotiana tabacum*) plants inoculated with disarmed *Agrobacterium tumefaciens* before infection by virulent *Pst* showed primed expression of SA as well as of *MAPK* pathway genes, rapid production of ROS, and an increase in callose deposition (Sheikh et al. 2014; Rico et al. 2010). In Arabidopsis, priming stimuli like BABA, the dicarboxylic acid azelaic acid, the tricarboxylic acids citrate and fumarate, the diterpenoid dehydroabietinal, and inoculation with plant growth-promoting rhizobacteria increased the plants' resistance against virulent *Pst* strains and concomitantly primed the accumulation of ROS, SA, and pathogenesis-related protein 1 (*PR1*) expression (Jung et al. 2009; Dempsey and Klessig, 2012; Balmer et al. 2018; Van de Mortal et al. 2012). Potentiation of the SA response and prevention of jasmonate-isoleucine (JA-Ile) accumulation on challenge with *Pst* was correlated with prior priming through hexanoic acid in tomato (*Solanum lycopersicum*) (Scalschi et al. 2013). In field experiments, volatile organic compounds (VOCs) produced by pepper (*Capsicum annuum*) plants on colonization with growth-promoting rhizobacteria significantly reduced disease severity against Xanthomonads through priming of SA-related and JA-dependent defenses (Choi et al. 2014).

Other plant hormones and metabolites are also involved in priming against bacteria. In the subsequent *T. asperellum*-primed Arabidopsis state following challenge with *Pst*, a substantial

increase in the number of upregulated genes was identified (Brotman et al. 2012). The most significantly induced genes were transcription factors involved in JA-mediated defense, regulation of indolic glucosinolate biosynthesis, and modulation of plant responses to auxin (Xu et al. 2006; Gigolashvili et al. 2007). Moreover, SA- and Et/JA-responsive genes also showed a massive increase in expression in *T. asperellum*-primed Arabidopsis infected by *Pst* (Brotman et al. 2012). In addition to gene expression analysis, *T. asperellum*-primed Arabidopsis were subjected to a metabolomic analysis that revealed an altered metabolic signature following infection with Pst compared with non-primed plants. Significant changes in amino acid composition such as leucine, valine, isoleucine, methionine, and glutamine, sugars, and citric acid intermediates in addition to polyamine levels became apparent (Brotman et al. 2012). Putative phenolic acid derivatives were also identified as important metabolites produced by plants primed during antibacterial defenses (Luzzato et al. 2007). A good example of an integrated approach is a study on priming Arabidopsis with *Pseudomonas fluorescens* where the integration of a genome-wide transcriptomic and metabolomic analysis revealed that camalexin and glucosinolates play an important role in protection against *Pst*. The results were validated with Arabidopsis mutants in the corresponding pathways (Van de Mortel et al. 2012).

In Calla lily (*Zantedeschia aethiopica*), priming with methyl jasmonate (MeJA) against *Pectobacterium carotovorum* is accompanied by an increase in secondary metabolites and oxidising enzyme activity (Luzzato et al. 2007). A non-targeted approach of label-free mass spectrometry (MS) to monitor protein levels revealed 215 proteins whose expression levels were altered following MeJA priming and following infection with the pathogen. Among these, proteins showing the highest accumulation included nucleic acid-binding proteins, ion-binding proteins, cofactor-binding proteins, transferases, hydrolases, and oxidoreductases. Additionally, *in planta* localisation of oxidoreductases revealed that increased enzymatic activities were restricted to the zones surrounding the pathogen penetration sites of *Z. aethiopica* with *P. carotovorum* (Luzzato-Knaan et al. 2014). This strongly suggests an involvement in plant defense.

c. Events in the transgenerational primed state

Although events in the transgenerational primed state remain largely unknown, some recent studies indicate an epigenetic basis regulating this state of defense priming. Jaskiewicz and colleagues 2011, described long-lasting priming following acibenzolar S-methyl treatment or bacterial infection that was regulated by chromatin remodelling and histone modifications. Further studies on the methylation status of promoters of defense-related genes in the offspring of BABA-treated or avirulent *Pst*-inoculated plants suggested that the hypomethylation of transcription factors regulating *PR1* expression may be a plausible mechanism to explain transgenerational priming in which non expressor of pathogenesis-related gene 1 (*NPR1*) plays a critical role. However, the authors concluded that an epigenetic basis of priming is just emerging and further research is needed (Slaughter et al. 2012; Luna et al. 2012). In summary, the available data reveal a multifaceted priming phase and post-challenge primed state against bacteria where the involved defense arsenal strongly depends on the given priming inducer and challenging pathogen.

Prime-omics in defense against oomycetes

Although priming against oomycetes has been known for a long time (Ton et al. 2005; Zimmerli et al. 2000), the omics knowledge in this field is rather scarce. In Arabidopsis, priming against *Hyaloperonospora arabidopsidis* can be achieved by various treatments such as BABA (Zimmerli et al. 2000) or phosphite (Massoud et al. 2012). A full 'omics' layout of this process in Arabidopsis is missing; nevertheless, recent work on *S. tuberosum* deciphering phosphite-induced resistance against *Phytophthora infestans* has provided insights into transcriptional and secretomic priming responses (Dhar Burra et al. 2014). One of the few proteomics priming studies explored the response of potato primed against *P. infestans* by phosphite (Lim et al. 2013), aiming to identify proteins that might orchestrate priming. It showed that phosphite induces a phenocopy of a hypersensitive response (HR). Recent studies dissected the leaf proteome of potato treated with BABA, GABA, laminarin, and 2,6-dichloroisonicotinic acid (INA) (Bengtsson et al. 2014; Arasimowicz-Jelonek et al. 2013). Twenty-five proteins commonly induced by these priming agents were identified, most of them belonging to the primary metabolism but also including redox-regulating enzymes. A proteomic analysis of pearl millet (*Pennisetum glaucum*) treated with BABA or inoculated with *P. fluorescens* demonstrated that BABA treatment led to a decrease in the abundance of most of the identified proteins involved in energy and primary metabolism,

secondary metabolism, cell structure, and signal transduction during the priming phase. Exposure to *P. fluorescens* had a much lesser effect on protein abundance (Anup et al. 2015). Interestingly, the protein accumulation pattern of such primed plants following inoculation with *Sclerospora graminicola* revealed that only a few proteins were common to the two priming treatments, suggesting that the defense mounted in the post-challenge primed state differed for the two priming treatments (Anup et al. 2015).

Table 1. Overview of the omics involved in **A.** priming phase in plants and **B.** post-challenged primed state in plants against bacteria and oomycetes.

	Plant	Priming by	Priming against	-omics gen-	-omics metabol-	-omics prote-	Suggested mechanism	Reference
A.								
Priming phase	*Nicotiana tabacum*	*Agrobacterium tumefaciens GV3101*	-	√		√	SA, ROS, MAPK	Sheikh et al. 2014
	Arabidopsis thaliana	*Trichoderma asperellum T203* BABA *Pseudomonas syringae*	-	√	√		Et/JA signalling, phytohormones, polyamines, amino acids, TCA, purines, indolic compounds, fatty acids	Brotman et al. 2012; Pastor et al. 2014
	Solanum tuberosum	Phosphite BABA GABA Laminarin INA	-	√	√	√	SA, PR proteins, PTI, HR, wall-associated kinase, primary metabolism, TCA, ROS, Ca2+-dependent pathway redox-regulating enzymes, sesquiterpene phytoalexin biosynthesis	Dhar Burra et al. 2013; Lim et al. 2014; Arasimowicz-Jelonek et al. 2013; Bengtsson et al. 2014.
	Hordeum vulgare	*Pseudomonas fluorescens*	-	√			Detoxification, lipid transfer, cell wall biosynthesis, JA	Petti et al. 2010
	Zea mays	Synthetic indole dispensers	-		√		HIPVs, ABA, JA, JA-Ile	Erb et al. 2015
B.								
			Bacteria					
Post-challenged primed state	*N. tabacum*	*Agrobacterium tumefaciens GV3101* Pipecolic acid	*P. syringae pv. tabaci.* *P. syringae pv. tomato* DC3000	√	√	√	Callose deposition, SA, nicotine	Sheikh et al. 2014; Rico et al. 2010; Vogel-Adghough et al. 2013
	A. thaliana	Azelaic acid *P. fluorescens* ABA *T. asperellum T203*	*P. syringae pv. maculicola PmaDG3* *P. syringae pv. tomato* DC3000	√	√		SA, camalexine, callose deposition, PR genes, Et/JA-responsive genes, indolic glucosinolate	Jung et al. 2009; Van de Mortel et al. 2012; Oide et al. 2013; Brotman et al. 2012
	Solanum lycopersicum	Hexanoic acid	*P. syringae pv. tomato DC300*	√	√		JA biosynthesis, SA-responsive genes	Scalschi et al. 2013
	Capsicum annuum	VOC 3-pentanol	*Xanthomonas axonopodis pv. vesicatoria*	√			SA, JA	Choi et al. 2014
	Zantedeschia aethiopica	MeJA	*Pectobacterium carotovorum*		√	√	Phenolic acids, nucleic acid-, cofactor-, and ion-binding proteins, transferases, hydrolases, oxidoreductases	Luzatto et al. 2007; Luzatto et al. 2014
			Oomycetes					
	Vitis. spp	Methionine	*Plasmopora viticola*	√	√		ROS	Boubakri et al. 2013
	A. thaliana	Phosphite	*Hyaloperonospora arabidopsidis*	√	√	√	SA	Massoud et al. 2012
	S. tuberosum	Phosphite BABA	*Phytophthora infestans*		√	√	Callose deposition, HR, TCA, PRs gene expression	Lim et al. 2014 Meller et al. 2018

Prime-omics in defense against fungi

All reports on priming against fungal diseases concentrate on the investigation of the post-challenge primed state. Besides the classical cases of priming of specific defense genes such as *PR1* or *PR5*, transcriptomic knowledge of primed plants is scarce. Only a few transcriptomes of plants primed against fungi are available.

Notably, the nature of priming inducers as well as the pathosystem strongly influence a specific primed state, which makes it elusive to define general rules of how primed plants react. However, recent results summarized below serve as a basis to understand the importance of the diverse plant defense – omics during priming and the primed phase. A transcriptomic analysis of ISR induced by the fungus *Trichoderma hamatum* T382 against *Botrytis cinerea* was conducted in Arabidopsis plants. Both the priming phase and the post-challenge primed state were studied to decipher the molecular basis for this particular interaction (Mathys et al. 2012). Rather than just describing the genes that are up- or downregulated by the fungus Trichoderma, the authors describe pathways implicated in the ISR, with the aim of giving a wider view of the ISR process. Interestingly, they show that the establishment of ISR during the priming phase is not dependent of JA/Et signaling pathways although PR1 is accumulated during this priming phase, supporting the relevance of PR proteins during this phase. In addition, the post-challenge primed state is characterized by an increased response to JA and wounding pathways, which is a typical hallmark of ISR (Mathys et al. 2012). Barley (*Hordeum vulgare*) plants inoculated with *Piriformospora indica* show faster induction of 22 defense-related genes when infected with *Blumeria graminis* compared with control plants (Molitor et al. 2011). Similarly, the transcriptome of barley head tissue inoculated with *P. fluorescens* MKB158, a non-pathogenic resistance inducer, displays primed expression of a set of 74 genes; among them, lipid transfer proteins, protease inhibitors, and, in general, JA-related genes were identified (Petti et al. 2010).

Table 1 (Continued). Overview of the omics involved in **B**. post-challenged primed state in plants against fungi, nematodes and arthropods. **C**. transgenerational primed state.

	Plant	Priming by	Priming against	-omics gen-	-omics metabol-	-omics prote-	Suggested mechanism	Reference
B. Post-challenged primed state								
			Fungi					
	A. thaliana	BABA	*Plectosphaerella cucumerina*	√	√		Callose deposition, ROS, indole-3- carboxylic acid, sugar metabolism, cell wall, amino acids, IAA, camalexin	Pastor et al. 2013; Gamir et al. 2012; Gamir et al. 2014
	Brassicia carinata	BABA	*Alternaria brassicicola*		√		ROS	Chavan et al. 2013
	Triticum spp. line *PmA/var.* Sahara	H_2O_2, Z-3-HAC	*Blumeria graminis Fusarium graminearum*	√	√		JA and/or Et signalling pathways, lipid metabolism JA	Li et al. 2011; Ameye et al. 2015
	Hordeum vulgare	*P. fluorescens Piriformospora indica*	*Fusarium culmorum B. graminis*	√	√		IAA, JA, ABA, PR genes, sugar cycling, TCA, detoxification, lipid transfer, cell wall biosynthesis	Petti et al. 2012; Molitor et al. 2011
	S. lycopersicum	*Trichoderma harzianum*	*B. cinerea*	√			JA, SA, ABA	Martinez-Medina et al. 2013
	Cucumis sativus	*P. azotoformans Paenibacillus elgii* strain MMB22 BABA	*Colletotrichum orbiculare*		√		HR, H_2O_2 defense-related enzymes accumulation	Sang et al. 2014
			Nematodes					
	S. lycopersicum	Arbuscular mycorrhizal fungi	*Meloidogyne incognita*	√			Phenylpropanoid pathway. ROS metabolism	Vos et al. 2013
	Vitis. spp	Arbuscular mycorrhizal fungi	*Xiphinema index*	√			Chitinase, PR-genes, shikimate enzyme pathway	Hao et al. 2012
	Oryza sativa	Silicon	*M. incognita*	√	√		Et pathway, H_2O_2, phenolic compunds	Zhan et al. 2018
			Arthropods					
	Phaseolus lunatus	JA (E)-β-Ocimene	*Tetranychus urticae*	√	√		PIOS, volatile emission, JA, predator attraction	Menzel et al. 2014; Muroi et al. 2011
	A. thaliana	MeSA + feeding larvae Caterpillar feeding	*Pieris brassicae*	√	√	√	ABA, JA, oviposition deterrence	Groux et al. 2014; Vos et al. 2013
	S. lycopersicum	Arbuscular mycorrhizal fungi Aphid feeding	*Helicoverpa arimigera Bemisia tabaci*	√	√		Larval deterrence, JA signalling, systemin signalling, HIPVs – indirect defence	Song et al. 2013
	Ammopiptanthus mongolicus	Conspecifics HIPVs	*Orgyia ericae*		√		TCA, amino acids, lipids, glycolate, sugars, quinolizidine alkaloids	Sun et al. 2014
	O. sativa	Silicon	*Cnaphalocrocis medinalis*		√		JA signalling	Ye et al. 2013
	Z. mays	(E)-β-Ocimene	*Mythimna separata*		√		Parasitoid attraction	Muroi et al. 2011
C. Transgenerational primed state								
	S. lycopersicum A. thaliana	MeJA/herbivory	*Pieris raphae Spodoptera exigua*	√			JA-signaling	Rasmann et al. 2012
	N. tabacum	*Tobacco mosaic virus*	*Tobacco mosaic virus*		√		Sugars, amino acids	Mandal et al. 2012
	A. thaliana	*P. syringae pv.* tomato *DC3000*	*P. syringae pv.* tomato *DC3000 H. arabidopsidis*	√	√		SA-dependent genes, histone modifications	Luna et al. 2012
	S. tuberosum	BABA	*Phytophthora infestans*	√			Histone modifications, PRs gene expression	Meller et al. 2018

Although various metabolomics studies on plant–fungus interactions shed light on defense-related metabolites, knowledge about metabolites contributing to antifungal priming again is scarce. Antifungal priming against *B. cinerea* by application of flavonoid derivatives enhances early hydrogen peroxide (H_2O_2) accumulation in Arabidopsis (Malolepsza, 2005) similarly to BABA-treated *Brassica carinata* when attacked by *Alternaria brassicicola* (Chavan et al. 2013). H_2O_2 also regulates callose deposition in Arabidopsis attacked by *Plectosphaerella cucumerina* or *A. brassicicola*. A recent transcriptomic analysis of wheat seedlings treated with H_2O_2 showed induction of JA- and Et-related genes that are potential regulators of resistance against *Blumeria graminis* (Li et al. 2011). In the same plant species, the green leaf volatile (GLV) Z-3- hexenyl acetate (Z-3-HAC), which is often associated with defense against herbivores, was tested as a priming inducer against the hemibiotrophic fungus *Fusarium graminearum*. Primed wheat plants showed slower fungal disease progress through enhanced JA-dependent defense induction during the necrotrophic growth phase (Ameye et al. 2015). Additional metabolites mediating priming against fungal pathogens are indole-3-acetic acid (IAA) in wheat plants primed by *P. fluorescens* against *Fusarium culmorum* (Petti et al. 2012) and indole-3-carboxylic acid primed by BABA in Arabidopsis infected by *P. cucumerina* (Gamir et al. 2012). All of these compounds share the common fact that their mode of action in defense priming is not yet fully understood.

Only a little is known about whole-metabolome responses during antifungal priming. Most metabolomics studies of inducible antifungal resistance decipher induced systemic resistance phenomena. In tomato, for example, hormonal networks of Trichoderma-induced systemic resistance against *B. cinerea* are well described (Martinez-Medina et al. 2013). One of the few characterized metabolomic fingerprints during antifungal priming is described for the Arabidopsis–*P. cucumerina* interaction. Here, metabolites such as tryptophan, uronic acid, and purine derivatives have been identified in BABA- primed plants and in mutants expressing constitutive priming (Gamir et al. 2014ab). An interesting approach could be to study the fungal metabolome in response to priming. This should also provide a valuable foundation for a better understanding of antifungal priming. Nitric oxide (NO) is well known to promote HR in plants. Intriguingly, *Magnaporthe oryzae* was found to produce NO and its synthesis was shown to be required for successful infection of rice (*Oryza sativa*) (Samalova et al. 2013). Hence, future omics studies of fungal attackers might clarify whether fungi also make use of chemical compounds to ‘prime’ a plant to allow more efficient infection.

Prime-omics in defense against nematodes

The priming of plant defenses against plant pathogenic nematodes has until now been investigated mostly at the transcriptional level. The priming capacity of arbuscular mycorrhizal fungi (AMF) has potential in biocontrol against several types of nematode. In tomato plants primed by AMF, infestation by *Meloidogyne incognita* was significantly decreased in mycorrhized roots, probably due to involvement of the phenylpropanoid pathway in concert with ROS metabolism (Vos et al. 2013). Similarly, reduced gall formation and load of *Xiphinema index* nematodes in AMF-primed grapevine (*Vitis vinifera*) plants has been reported (Hao et al. 2012). Several defense genes were upregulated during AMF-induced control of *X. index* in Vitis, including genes encoding PR proteins and the enzyme 5-enolpyruvylshikimate 3-phosphate synthase (Hao et al. 2012). In rice (*Oryza sativa* cv Nipponbare) plants, root-applied silicon (Si) significantly reduce *M. incognita* infestation. This increase resistance was correlated with the production of phenolic compounds, hydrogen peroxide and Et pathway (Zhang et al. 2018). To our knowledge, no further omics studies have been conducted that elucidate priming responses against nematodes; therefore, further studies to obtain a complete picture of prime-omics against nematodes are needed.

Prime-omics in defense against arthropods

Plant responses to arthropod pests are highly dependent on the feeding habits. The way to perceive and respond to a chewing insect differs substantially from phloem-sucking to cell-content-feeding pests (Howe and Jander, 2008). In addition to the feeding style, plants can also respond specifically to different insect species with the same feeding habits. Notably, specific elicitation and specific responses were also proposed as components of defense priming against specialist lepidopteran (Tobacco hornworm) and coleopteran (Colorado potato beetle) species (Chung et al. 2011). As a reaction to chewing insects (tissue loss and oral secretions), specific molecular cues, besides inducing direct plant responses, can also prime systemic plant tissues and neighbouring plants and cause more efficient attraction towards natural enemies (Heil et al. 2006; Erb et al. 2015).

The speed with which an insect feeds on a plant requires faster responses of the plant compared with pathogens. This can be overcome by the release of herbivore-induced plant volatiles (HIPVs)

that can move through plant vessels but can also be released to the air and hence trigger faster priming in distal tissues and also in conspecific plant species in the nearby surroundings (Heil and Silva Bueno, 2007). Numerous such organic volatile priming compounds have been reported (Howe and Jander, 2008; Farmer and Ryan 1990; Matthes et al. 2010; Shulaev et al. 1997; Engelberth et al. 2004; Arimura et al. 2009). In addition to the production of HIPVs, following first contact with arthropods (Kim and Felton 2013) priming can be induced by overexpressing the ocymene synthase gene (Muroi et al. 2011) or by chemicals such as BABA, Si, or synthetic indole dispensers (Erb et al. 2015; Zhong et al. 2014; Ye et al. 2013).

Prime-omics of direct defenses against arthropods

a. In the priming phase

Mechanical damage, herbivores (specialist/generalist), and phytohormone treatments were shown to induce subtle changes in *Plantago lanceolata* systemic leaves (Sutter et al. 2011). Unlike the response to pathogen attack, plants induce primary metabolism to cope with aphids. Inducible metabolites against the foxglove aphid *Aulacorthum solani* were identified in constitutively resistant soybean cultivars (Sato et al. 2013). Resistant genotypes accumulated higher levels of various amino acids and of tricarboxylic acid (TCA) cycle intermediates compared with susceptible genotypes.

A known direct priming signal is insect egg oviposition. Tomato plants can perceive *Helicoverpa zeae* eggs, resulting in a slight but direct induction of pin-formed 2 (*PIN2*) in the priming phase. However, *PIN2* expression and JA accumulation are strongly primed if the plant is wounded within the hour following this priming cue (**Figure 2**) (Kim et al. 2012). Interestingly, priming seems to be mediated by different signals depending on the plant species and the insect (Gouhier-Darimont et al. 2013; Groux et al. 2014).

b. In the post-challenge state

Crosstalk between insect and pathogen signals has also been reported (Hilfiker et al. 2014). Following *Pieris brassicae* oviposition, SAR mediated by AGD2-like defense response protein1 (ALD1) and flavin-dependent monooxygenase (FMO) and transmitted by pipecolic acid is induced.

This egg-induced SAR primes systemic responses that confer resistance to *Pst*. The crosstalk seems to be reciprocal although less pronounced: *Pst* infection leads to reduced *P. brassicae* performance compared with mock- inoculated plants (Hilfiker et al. 2014). The finally accumulating toxic compounds such as glucosinolates (Bednarek et al. 2009) reduce pest growth and the plants' palatability. Regarding defense priming against pathogens, chemicals have also been shown to prime plants to be more resistant against arthropods. In rice, pretreatment with Si primes JA-dependent signaling leading to increased resistance against the caterpillar *Cnaphalocrocis medinalis*. *C. medinalis* feeding activates transcription of genes encoding Si transporters, suggesting an attempt of the plant to take up more Si from the soil and to influence down- stream defense priming (Ye et al. 2013). Abscisic acid (ABA) has been proposed as the starting signal that primes JA-dependent signaling in Arabidopsis against *Pieris rapae* infestation. Because ABA and oxophytodienoic acid (OPDA) rise only in local leaves whereas JA-Ile is induced in local damaged and distal undamaged leaves, ABA is a key regulator of herbivore-induced resistance priming of JA-dependent defenses on a second herbivore challenge in Arabidopsis (Vos et al. 2013). Another remarkable chemical priming inducer against insects is BABA. In BABA-treated soybean plants, enzymatic activities as well as expression of genes involved in SA signaling and ROS scavenging are primed in the presence of *Aphis glycines* and the plants display enhanced resistance against the soybean *A. glycines* Matsumura (Zhong et al. 2014).

Additionally, beneficial organisms such as arbuscular mycorrhizae also induce primed defenses against arthropods (Song et al. 2013). Tomato plants mycorrhized by *Funneliformis mosseae* (formerly *Glomus mosseae*) display enhanced resistance, reducing the performance of *Helicoverpa arimigera*. *F. mosseae* does not directly induce gene expression; however, on infestation the defense response is strongly primed. Here again, priming is mediated by JA-dependent signaling (Song et al. 2013). Similarly to defense priming against bacteria, epigenetic mechanisms have also been suggested to play a role in the transgenerational priming state against arthropods, where the inheritance of resistance against caterpillar herbivory was shown to be mediated by siRNAs in both Arabidopsis and tomato plants (Rasmann et al. 2012).

Prime-omics of indirect defenses by and against arthropods

A special priming mechanism by mirid insects was recently described (**Figure 2**) (Pérez-Hedo and Urbaneja, 2014). The entomophytophagous mirid *Nesidiocoris tenuis* pierces tomato plants thereby stimulating the release of HIPVs repellent to *Bemisia tabaci* (whitefly). Accelerated ABA- and JA-dependent signaling precedes the release of HIPVs. Interestingly, the mirid- pierced tomato plants are also more attractive to *Encarsia formosa*, a natural enemy of *B. tabaci*. The molecular mechanisms governing these surprising observations remain unknown but this multitrophic system that combines indirect defenses with priming is fully effective in the biocontrol of white flies. Although from an ecological and evolutionary view- point the effect of HIPVs on neighbouring plants remains controversial (Heil et al. 2014; Sun et al. 2014), it is clear that VOCs induced after an insect attack prime plants rather than directly induce changes in defense metabolism, as shown for Lima bean (*Phaseolus lunatus*), maize (*Zea mays*), and tobacco (Heil and Kost, 2006; Ton et al. 2007). Regarding plant–pathogen interactions, priming against insects can also be stable during several generations (Rasmann et al. 2012). In *Phaseolus lunatus*, JA treatment followed by minor herbivory primes for enhanced attraction of *Phytoseiulus persimilis*; hence, indirect defenses can be also primed (Gols et al. 2003). In such primed plants the ocimene synthase gene is strongly induced, giving rise to high levels of β-ocimene that repel spider mites and attract entomophagous mites such as *P. persimilis* (Arimura et al. 2004). Plant–plant priming has also been shown for mycelial networks of arbuscular mycorrhiza when warning neighbouring plants about aphid attacks (Babikova et al. 2013).

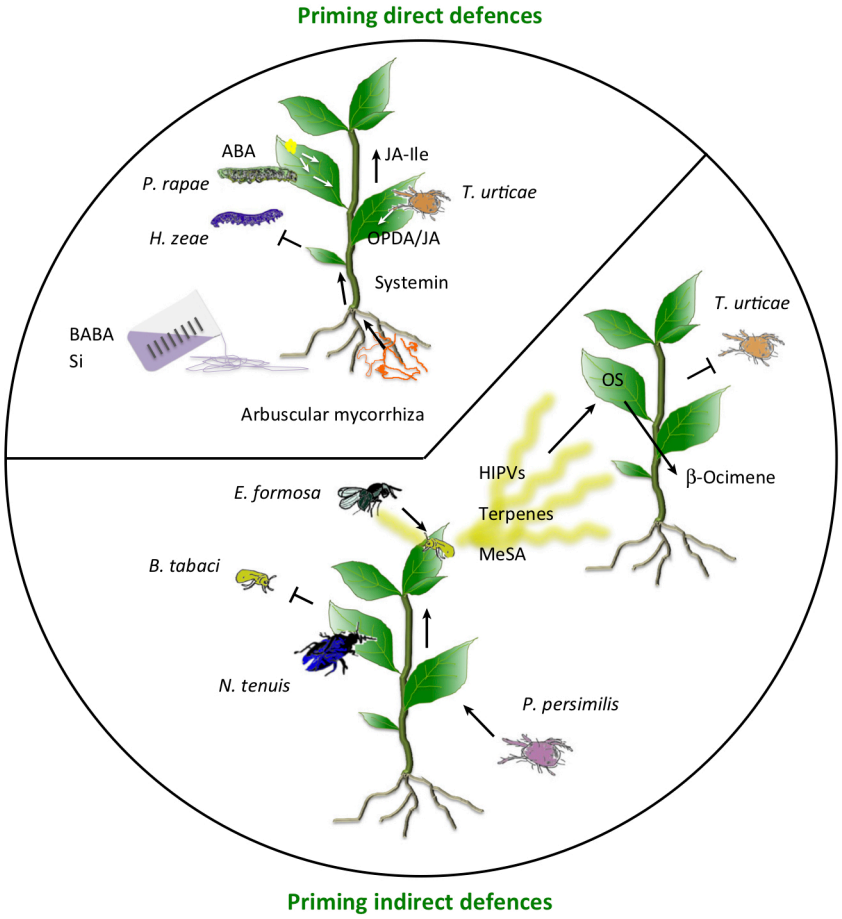


Figure 2. Direct and indirect priming against arthropods. Direct priming can be achieved through either chemical or natural stimuli. Natural stimuli can stem from arbuscular mycorrhiza, nonpathogenic rhizobacteria, or various arthropod cues such as oviposition or insect wounding. In priming against arthropods, abscisic acid (ABA) is a key regulator that activates defenses co-ordinately with jasmonic acid (JA)- and systemin-dependent signaling. Indirect defense priming is more diverse. It involves the enhancement of defense responses in neighbouring plants and also more efficient attraction of predators such as *Nesidiocoris tenuis* and *Phytoseiulus persimilis* and parasitoids as *Encarsia formosa*, but also strong antixenosis through the release of volatiles that repel *Bemisia tabaci*. Primed defenses in surrounding plants activate ocymene synthase (OS), which catalyses the accumulation of β-ocimene. Additionally, JA signallng is enhanced and, as a final consequence, insect performance is reduced in primed plants. Positive and negative regulatory actions are indicated by arrows and lines with bars, respectively. Abbreviations: BABA, β-aminobutyric acid; HIPV, herbivore-induced plant volatile; JA-Ile, jasmonate isoleucine; MeSA, methyl salicylate; OPDA, oxophytodienoic acid; Si, silicon.

Concluding remarks and future outlook

At the peak of the genomics era at the turn of the millennium, Trethewey and colleagues (1999) had already indicated the necessity of expanding the study of functional genomics towards metabolomic profiling, although they were wondering whether this might ever be technically possible. The study of gene expression only rarely gives holistic information, because often there is no clear link between changes in gene expression or protein synthesis and observed alterations in phenotype. Because of a potentially rapidly changing environment, plants rely on efficient mechanisms to quickly adapt their own metabolism to altered situations in order to achieve a new state of equilibrium.

The priming phase as a 'silent' moment before encountering a stress is characterized by minimal changes in primary metabolism (Pastor et al. 2014) that switch the plant defense mechanisms to a standby modus. The ideal situation would be to have a complete picture of the events occurring during the onset, persistence, and transmission of the primed state to the progeny. However, it seems not to be possible to assign a clearly defined, ubiquitously applicable gene expression or protein/metabolite accumulation pattern to a specific stage of priming. The reaction of the primed plant during the post-challenge stages depends strongly on the plant/challenger combination and is most certainly also influenced by other factors such as developmental stage or environmental inputs. In this context, it would be worth investigating whether there are any attempts from the pathogen or pest to influence the reaction of the plant regarding primed responses. Priming can reasonably be assumed to have developed over a long period in time to help plants rapidly adapt to new situations. Therefore, some adaptations on the attacker side are expected and these could comprise the manipulation of the reactions of primed plants to alleviate their impact on the pathogen or pest.

Another field where information is lacking is how priming is affected in the situation of combined stresses, either of the same type or between biotic and abiotic stresses. Extrapolating the results of studies with single stress conditions to such situations might lead to false conclusions. It has been commonly assumed that when similar gene expression or metabolite accumulation patterns are shared between plants, common pathways might be implicated (Quackenbush, 2003). This could explain why priming is effective against many different stresses. However, in the case of

combinations between biotic and abiotic stressors or different biotic stressors where negative crosstalk between the defense signaling pathways is known, much research remains to be done to find a satisfactory explanation for the observed primed phenotype.

Despite these open questions, by putting together the available pieces of the puzzle a holistic picture of priming is slowly beginning to materialize. The main part of this task still lies ahead of us, but with the help of technical advances and the development of metadata the goal now seems reachable. The creation of shared spaces such as the Data-Enabled Life Sciences Alliance (DELSA Global) (Kolker et al. 2014; Kolker and Stewart, 2014) that allow sharing of the available omics information by creating a multi-omics metadata checklist will substantially contribute to reaching this goal and also incite other groups to establish such integrated platforms.

Thesis outline

In this PhD thesis, the priming phenomenon was studied using distinct approaches in order to improve our understanding of chemical vocabulary of priming using Arabidopis, BABA and the TCAs citrate and fumarate as models.

In chapter II I adressed the question whether the underlying mechanism by which changes in carboxylic acid abundances induced by BABA may play a role during the priming phase. Herein I analyzed the capacity of citrate and fumarate - two principal TCA flux components - to induce priming in Arabidopsis against the phytopathogenic bacteria *Pseudomonas syringae* pv. tomato DC3000. Expression patterns of selected defenses genes, as well as perturbations in phytohormone levels as well as the phytoalexin camalexin were analyzed to identify possible defense pathways involved during primed plant defense responses. These experiments were conducted at different time points in order to cover the two characteristic priming phases, namely the primed stage and the priming phase.

Considering the eminent role of synthetic BABA as a chemical priming agent and the lack of data about the behavior of this molecule in Arabidopsis during the primed stage, we analyzed the kinetics of synthetic BABA in leaves and roots of Arabidopsis plants in the following chapter III. Subsequently, based on the accumulation pattern of synthetic BABA specific time points were selected in order to analyze the general proteomic changes induced by BABA at early and late time points after treatments of both leaves and root tissues.

Finally, recent results obtained in our group provide evidence that plants can naturally synthesize BABA. These exciting results rise the question about the mode of action of endogenous BABA in different plant tissues. The last section of this thesis (Chapter IV) aims to decipher the tissue-specific distribution of natural BABA during normal plant development and under salt stress conditions. This is complemented by answering underlying question about the possible systemic accumulation of endogenous BABA after pathogen stress as well as the implication of BABA during plant aging processes.
A general conclusion of the thesis and suggested perspectives for the project are addressed in chapter V.

References

Ameye, M. et al. (2015) Priming of wheat with the green leaf volatile Z- 3-hexenyl acetate enhances defense against *Fusarium graminearum* but boosts deoxynivalenol production. Plant Physiol. 167, 1671–1684

Anup, C.P. et al. (2015) Proteomic analysis of elicitation of downy mildew disease resistance in pearl millet by seed priming with β- aminobutyric acid and *Pseudomonas fluorescens*. J. Proteomics 120, 58–74

Arasimowicz-Jelonek, M. et al. (2013) The proteome response of potato leaves to priming agents and S-nitrosoglutathione. Plant Sci. 198, 83–90

Arimura, G. et al. (2004) Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (E)-β-ocimene and transcript accumulation of (E)-β-ocimene synthase in *Lotus japonicus*. Plant Physiol. 135, 1976–1983

Arimura, G. et al. (2009) Chemical and molecular ecology of herbivore- induced plant volatiles: proximate factors and their ultimate functions. Plant Cell Physiol. 50, 911–923

Babikova, Z. et al. (2013) Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. Ecol. Lett. 16, 835–843

Baccelli, I., Mauch-Mani, B. (2016) Beta-aminobutyric acid priming of plant defense: the role of ABA and other hormones. Plant Mol. Biol. 91, 703-711

Balmer, A. et al. (2018) Tricarboxylates induce defense priming against bacteria in *Arabidopsis thaliana*. Front. Plant Sci. 10.3389/fpls.2018.01221

Bednarek, P. et al. (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323, 101–106

Bengtsson, T. et al. (2014) Proteomics and transcriptomics of the BABA-induced resistance response in potato using a novel functional annotation approach. BMC Genomics 15, 31

Berendsen, R.L. et al. (2012) The rhizosphere microbiome and plant health. Trends Plant Sci. 17, 478–486

Birch, P. et al. (2018) Programmed cell death in plants in response to pathogen attack. Annu. Plant Rev. 184-208

Birkenbihl, R. et al. (2017) Transcriptional events defining plant immune responses. Curr. Opin. Plant Biol. 38, 1-9

Boubakri, H. et al. (2013) Methionine elicits H_2O_2 generation and defense gene expression in grapevine and reduces *Plasmopara viticola* infection. J. Plant Physiol. 170, 1561–156889

Brotman, Y. et al. (2012) Transcript and metabolite analysis of the Trichoderma-induced systemic resistance response to *Pseudomonas syringae* in *Arabidopsis thaliana*. Microbiology 158, 139–146

Chavan, V. et al. (2013) Temporal modulation of oxidant and antioxidative responses in *Brassica carinata* during β-aminobutyric acid-induced resistance against *Alternaria brassicae*. Physiol. Mol. Plant Pathol. 83, 35–39

Choi, H.K. et al. (2014) Field evaluation of the bacterial volatile derivative 3-pentanol in priming for induced resistance in pepper. J. Chem. Ecol. 40, 882–892

Chung, S.H. and Felton, G.W. (2011) Specificity of induced resistance in tomato against specialist lepidopteran and coleopteran species. J. Chem. Ecol. 37, 378–386

Conrath, U. et al. (2009) Priming of induced plant defense responses. Adv. Bot. Res. 51, 361–395

Conrath, U. et al. (2015) Priming for enhanced defense. Annu. Rev. Phytopathol. 53

Coruzzi, G. and Last, R. (2000) Amino acids. Biochem. Mol. Biol. Plant 358–410

Couto, D., Zipfel, C. (2016) Regulation of pattern recognition receptor signaling in plants. Nat. Rev. Immunol. 16, 537

Dhar Burra, D. et al. (2014) Phosphite-induced changes of the transcriptome and secretome in *Solanum tuberosum* leading to resistance against *Phytophthora infestans*. BMC Plant Biol. 14, 254

Dempsey, D.A. and Klessig, D.F. (2012) SOS – too many signals for systemic acquired resistance? Trends Plant Sci. 17, 538–545

Engelberth, J. et al. (2004) Airborne signals prime plants against insect herbivore attack. Proc. Natl. Acad. Sci. U.S.A. 101, 1781–1785

Erb, M. et al. (2015) Indole is an essential herbivore-induced volatile priming signal in maize. Nat. Commun. 6, 6273

Farmer, E.E. and Ryan, C.A. (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. U.S.A. 87, 7713–7716

Fernández-Crespo, E. et al. (2017) Hexanoic acid treatment prevents systemic MNSV movement in

Cucumis melo plants by priming callose deposition correlating SA and OPDA accumulation. Front. Plant Sci. 8, 1793

Gamir, J. et al. (2012) Identification of indole-3-carboxylic acid as mediator of priming against *Plectosphaerella cucumerina*. Plant Physiol. Biochem. 61, 169–179

Gamir, J. et al. (2014) Molecular and physiological stages of priming: how plants prepare for environmental challenges. Plant Cell Rep. 33, 1935–1949

Gamir, J. et al. (2014) Targeting novel chemical and constitutive primed metabolites against *Plectosphaerella cucumerina*. Plant J. 2, 227–240

Gigolashvili, T. et al. (2007) The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J. 50, 886–901

Gols, R. et al. (2003) Induction of direct and indirect plant responses by jasmonic acid, low spider mite densities, or a combination of jasmonic acid treatment and spider mite infestation. J. Chem. Ecol. 29, 2651–2666

Gouhier-Darimont, C. et al. (2013) Signaling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP- triggered immunity. J. Exp. Bot. 64, 665–674

Groux, R. et al. (2014) Role of methyl salicylate on oviposition deterrence in *Arabidopsis thaliana*. J. Chem. Ecol. 40, 754–759

Hao, Z. et al. (2012) Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defense gene responses in grapevine. J. Exp. Bot. 63, 3657–3672

Heil, M. and Kost, C. (2006) Priming indirect defenses. Ecol. Lett. 9, 813–817

Heil, M. and Silva Bueno, J.C. (2007) Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proc. Natl. Acad. Sci. U.S.A. 104, 5467–5472

Heil, M. (2014) Herbivore-induced plant volatiles: targets, perception and unanswered questions. New Phytol. 204, 297–306

Hilfiker, O. et al. (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J. 80, 1085–1094

Howe, G.A. and Jander, G. (2008) Plant immunity to insect herbivores. Annu. Rev. Plant Biol. 59, 41–66

Jaskiewicz, M. et al. (2011) Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. EMBO Rep. 12, 50–55

Jung, H.W. et al. (2009) Priming in systemic plant immunity. Science 324, 89–91

Kim, J. and Felton, G.W. (2013) Priming of antiherbivore defensive responses in plants. Insect Sci. 20, 273–285

Kim, J. et al. (2012) Insect eggs can enhance wound response in plants: a study system of tomato *Solanum lycopersicum* L. and *Helicoverpa zea* Boddie. PLoS ONE 7, e37420

Kolker, L. et al. (2014) Toward more transparent and reproducible omics studies through a common metadata checklist and data publications. OMICS 18, 10–14

Kolker, E. and Stewart, E. (2014) OMICS studies: how about metadata checklist and data publications? J. Proteome Res. 13, 1783–1784

Li, A. et al. (2011) Transcriptome analysis of H_2O_2-treated wheat seedlings reveals a H_2O_2-responsive fatty acid desaturase gene participating in powdery mildew resistance. PLoS ONE 6, e28810

Lim, S. et al. (2013) Proteomics analysis suggests broad functional changes in potato leaves triggered by phosphites and a complex indirect mode of action against *Phytophthora infestans*. J. Proteomics 93, 207–223

Luna, E. et al. (2012) Next-generation systemic acquired resistance. Plant Physiol. 158, 844–853

Luzzatto, T. et al. (2007) Priming of antimicrobial phenolics during induced resistance response towards *Pectobacterium carotovorum* in the ornamental monocot *Calla lily*. J. Agric. Food Chem. 55, 10315–10322

Luzzato-Knaan, T. et al. (2014) Priming of protein expression in the defense response of *Zantedeschia aethiopica* to *Pectobacterium carotovorum*. Mol. Plant Pathol. 15, 364–378

Mauch-Mani, B. et al. (2017) Defense priming: an adaptive part of induced resistance. Ann. Rev. Plant Biol. 68, 485-512

Małolepsza, U. (2005) Spatial and temporal variation of reactive oxygen species and antioxidant enzymes in o-hydroxyethylorutin- treated tomato leaves inoculated with *Botrytis cinerea*. Plant Pathol. 54, 317–324

Mandal, R. et al. (2012) Progeny of tobacco mosaic virus-infected *Nicotiana tabacum* plants exhibit trans-generational changes in metabolic profiles. Biocatal. Agric. Biotechnol. 1, 115

Martinez-Medina, A. et al. (2013) Deciphering the hormonal signaling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. Front. Plant Sci. 4, 206

Mathys, J. et al. (2012) Genome-wide characterization of ISR induced in *Arabidopsis thaliana* by *Trichoderma hamatum* T382 against *Botrytis cinerea* infection. Front. Plant Sci. 3, 108

Matthes, M.C. et al. (2010) The transcriptome of cis-jasmone-induced resistance in *Arabidopsis thaliana* and its role in indirect defense. Planta 232, 1163–1180

Massoud, K. et al. (2012) Dissecting phosphite-induced priming in Arabidopsis infected with *Hyaloperonospora arabidopsidis*. Plant Physiol. 159, 286–298

Meller, B. et al. (2018) BABA-primed histone modifications in potato for intergenerational resistance to *Phytophthora infestans*. Front. Plant Sci. 9, 1228

Molitor, A. et al. (2011) Barley leaf transcriptome and metabolite analysis reveals new aspects of compatibility and *Piriformospora indica*-mediated systemic induced resistance to powdery mildew. Mol. Plant Microbe Interact. 12, 1427–1439

Muroi, A. et al. (2011) The composite effect of transgenic plant volatiles for acquired immunity to herbivory caused by inter-plant communications. PLoS ONE 10, e24594

Oide, S. et al. (2013) A novel role of *PR2* in abscisic acid (ABA) mediated, pathogen-induced callose deposition in *Arabidopsis thaliana*. New Phytol. 200, 1187–1199

Pastor, V. et al. (2013) Primed plants do not forget. Environ. Exp. Bot. 94, 46–56

Pastor, V. et al. (2014) Preparing to fight back: generation and storage of defensive compounds during the priming phase in Arabidopsis. Front. Plant Sci. 5, 295

Petti, C. et al. (2010) Lipid transfer proteins and protease inhibitors as key factors in the priming of barley responses to Fusarium head blight disease by a biocontrol strain of *Pseudomonas fluorescens*. Funct. Integr. Genomics 4, 619–627

Petti, C. et al. (2012) Auxin as a player in the biocontrol of Fusarium head blight disease of barley and its potential as a disease control agent. BMC Plant Biol. 12, 224

Perez-Hedo, M. and Urbaneja, A. (2014) Prospects for predatory mirid bugs as biocontrol agents of aphids in sweet peppers. J. Pest Sci. 88, 65–73

Pieterse, C.M. et al. (2014) Induced systemic resistance by beneficial microbes. Annu. Rev. Phytopathol. 52, 347–375

Quackenbush, J. (2003) Microarrays – guilt by association. Science 302, 240–241

Rasmann, S. et al. (2012) Herbivory in the previous generation primes plants for enhanced insect resistance. Plant Physiol. 158, 854–863

Rico, A. et al. (2010) Agroinfiltration reduces ABA levels and suppresses *Pseudomonas syringae*-elicited salicylic acid production in *Nicotiana tabacum*. PLoS ONE 5, e8977

Samalova, M. et al. (2013) Nitric oxide generated by the rice blast fungus *Magnaporthe oryzae* drives plant infection. New Phytol. 1, 207–222

Sang, M.K. et al. (2014) Priming-mediated systemic resistance in cucumber induced by *Pseudomonas azotoformans* GC-B19 and *Paenibacillus elgii* MM-B22 against *Colletotrichum orbiculare*. Phytopathology 104, 834–842

Sato, D. et al. (2013) Metabolomic profiling of the response of susceptible and resistant soybean strains to foxglove aphid *Aulacorthum solani* Kaltenbach. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 925, 95–103

Scalschi, L. et al. (2013) Hexanoic acid is a resistance inducer that protects tomato plants against *Pseudomonas syringae* by priming the jasmonic acid and salicylic acid pathways. Mol. Plant Pathol. 14, 342–355

Schwanhausser, B. et al. (2011) Global quantification of mammalian gene expression control. Nature 473, 337–342

Sheikh, A.H. et al. (2014) Agroinfiltration by cytokinin-producing *Agrobacterium sp*. strain GV3101 primes defense responses in Nicotiana tabacum. MPMI 27, 1175–1185

Shulaev, V. et al. (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. Nature 385, 718–721

Slaughter, A. et al. (2012) Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. Plant Physiol. 158, 835–843

Song, Y.Y. et al. (2013) Priming of anti-herbivore defense in tomato by arbuscular mycorrhizal fungus and involvement of the jasmonate pathway. J. Chem. Ecol. 39, 1036–1044

Sun, J. et al. (2014) Similar metabolic changes induced by HIPVS exposure as herbivore in *Ammopiptanthus mongolicus*. PLoS ONE 9, e95474

Sutter, R. and Müller, C. (2011) Mining for treatment-specific and general changes in target compounds and metabolic fingerprints in response to herbivory and phytohormones in *Plantago lanceolata*. New Phytol. 191, 1069–1082

Ton, J. et al. (2005) Dissecting the β-aminobutyric acid-induced priming phenomenon in Arabidopsis. Plant Cell 17, 987–999

Ton, J. et al. (2007) Priming by airborne signals boosts direct and indirect resistance in maize. Plant J. 49, 16–26

Trethewey, R.N. et al. (1999) Metabolic profiling: a Rosetta stone for genomics? Curr. Opin. Plant Biol. 2, 83–85

Van Hulten, M. et al. (2006) Costs and benefits of priming for defense in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 103, 5602–5607

Van de Mortel, J.E. et al. (2012) Metabolic and transcriptomic changes induced in Arabidopsis by the rhizobacterium *Pseudomonas fluorescens* SS101. Plant Physiol. 160, 2173–2188

Vogel-Adghough, D. et al. (2013) Pipecolic acid enhances resistance to bacterial infection and primes salicylic acid and nicotine accumulation in tobacco. Plant Signal. Behav. 8, e26366

Vos, C. et al. (2013) Mycorrhiza-induced resistance against the root-knot nematode *Meloidogyne incognita* involves priming of defense gene responses in tomato. Soil Biol. Biochem. 60, 45–54

Vos, I.A. et al. (2013) Onset of herbivore-induced resistance in systemic tissue primed for jasmonate-dependent defenses is activated by abscisic acid. Front. Plant Sci. 4, 539

Wang, K. et al. (2015) Response of direct or priming defense against *Botrytis cinerea* to methyl jasmonate treatment at different concentrations in grape berries. Int. J. Food Microbiol. 194C, 32–39

Xu, X. et al. (2006) Physical and functional interactions between pathogen-induced Arabidopsis *WRKY18, WRKY40*, and *WRKY60* transcription factors. Plant Cell 18, 1310–1326

Ye, M. et al. (2013) Priming of jasmonate-mediated antiherbivore defense responses in rice by silicon. Proc. Natl. Acad. Sci. U.S.A. 110, 3631–3639

Zhan, L.et al. (2018) Priming effect of root-applied silicon on the enhancement of induced resistance to the root-knot nematode *Meloidogyne graminicola* in rice. BMC plant biology, 18, 50

Zimmerli, L. et al. (2000) Potentiation of pathogen-specific defense mechanisms in Arabidopsis by β-aminobutyric acid. Proc. Natl. Acad. Sci. U.S.A. 97, 12920–12925

Zhong, Y. et al. (2014) DL-β-Aminobutyric acid-induced resistance in soybean against *Aphis glycines* Matsumura (Hemiptera: Aphididae). PLoS ONE 9, e85142

Chapter II- **The quest for novel priming blends**

Tricarboxylates induce defense priming against bacteria in *Arabidopsis thaliana*

Balmer, A., Pastor, V., Glauser, G., & Mauch-Mani, B. (2018). Tricarboxylates induce defense priming against bacteria in *Arabidopsis thaliana*. Frontiers in plant science, doi: 10.3389/fpls.2018.01221.

Keywords
Priming, TCA, induced resistance, citrate, fumarate, primary metabolism, carboxylic acids.

Tricarboxylates induce defense priming against bacteria in *Arabidopsis thaliana*

1. Introduction

The continuous exposure of plants to biotic stressors and environmental changes forces them to constantly remodel their defense strategies as well as their metabolism (Van Aken et al. 2016; Stael et al. 2011; Nomura et al. 2012; Zhang et al. 2014). Hence, there is a massive reprogramming of the plant cell in order to activate and deploy an efficient immune reaction in response to different kinds of stress. In this context, the phenomenon of priming has been well described to be part of the resulting intricate networks of inducible defenses. In this regard, priming is defined as an induced state whereby basal and further layers of defense are potentiated to react more rapidly and more efficiently to a stress (Conrath et al. 2002; Pastor et al. 2013a). The induction of these defense mechanisms involves the regulation of defense gene expression, the release of plant hormones like auxin, abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and/or the induction of secondary metabolites (Reviewed by Balmer et al. 2015) as well as a massive redistribution of energy (Bolton 2009). During the priming phase induced by β-aminobutyric acid (BABA), a series of metabolic changes occur which are generally characterized by a massive boost of the primary metabolism through a specific accumulation of tricarboxylic acids (TCAs) such as citrate, fumarate, (S)-malate and 2-oxoglutarate (Pastor et al. 2014). Hence, it is possible that BABA-induced priming functions through the potentiation of the TCA flux and that the primary metabolism plays an important role in BABA-induced priming.

The TCA flux could occur in a cyclic or no-cyclic fashion depending on the physiological and metabolic demands of the plant cells (Reviewed by Sweetlove et al. 2010) and is a central pathway for the generation of primary metabolites. Especially in terms of energy metabolism, it is commonly thought of being important during aerobic processes and also responsible for oxidation of a major part of carbohydrate, fatty acid, amino acid and respiratory substrates to drive ATP synthesis and energy production (Fernie et al. 2004). Significant research efforts have concentrated on exploring by which means plants are able to recruit and redistribute energy flows. However, the specific role of most of the primary metabolism compounds during plant defense responses is still not fully understood. The association between primary metabolism and plant defenses has been often

elucidated by analyzing the expression of genes encoding transcription factors, metabolic enzymes or by metabolomic analysis (Bolton et al. 2009). For example, an exposure of Arabidopsis plants to biotic stresses such as a virulent pathogen (*Phytophthora infestans*), avirulent pathogens (*Pseudomonas syringae* pv. tomato *Hrc-* and *AvrRpm1*), and to pathogen-derived elicitors (flagellin and Hairpin elicitor protein) led to up-regulation of transcripts from specific functional categories associated with primary metabolic pathways such as synthesis or degradation of carbohydrates, amino acids and lipids (Rojas et al. 2014; Schwachtje et al. 2018). These findings suggest that the primary metabolism could modulate signal transduction cascades that lead to plant defense responses (Less et al. 2011). In response to biotic stress, the enhanced demand for carbon can be provided by TCA intermediates through different pathways. Starting from amino acids, the enzyme glutamate dehydrogenase (GDH) can release amino nitrogen to yield a keto-acid that can be used in the TCA flux. In fact, the 20 proteinogenic amino acids can be metabolized into some of the seven intermediates (α-ketoglutarate, acetoacetate, acetyl-CoA, fumarate, oxaloacetate, pyruvate, and succinyl-CoA), which are critical for energy generation in plants (Miflin and Habash, 2002). On the other hand, using either glutamate or α-ketoglutarate as substrates, the γ-aminobutyric acid (GABA) pathway produces succinate, a component of the TCA flux (Shelp et al. 1999). GABA is known to be involved in the resistance response to pathogens (Bolton et al. 2008b). Under particular energetically demanding conditions, the GABA shunt can provide means to utilize excess of pyruvate for energy production. Moreover, during a hypersensitive response (HR), GABA induction may also provide a way of keeping NADH-generation unaltered through the TCA flux by avoiding enzymes like aconitase, succinyl-CoA ligase, and α-ketoglutarate dehydrogenase which are inactivated under oxidative stress conditions (Sweetlove et al. 2002; Tretter and Adam-Vizi, 2000). Another potential source to reply to the high energy demand during plant defense is the degradation of fatty acids during β-oxidation. This reaction takes place in the glyoxylate cycle that mediates the conversion of acetyl-CoA to succinate; the latter is transported from the glyoxysome to the mitochondria, where it can be employed in the TCA flux. This reaction has been shown to be characteristic of Arabidopsis defense responses to *Pseudomonas syringae* (Scheideler et al. 2002). Besides supporting higher energy demand during biotic stress situations, TCA flux intermediates such as citrate are presumed to be important players in gene expression and metabolite signaling in various prokaryotic and eukaryotic organisms (McCammon et al. 2003; Wellen et al. 2009; Yang et al. 2012). Hence, tricarboxylates could act as direct defense signals. In Arabidopsis plants, manipulating levels of TCA flux intermediates induced strong changes in

transcript abundances (Finkemeier et al. 2013). However, transcriptional changes caused by TCA intermediates are specific for each metabolite. For example, supplying tobacco leaves with 40mM 2-oxoglutarate led to a strong induction of NITRATE REDUCTASE but only to a faint upregulation by 40mM malate or citrate (Müller et al. 2001). Additionally, high similarity in the transcript response was observed when comparing microarray data sets of Arabidopsis plants treated with citrate with arrays of several biotic stress experiments like *Pseudomonas syringae* infection or *flg22* treatment. The behavior of specific transcript induction or repression by the intermediates of the TCA cycle confirms that these metabolites can act as signaling molecules and strongly supports a positive interconnection between TCA components and plant defenses (Finkemeier et al. 2013).

Changes in carboxylic acid levels were demonstrated to be perceived in plants during stress responses. Additionally, during priming induced by BABA, the specific induction of TCAs indicates that components of this pathway may play a role during priming mechanisms. So far it remains ambiguous if TCAs are pivotal players in the chemical orchestra of BABA-induced priming, or whether TCAs could act alone as novel priming signals. Hence, the present study aims to assess the relationship between specific TCAs and priming in order to find out if TCAs are necessary components for an accurate deployment of priming defenses. Here, the TCAs citrate and fumarate are identified as inducers of BABA-independent priming of resistance against the bacterial pathogen *Pseudomonas syringae*. Expression patterns of selected defense genes and phytohormone levels were analyzed to identify possible defense pathways involved during TCA-induced priming and defense responses. Our results advocate adding TCA intermediates, in particular citrate and fumarate, to the chemical spectrum of plant priming inducers. A better understanding of a putative role of TCAs in priming could provide a means to enhance stress tolerance and, thus, productivity in crop species.

2. Material and methods

2.1 Plant material and growth conditions

Arabidopsis thaliana genotypes *sid1-2, jin1, coi1* and Col-0 (Provided by C. Nawrath, University of Lausanne, Switzerland; J. Turner, University of East Anglia, Norwich, UK and Lehle Seeds, Round Rock, TX, respectively) were germinated in 33 mL hydrated Jiffy pellets maintained at 21°C day/19°C night, with 9 hours of light (120 µE m^{-2} s^{-1}) and 60% of relative humidity. One week after germination seedlings were individually transferred to Jiffys and kept in the same conditions until the chemical treatments and infection essays. All biological assays were performed with four to five weeks old plants.

2.2 Treatment of plants with chemicals

All the chemicals used in this study were obtained from SIGMA-ALDRICH (www.sigmaaldrich.com). Four to five week-old Col-0 plants were soil-drenched with a final concentration of the carboxylic acids citrate (Trisodium citrate dihydrate, reference No. S1804) and fumarate (Fumaric acid, reference No. A 47910) at 0.1, 1, 5, 10 mM, hydrochloric acid (Hydrochloric acid solution, reference No. H9892; HCl at pH 2.5 as control), 250 µM of BABA (DL-3-Aminobutyric acid, reference No. A44207, racemate) or water (control), were applied as soil drench 2 days prior to inoculation with bacteria (Slaughter et al. 2011). Samples were taken at 48 hours post treatment (hpt). Concentrations of exogenous TCAs treatments were selected beforehand based on the fact that citrate is one of the most abundant carboxylic acids in plant cells with concentrations of about 1 to 5 mM in the cytosol (Martinoia and Rentsch, 1994).

2.3 Pathogen cultivation and inoculation

The virulent bacterial strain *Pseudomonas syringae* pv tomato DC3000 (*Pst* DC3000) was grown overnight in liquid King's medium B (King et al. 1954) amended with the antibiotic rifampicin (50 µg mL^{-1}) for selection (Flors et al. 2008). Plants were inoculated two days after the chemical treatments by dipping the leaves in a *Pst* DC3000 suspension containing 10^6 colony-forming units (cfu) mL^{-1} in 10 mM $MgSO_4$ and 0.001% v/v Silwet L-77 (www.momentive.com) for 4 seconds. Mock-treated plants were dipped in the same solution without bacteria. Samples were taken at 6, 24, 48 and 72 hours post infection (hpi).

2.4 Monitoring of disease symptoms and direct toxic effects

The disease phenotype was assessed 24, 48 and 72hpi by counting the cfu of bacteria per gram of fresh material using the serial dilution method as described by Flors et al. 2008, or at 72hpi by calculating the percentage of symptomatic leaves. To test the effect of TCAs on bacterial growth, King's medium B was enriched with TCAs (citrate and fumarate, respectively) at 0.1, 1, 5, 10 mM final concentration, control medium containing rifampicin (50µg mL^{-1}) and sterile water. To maintain a stable pH, the culture medium was buffered by adding MES. Twenty µL from the stock culture of *Pst* DC3000 ($2x10^9$cfu mL^{-1}) were added to each replicate (12 mL tube), and inoculated tubes were incubated overnight in a horizontal shaker at 28°C. Subsequently, *Pst* DC3000 growth was measured by optical density at 600nm (OD_{600}).

2.5 TCAs measurement

Plant samples were powdered and freeze dried for TCA quantification. Five milligrams of powdered dry material were transferred into a 1.5 mL microcentrifuge tube. Samples were hydrated and homogenized with 1 mL of HCOOH 0.1% and the extraction was performed twice, with a final volume of 2 mL. Sample homogenization and hydration were made with a solution of HCOOH 0.1% containing a mix of internal standards $^{13}C_6$ –citric acid, $^{13}C_4$ –succinic acid and $^{13}C_4$ – fumaric acid that were added during the first extraction with 100 µg L^{-1} as a final concentration. The extraction was performed introducing glass beads (2 mm Ø) into each tube and using a mixer mill at a frequency of 30 Hz during 3 minutes. Tubes were centrifuged at 14000 rpm at 10°C and the supernatant was recovered and placed into a new tube. The two extractions were joined and filtered through a 0.2 µm cellulose acetate filter. An aliquot of 20 µL was injected into the LC-MS/MS instrument, an Acquity Ultraperformance Liquid Chromatography system connected to a triple quadrupole mass spectrometer (UPLC-TQD, Waters, Manchester UK). The separation of the TCAs was performed with a column (Acquity UPLC HSS T3 2.1 x 100 mm, 1.8 µm) maintained at 40°C. A gradient of methanol and water containing 0.1% HCOOH was used for analyte elution. The gradient elution started with a flow of 0.3 mL min^{-1} and kept in isocratic conditions during 4 minutes at 95% aqueous mobile solvent, that reached 60% during two minutes and left to recover for one more minute at initial conditions. The column was allowed to equilibrate for 1 minute, giving 8 min per sample. Ion detection was set in negative electrospray ionization (ESI) applying 3.3 kV capillarity voltage, and using multiple reaction monitoring (MRM) mode. Drying, nebulizing

and cone gas was nitrogen and for the collision gas Ar (Praxair, Valencia, Spain) was used. The desolvation gas was set at 800 L/ h and 60 L/h for the cone gas flow. Temperatures were fixed at 350°C for desolvation and 120°C for the source. The cone and collision energies were 15 V and 10 eV for all analytes. The transitions selected for citric, fumaric, malic, succinic and 2-oxoglutaric acid (Sigma) were *m/z* 191>111, 115>71, 133>115, 117>73, 145>101, and for the internal standards, $^{13}C_4$- fumaric acid *m/z* 119>74; $^{13}C_4$- succinic acid *m/z* 121>76 and $^{13}C_6$- citric acid *m/z* 197>116 (Cambridge Isotope Laboratories, Inc). Analyte quantification was achieved by internal calibration. For malic acid quantification, $^{13}C_4$- succinic acid was used as internal standard and $^{13}C_4$- fumaric acid for 2-oxoglutaric acid. The LOQ for citric, fumaric, succinic, malic acid, and 2-oxoglutaric acids were 0.31, 0.24, 0.14, 0.27 and 0.19 µg mL^{-1} correspondingly.

2.6 Hormone quantification

For hormone analysis, salicylic acid (SA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile) and abscisic acid (ABA) were quantified simultaneously from leaf material by UHPLC-MS/MS as previously described (Glauser et al. 2014). Hormone measurements were performed in material from plants treated with chemical inducers as well as after infection with *Pst* DC3000. To analyze each condition, three independent biological replicates per sample were generated and three independent experiments were conducted.

2.7 Camalexin quantification

For the analysis of camalexin, fresh frozen plant material was ground to a fine powder using mortars and pestles cooled with liquid nitrogen. A 100 mg aliquot was weighed and transferred to a 1.5 mL microcentrifuge tube, to which 500 µL were added of extraction solvent (methanol:H_2O:formic acid, 80:19.5:0.5, v/v) and 5-6 glass beads (2mm diameter). Samples were extracted in a Retsch mixer mill for 4 min at 30 Hz, after which the extract was centrifuged and the supernatant recovered and transferred to an HPLC vial. The analysis of camalexin was performed by UHPLC-MS/MS using an Ultimate 3000 RSLC (Dionex, Thermo Fisher Scientific) coupled to a 4000 QTRAP (AB Sciex). The column was an Acquity UPLC BEH C18 (50x2.1mm, 1.7 µm, Waters) maintained at 25°C and the mobile phases were H_2O+formic acid 0.05% (phase A) and acetonitrile+formic acid 0.05% (phase B). The following gradient program was used at a flow rate of 0.4 mL/min: 5-60% phase B in 4 min, 60-100% phase B in 2 min, holding at 100% phase B for 2 min and reequilibration at 5% phase B for 3 min. The injection volume was 3.5 µL. Detection was performed in electrospray positive ionization using the multiple reaction monitoring (MRM) mode. The transition *m/z* 201/59

was used as quantifier while the transitions *m/z* 201/160, 201/142, 201/130, 201/116 and were used as qualifiers. For the quantifier transition (*m/z* 201/59), the declustering potential (DP), the collision energy (CE) and the collision exit potential (CXP) were set to 100V, 51 V, and 10 V, respectively. Source parameters were set as follows: ion spray (IS) voltage +5.5 kV, gas temperature (TEM) 500°C, nebulizing gas (GS1), drying gas (GS2) and curtain gas (CUR) 60, 40 and 25 psi, respectively. Quantification was achieved by external calibration using camalexin (www.sigmaaldrich.com) concentrations at 0.2, 1, 5, 20, 100 and 500 ng/mL. The lowest limit of quantification was 0.2 ng/mL or 1 ng/g FW.

2.8 Gene expression analysis

Arabidopsis plants that had been subjected to chemical treatments and inoculated with *Pst* DC3000 as described above were evaluated. RNA extraction and cDNA synthesis was performed as described before (Balmer et al. 2013). The primers used in this study and their efficiencies are listed in **Table S1**; primer efficiency was calculated with the help of a dilution curve. Quantitative real-time PCR was performed using the SensiMix SYBR kit (Bioline, http://www.bioline.com) on a ROTOR GENE 6000 cycler (Qiagen). PCR reactions were conducted using three independent biological replicates per sample. PCR reactions were done in technical duplicates as a three-step reaction (initial hold step, 95°C for 10 min; 40 cycles of amplification comprising 95°C for 15 sec, 60°C for 20 sec and 72°C for 20 sec), with a final melting curve analysis (68–95°C). Melting curve and cycle threshold analysis were performed using rotor gene 6000 software version 1.7. Gene expression of infected tissue and control plants were calculated relative to the expression of the housekeeping genes *Actin* and *SAND* using Ct delta-delta method.

2.9 Extraction and liquid chromatography-tandem mass spectrometry analysis of BABA

BABA was quantified in material from plants treated with chemical inducers as well as after infection with *Pst* DC3000. Plant material was harvested, flash-frozen and ground to fine powder in liquid nitrogen. The extraction protocol was conducted as described by Thevenet et al. 2017; in brief 100 mg of ground tissue was extracted in 500 µL of 0.1% HCOOH/H_2O (v/v) containing the deuterium labeled internal standard (BABA-d_3) using a Retsch mixer mill. After centrifugation of the extract at 18400 g during 4 min, the supernatant was purified by solid phase extraction on an Isolute SCX-2 cartridge (1 mL, 100 mg). The eluate was concentrated to dryness in a centrifugal evaporator (Speedvac) at 35°C. Samples were finally resuspended in 300 µL (1: 3) organic mobile

phase B/EtOH 80% (v/v) leading to a final concentration of internal standards of 50 ng mL^{-1}. Extracted BABA was quantified using an Ultimate 3000 RSLC (Dionex, Thermo Fisher Scientific) interfaced with a 4000 QTRAP (AB Sciex) by injecting 3.5 µL of extract on an Acquity UPLC BEH HILIC column (100 mm x 2.1 mm, 1.7 µm, Waters).

2.10 Statistical analysis

Variances of quantified levels of bacterial growth, transcript abundance, fold induction of gene expression and phytohormones were analyzed by a *t*-test; a *P* value <0.05 was considered significant. All statistical analyses were performed using GraphPad Sofware (www.graphpad.com).

3. Results

3.1 Citrate and fumarate induce resistance in Arabidopsis against Pst DC3000 in a dose-dependent manner

It has been previously demonstrated that TCAs are upregulated during the BABA-induced priming phase, respect to the *Pst* Rpt2-induced resistance (Pastor et al. 2014). Therefore, the present study aimed to ask whether these compounds can also act as defense and/or priming inducers, as previously demonstrated for other acidic chemical signals such as azelaic acid, pipecolic acid and recently acetic acid (Jung et al. 2009; Návarová et al. 2012; Kim et al. 2017). To test the defense inducing capacity of carboxylic acids, citrate and fumarate were chosen. Their defense and priming potential were assessed in a bioassay using Arabidopsis plants treated by soil drench with both chemicals at various concentrations (0.1, 0.5, 1, 5 and 10 mM final concentration in the soil), as well as water, HCl (pH 2.5) and BABA (0.25 mM) as controls. Two days after the chemical treatments the plants were inoculated with the hemibiotrophic pathogen *P. syringae* pv. tomato DC3000. Three days post bacterial infection the disease rate was measured as percentage of symptomatic leaves (**Figure 1**) and as cfu per gram of fresh material at 24, 48 and 72hpi (**Figure S1**). The treatment of Arabidopsis plants with 5 mM and 10 mM of citrate resulted in an induction of resistance against *Pst* DC3000 compared to water- and HCl-treated plants (**Figure 1A**). Notably, the disease rate on plants pre-treated with citrate was similar to the one observed for BABA-treated plants. Five days after inoculation, leaves from 5 mM and 10 mM citrate-treated plants were less affected by the bacterial infection than the corresponding controls (**Figure 1A**). Fumarate treatment at 0.1 mM also seemed to protect the plants from bacterial infection compared to water treatment. However, this difference was not statistically significant with respect to HCl treatment and could not be observed with higher concentrations between 0.5-5 mM (**Figure 1B**). In contrast, fumarate treatment at 10 mM induced resistance to a similar extent as citrate. Importantly, HCl (pH2.5), citrate and fumarate treatments at the highest concentration (10 mM) did not affect plant growth or trigger phytotoxicity symptoms, excluding that a chemical stress induced antibacterial resistance (**Figure 1C**).

As shown in **Figure 1**, resistance induced by citrate and fumarate were dose-dependent, with 10 mM citrate and 10 mM fumarate being the most efficient concentrations for priming bacterial resistance.

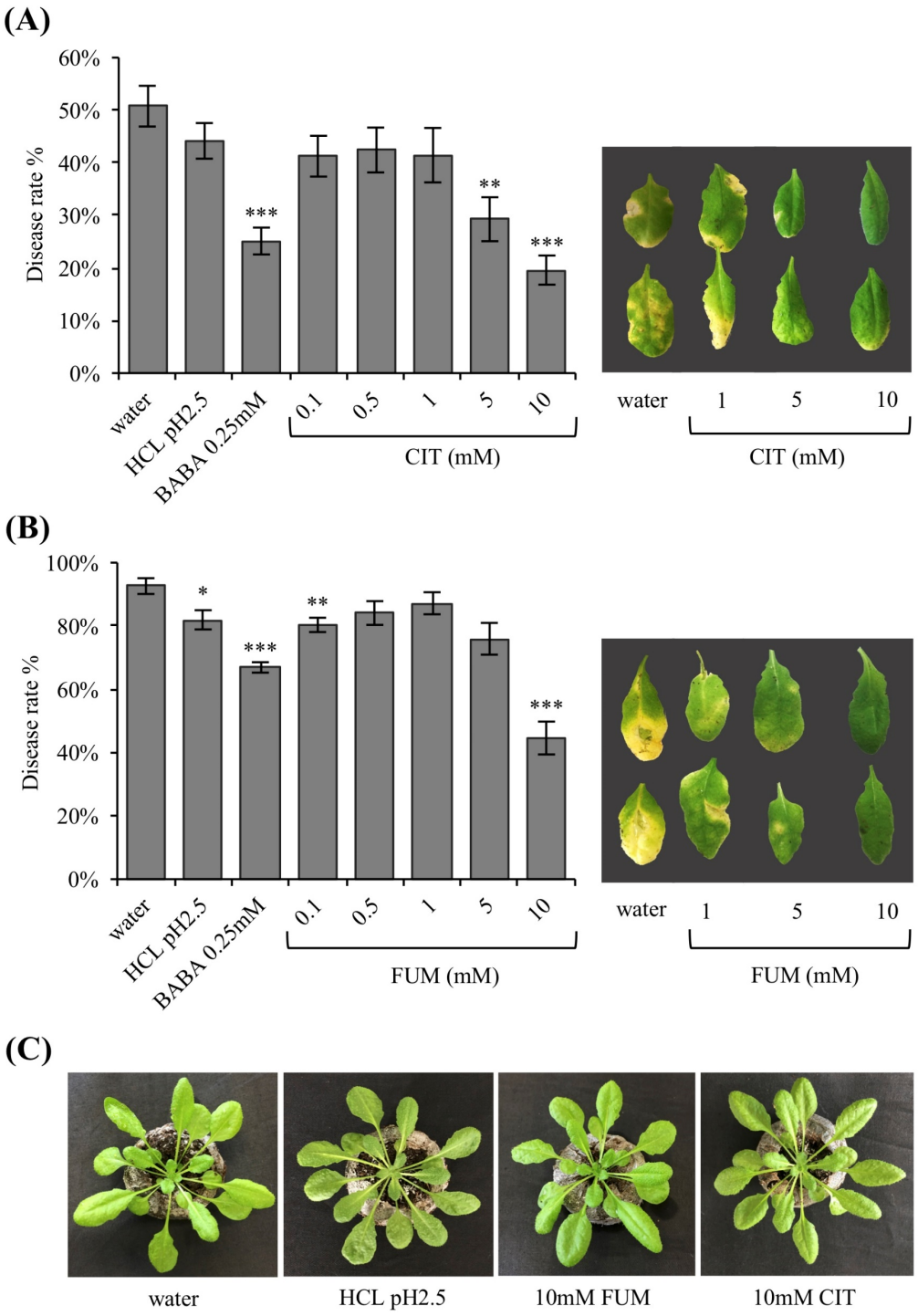


Figure 1. In Arabidopsis, the TCAs citrate and fumarate confer resistance to *Pst* DC3000. (A) Disease rate in percentage (3dpi) of Arabidopsis plants soil drenched with different concentration of citrate, water, HCl and BABA as a controls 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}). Depicted leaves were collected 5dpi. (B) Disease rate of Arabidopsis plants soil-drenched with various concentrations of fumaric acid, water, HCl and BABA as a controls 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}) is shown in percentage (3pdi bacterial challenged). Leaves from pictured were collected 5dpi. (C) Arabidopsis Col-0 plants 3 days after being soil drenched with HCl (pH 2.5), citrate 10mM, fumarate 10mM and water. Data represent the mean ± SEM (n = 12 biological replicates). The experiments were repeated three times and a representative replicate is shown. Asterisks indicate significant differences: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (treatments vs. water) as determined by t-test.

3.2 Citrate and fumarate have no direct toxicity against Pst DC3000

To determine whether carboxylic acid compounds display direct antimicrobial activity, we performed an in vitro growth assay, cultivating *Pst* DC3000 in liquid King's medium B amended with different concentrations of citrate and fumarate. Neither of the treatments reduced bacterial growth (**Figure 2**) compared to the control. As a consequence, enhanced bacterial resistance found in planta is solely based on the induction of the plant defense system, and not triggered by secondary antimicrobial effects of TCAs.

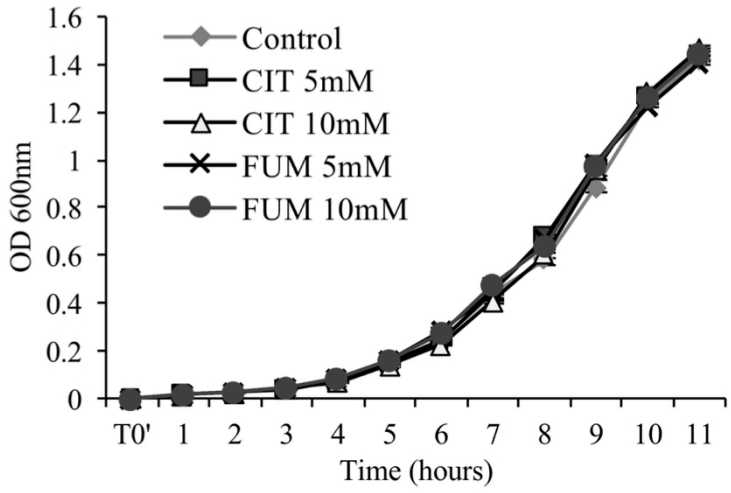


Figure 2. Assessment of antimicrobial activity of citrate and fumarate against *Pst* DC3000. Liquid King's medium B was enriched with citrate and fumarate at different concentrations (5 mM and 10 Mm) and water as a control. *Pst* DC3000 growth was measured every hour during 11 hours post inoculation by optical density at 600nm. Data represent the mean ± SEM (n = 6 biological replicates).

3.3 TCAs induce defenses independently of endogenous BABA

Endogenous levels of BABA have been recently demonstrated to be specifically induced by the plant's immune system under particular biotic stress conditions such as *Pst* DC3000 infection, suggesting an involvement of endogenous BABA in plant defense responses (Baccelli et al. 2017). Furthermore, synthetic BABA treatment has been shown to prime for enhanced plant resistance, concomitantly with a boost of TCA levels (Pastor et al. 2014). In order to analyze whether TCAs could be linked to the induction of endogenous BABA, the levels of BABA were analyzed in plants pre-treated with citrate and fumarate, both at 10 mM. Leaf material was collected two days post chemical treatment (priming phase) and 6h, 48h and 72h post- *Pst* DC3000 infections (the primed state for BABA measurements). During the priming phase, neither citrate nor fumarate resulted in

any difference in the levels of BABA compared to water-treated plants (**Figure 3A**). Furthermore, BABA levels gradually increased during the time course of infection (**Figure 3B**). However, no difference of BABA induction was detected when comparing citrate and fumarate treatments to the water-treated controls.

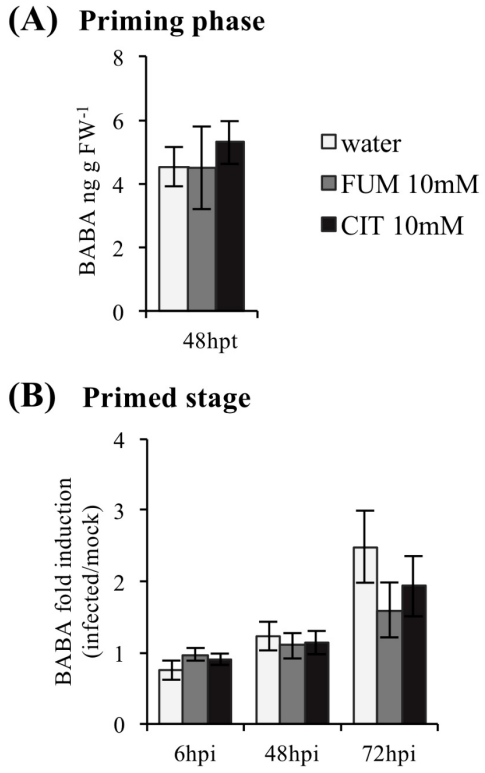


Figure 3. BABA induction upon treatment with citrate and fumarate in the priming phase and the primed stage. (A) Arabidopsis leaves were collected 48h after soil drench with citrate and fumarate or water as a control. BABA levels are expressed in ng gFW^{-1}. (B) BABA fold induction (infected/mock) were analyzed in plants pretreated with citrate 10 mM, fumarate 10 mM or water as a control 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}). Data represent the mean ± SEM (*n* = 6 biological replicates). A t-test was applied (water vs. treatments).

3.4 TCA levels during the priming phase induced by citrate and fumarate treatments

In order to test if exogenous fumarate and citrate treatments stimulate changes in TCA intermediate composition in leaves of treated plants, concentrations of fumarate, citrate, 2-oxoglutarate, malate and succinate were measured 24 and 48 hours post treatments (hpt). The time points for these measurements were selected in order to analyze the TCA levels in leaves before inoculation with the bacteria (48hp citrate and fumarate treatments). As shown in **Figure 4** and **Figure S2**, 10 mM fumarate treatment did not trigger any changes in TCA intermediates at 24hpt. At 48hpt, fumarate treatment led to a slight reduction of malate levels compared to the water treatment (**Figure S2**). On the other hand, exogenous citrate supplied by root drench only led to an increase of fumarate and citrate levels at 24hpt while at 48hpt no significant changes in TCA intermediates were found compared with water-treated plants. Additionally, upon citrate treatment, a depletion of 2-oxoglutarate and citrate was observed when comparing 24hpt with

48hpt. The similarity between the pattern of both TCAs was expected as citrate is one of the precursors of 2-oxoglutarate synthesis, therefore both citrate and 2-oxoglutarate levels are likely to be similarly tuned (Hodges 2002).

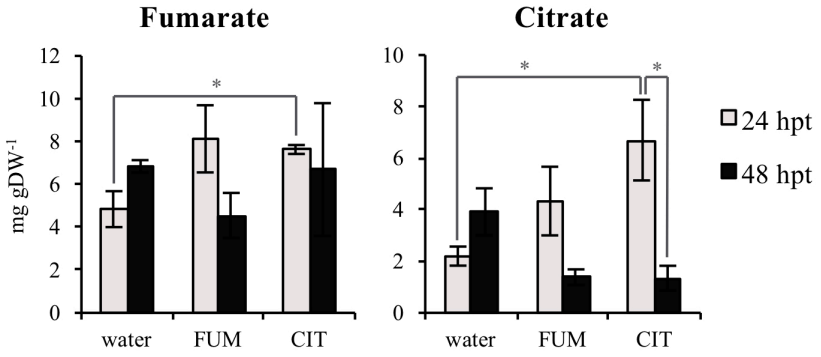


Figure 4. TCAs levels upon citrate and fumarate treatment during the priming phase (24hpt and 48hpt). Arabidopsis leaves were collected 24 and 48h after soil drench with 10 mM citrate and 10 mM fumarate or water as a control. TCAs levels are expressed in mg gDW^{-1}. Data represent the mean ± SEM (n = 3 biological replicates) of one from three independent experiments. Asterisks indicate significant differences (treatments vs. control or 24hpt vs. 48hpt) as determined by t-test: *P ≤0.05.

3.5 Defense-related genes are differentially regulated in TCA-treated plants during the distinct priming phases

To investigate putative transcriptional mechanisms by which citrate and fumarate may alter pathogen susceptibility in Arabidopsis, a comparative analysis of the leaf transcriptome of citrate-, fumarate- and water-treated plants under *Pst* DC3000 and mock inoculation was undertaken. Sampling was performed during the priming phase and the primed state. These two phases provide information about the mode of action of both TCAs: as inducers of resistance or/and as priming agents. Known defense genes were chosen as transcriptional markers for SA (*PR1, PR5 WRKY70*), JA (*LOX2, PDF1.2*) and camalexin (*PAD3*). During the priming phase, treatment with 10 mM fumarate reduced transcript abundance of *PR5* (Pathogenesis-Related Protein5) compared to water-treated plants (**Figure 5A**). However, the other monitored genes showed no significant changes in transcript abundance following 10 mM fumarate and 10 mM citrate treatments compared to water-treated plants (**Figure 5A**). During the priming state however, treatment with 10mM citrate resulted in a strong induction of *PR1* (Pathogenesis-Related Protein1) at early time-points (6hpi and 24hpi) compared to water-treated plants while at 72hpi, *PDF1.2* was found to be upregulated (**Figure 5B**). Interestingly, citrate-treated plants showed a significant reduction of the WRKY transcription factor 70 (*WRKY70*) at 72 hpi, and of *LOX2* which codes for the Lipoxygenase

2, (**Figure 5B**). Moreover, plants treated with 10 mM fumarate showed a different gene induction pattern compared to 10 mM citrate-treated plants. Fumarate treatment slightly increased the induction levels of *PR1*, *LOX2* and *PAD3* genes at early time points after *Pst* DC3000 infection. Levels of *PR5* were also slightly increased at 72h after *Pst* DC3000 infection (**Figure 5B**). *LOX2*, *PAD3* and *WRKY70* were found to be down-regulated in fumarate treatments at later time points compared to water.

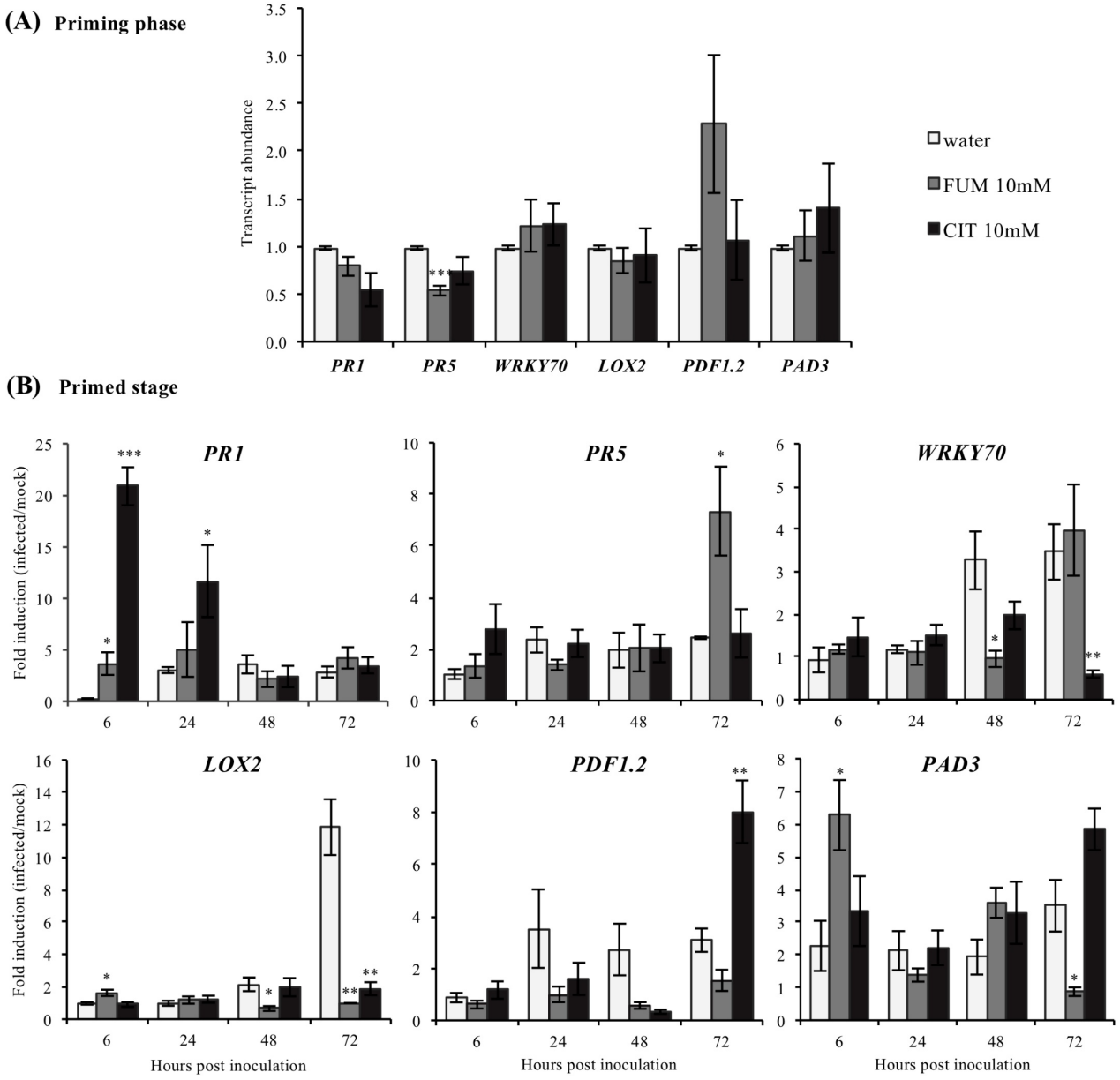


Figure 5. Effect of the TCAs citrate and fumarate and of *Pst* DC3000 infection on the expression of defense-related genes. (A) Priming phase: transcript abundance for *PR1*, *PR5 WRKY70*, SA signaling pathway; *LOX2* and *PDF1.2*, JA/ET biosynthesis and signaling pathway and *PAD3* camalexin biosynthesis. For the priming phase, samples consist of leaves from Arabidopsis plants soil-drenched with 10 mM citrate, 10 mM fumarate and water as a control, collected 48 hours after chemical treatments. Gene expression was measured by qRT-PCR and normalized by the housekeeping gene *Actin*. (B) Primed stage: fold induction (infected/mock) of gene expression for *PR1*, *PR5*, *WRKY70*, *LOX2*, *PDF1.2*, as well as for *PAD3* were analyzed. Arabidopsis plants were soil-drenched with 10mM citrate, 10mM fumarate and water 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}). Gene expression was analysed in a time-course of 6h, 24h, 48h and 72h after infection. Data represent the mean ± SEM (n = 6 biological replicates). Asterisks indicate significant differences (treatments vs. control) as determined by t-test: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.6 Analysis of Arabidopsis phytohormones and camalexin during the priming phase and the primed stage induced by citrate and fumarate

Plant hormones are well-known to be key players in plant-induced responses by virulent Pseudomonas bacteria. *Pst* DC3000 interaction with Arabidopsis is mediated by interplay of both SA and JA (Brooks et al. 2005). Moreover, our results shown here demonstrate that both citrate and fumarate treatments induced specific changes at the transcriptomic level of several phytohormone-related genes. Subsequently, in order to determine if citrate and fumarate could modulate phytohormone induction during both the priming phase (**Figure 6**; 0 hours post inoculation) and the primed stage (upon *Pst* DC3000 infection), we measured the levels of SA, JA and jasmonoyl-isoleucine (JA-Ile) in Arabidopsis plants after 10 mM citrate, 10 mM fumarate and water treatments and upon mock and *Pst* DC3000 infection. During the citrate- and fumarate-induced priming phase (48 hours post treatments with the chemicals), SA, JA and JA-Ile levels did not exhibit any statistically significant differences compared to water-treated plants. Besides, under mock conditions, JA accumulated to a slightly higher extent in citrate- than in water-treated plants (**Figure 6B**). On the other hand, in fumarate and citrate-treated plants, *Pst* DC3000 inoculation significantly induced SA accumulation at 24hpi. Fumarate-treatment reduced JA and JA-Ile levels at later time point after infection compared to water-treated plants (**Figure 6B, C**). Additionally, at 72hpi, JA-Ile accumulated in citrate-treated plants infected with *Pst* DC3000. These results suggest that citrate and fumarate did not affect SA, JA or JA-Ile levels in absence of bacterial infection. However, priming by both TCAs resulted in changes in SA, JA and JA-Ile at early and later times points post *Pst* DC3000 infection. In parallel to the above described phytohormones, levels of the camalexin were also measured in the same experimental conditions. Camalexin is one of the main phytoalexins produced by Arabidopsis. Its accumulation in plants under pathogen attack is mediated by a complex phytohormone balance and inhibits the growth of virulent strains of Pseudomonas (Heck et al. 2003). During the priming phase induced by citrate and fumarate and under mock conditions, camalexin levels did not show any statistically significant differences compared to water-treated plants (**Figure 6D**). Notably, basal levels of camalexin in Arabidopsis plants were found to be around 0.3 ng gFW^{-1} as show in **Figure S3**. In the experimental conditions applied in the assays above, plants from priming phase (**Figure 6D**; 0 hours post inoculation) samples were exposed to 100% relative humidity and mock samples to 100% relative humidity, 10 mM $MgSO_4$ and 0.001% Silwet. These factors could explain the augmented camalexin levels (~10 ng gFW^{-1}) measured at PP and mock time points (**Figure 6B**). Subsequently, camalexin levels gradually increased during the time course of infection under all treatments. Altogether, only

citrate induced significantly camalexin at an early time point upon *Pst* DC3000 infection compared to water-treated plants (**Figure 6D**).

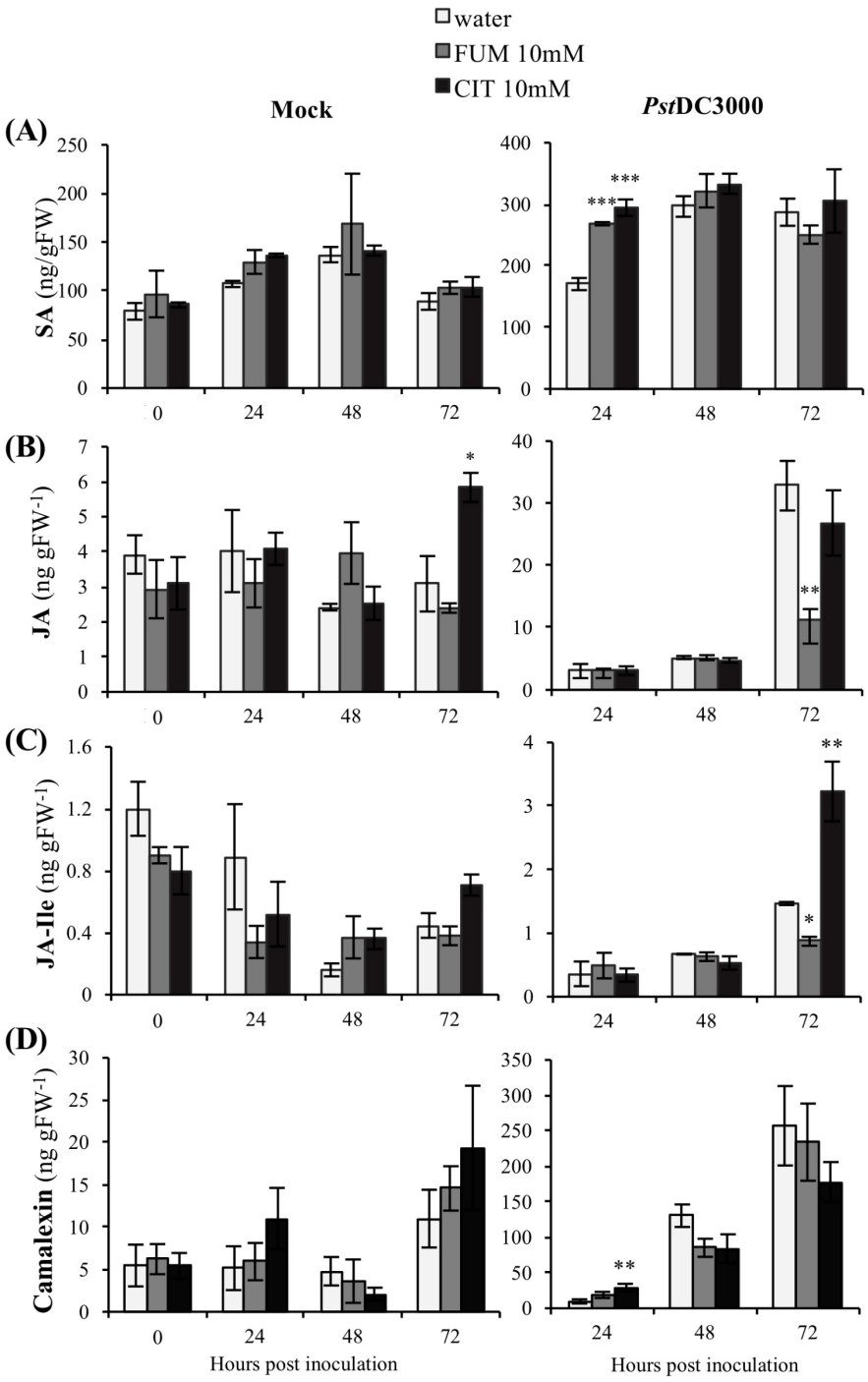


Figure 6. Effect of the TCAs citrate and fumarate and *Pst* DC3000 infection on the accumulation of SA, JA, JA-Ile and camalexin. (A) SA levels (ng gFW^{-1}), (B) JA levels (ng gFW^{-1}), (C) JA-Ile levels (ng gFW^{-1}) and (D) camalexin (ng gFW^{-1}) during the priming phase (0 hours post inoculation), 24h, 48h and 72h post mock and *Pst* DC3000 inoculation. Priming phase samples consist of soil-drenched Arabidopsis plants with 10 mM citrate, 10 mM fumarate and water as a control. The material was collected 48 hours post chemical treatments. Mock and *Pst* DC3000 samples are Arabidopsis plants soil-drenched with 10 mM citrate, 10 mM fumarate and water 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}). Data represent the mean ± SE (n = 3 biological replicates). These experiments were repeated on three independent occasions and a representative replicate is shown. The Asterisks indicate significant differences (treatments vs. control) as determined by t-test: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3.7 Priming induced by citrate and fumarate is SA- and JA-dependent

Upon bacterial infection, *Pst* DC3000 virulence factors like coronatine (COR) are well known to act as manipulators of host phytohormone signaling pathways (Yi et al. 2014), demonstrating the cardinal role of plant hormones in bacterial defense. Therefore, we investigated if modifications of phytohormone synthesis and signaling were crucial in triggering the phenotype of priming induction exhibited under both treatments. A set of established Arabidopsis mutants impaired in SA and JA signaling and synthesis were selected to monitor the efficiency of TCA-induce priming. Both mutants and wild type (Col-0) plants were treated with 10 mM citrate and 10 mM fumarate and subsequently inoculated with *Pst* DC3000. To assess the importance of JA, the *jin1* mutant (*jasmonate-insensitive 1*; also known as *MYC2*),– a mutant well described to exhibit reduced susceptibility to *Pst* DC3000 compared to wild-type (Lorenzo et al. 2004) was tested first. As expected, this mutant showed reduced susceptibility to *Pst* DC3000 infection upon water treatment compared to wild type Col-0. Notably, *jin1* was impaired in mounting citrate- and fumarate-induced priming against *Pst* DC3000 at all measured time points (**Figure 7A, B, C**). Similarly, *coi1* (*coronatine insensitive1*, a mutant which shows increased resistance to *Pst* DC3000, is jasmonate insensitive and has increased level of SA upon infection) (Thines et al. 2007), showed a slightly lowered capacity to be primed by citrate and fumarate. This was obvious at all the time points. In particular, at 72hpi this mutant did not induce resistance upon citrate treatment and displayed a higher bacterial pathogen load in fumarate- than in water-treated plants (**Figure 7C**). Furthermore, to address the importance of the SA pathway, the *enhanced disease susceptibility5* (*eds5*; also known as *sid1*) mutant was subjected to TCA-induced priming. *eds5/sid1* is an essential component of salicylic acid-dependent signaling for disease resistance and is salicylic acid-deficient (Nawrath and Metraux, 1999). As expected, *sid1* showed high susceptibility to *Pst* DC3000 in water-treated plants at 48hpi and 72hpi (**Figure 7B, C**). At early time point (24hpi), *sid1* was unable to mount a priming response triggered by citrate or fumarate (**Figure 7A**).

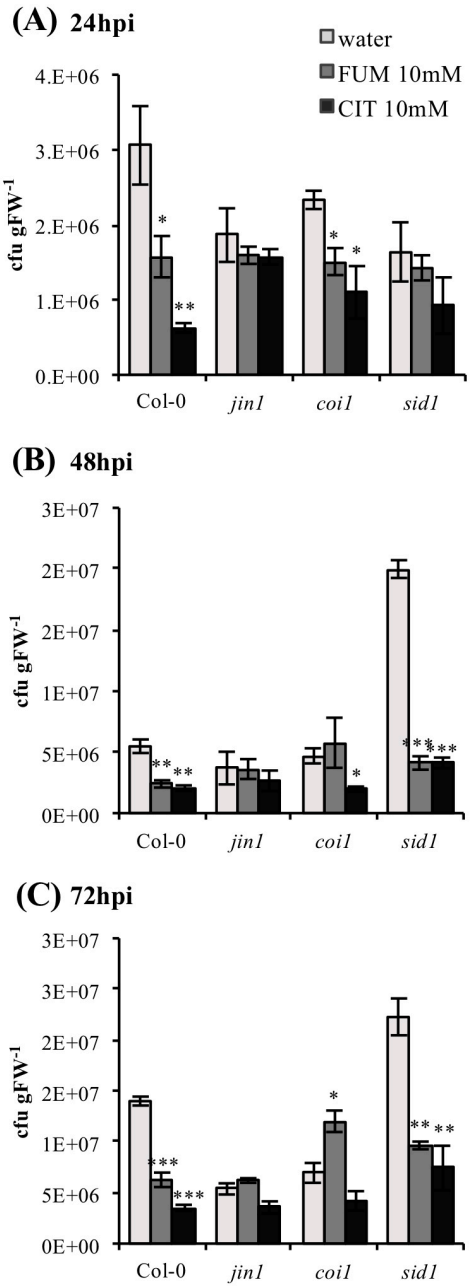


Figure 7. Citrate- and fumarate-induced priming against *Pst* DC3000 in Arabidopsis mutants impaired in SA and JA signaling. Arabidopsis genotypes *jin1, coi1, sid1* and the wild-type Col-0 were soil drenched with 10 mM citrate, 10 mM fumarate and water as a control 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}). The disease rate was expressed as cfu gFW^{-1} and analysed in a time-course of 24h (A), 48h (B) and 72h (C) after infection. Data represent the mean ± SE (n = 3 biological replicates). This experiment was repeat three time with similar results. Asterisks indicate significant differences (treatments vs. control) as determined by t-test: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

4. Discussion

The chemical profile during priming described in a previous study (Pastor et al. 2014) offered intriguing information about metabolic pathways that could be implicated in priming. Since a general upregulation of TCAs was observed during BABA-induced priming it was hypothesized that they might act as important players during priming responses. TCA flux intermediates such as citrate have been identified as important players in gene expression and metabolite signaling in plants (Finkemeier et al. 2013). In the present study, citrate and fumarate were shown to have the potential to prime for augmented resistance against *Pst* DC3000 in Arabidopsis. Moreover, these compounds did not display *in vitro* antibacterial activity, suggesting that they exert their mode of action through the plant immune system. Until now, the role of TCAs during induced resistance has remained unclear, but recent work started to uncover direct links between TCAs and plant defense. Often, alterations of TCA levels are characteristically associated with a pathogen challenge, but it was not clear if these alterations played a direct defensive role by interfering with pathogen development, if they mirrored a metabolic phenotype during biotic stress situations, or simply reflected a physiological status of high energy demand during plant defense. Notably, an enhanced resistance to *Pst* DC3000 infection was observed in knockout lines of the cytosolic NADP-isocitrate dehydrogenase (Mhamdi et al. 2010). These plants were shown to accumulate up to 2-fold more citrate, suggesting a resistance enhancing effect of higher citrate levels. Recently, enhanced levels of TCA intermediates in the Arabidopsis mutant *Chi-CWP2* (expressing a class-I chitinase and a *Fusarium*-specific recombinant antibody gene) could be linked to resistance against *Fusarium graminearum* (Chen et al. 2017). On the other hand, TCA cycle inhibition by monofluoroacetate (MFA) triggers citrate accumulation and increases the susceptibility of treated plants to *Pseudomonas syringae* pv. DC3000 *hrcC* suggesting a crucial role in energy supply during early stages of Pattern-Triggered immunity (PTI) establishment (Park et al. 2015).

Bearing in mind the priming potential of TCAs, and the positive link between BABA, plant defense responses and TCA components (Thevenet et al. 2017 and Pastor et al. 2014), it was assessed here whether citrate and fumarate are also able to trigger the induction of endogenous BABA. The present data show that citrate treatment does not affect levels of BABA in the priming phase, therefore it can be hypothesized that the elevated TCA levels observed during BABA-induced priming are likely not responsible for the potentiation of a putative endogenous BABA pathway.

As a consequence, TCAs seem to act as priming agent by a non-classical priming pathway independent of endogenous BABA.

As a next step, an attempt was made to characterize the molecular and chemical mechanisms of TCA-mediated priming, in order to build a hypothetic signaling scheme (**Figure 8**). Generally, in Arabidopsis plants, altered levels of TCA cycle intermediates induce strong alterations in transcript abundances (Finkemeier et al. 2013), and transcriptional changes caused by TCAs are known to be specific for each metabolite (Müller et al. 2001). The behavior of specific transcript induction or repression by the intermediates of the TCA cycle confirm that these metabolites can act as a signaling molecules and strongly indicates a positive relationship between TCA components and plant defenses. Consequently, the question remains whether citrate and fumarate induce resistance by triggering the expression of defense genes only, or if they also prime plants for an augmented defense gene expression. Data shown here corroborate the fact that citrate and fumarate act as modifiers of transcriptional signaling during priming (**Figure 8**). Here, a group of phytohormone-related genes involved in Arabidopsis defenses was analyzed.

The present findings indicate that citrate and fumarate act as priming inducers, since no significant transcriptional reprogramming occurs. Both chemical treatments are presumably triggering no additional costs to plants when no challenge or stress is present. Intriguingly, fumarate pre-treatment induces a different pattern of gene induction upon *Pst* DC3000 infection than citrate. Fumarate down-regulated *WRKY70* and *LOX2* gene expression at later time points after infection whereas *PR1* and *LOX2* were slightly induced at early time points upon *Pst* DC3000 infection. The distinct gene expression patterns observed in citrate- and fumarate-primed plants are in line with previous conclusions that alterations in specific TCA component levels result in unique transcriptome responses (Finkemeier et al. 2013). Especially, citrate pre-treatment primed for an enhanced *PR1* expression at early time points and a *PDF1.2* expression at later time points upon *Pst* DC3000 infection. This observation highlights the importance of the SA signaling pathway during priming and early defense responses (**Figure 8**), similarly as shown by Mhamdi and colleagues (2010). There, Arabidopsis mutants accumulating higher levels of citrate were more resistant to *Pst* DC3000 and showed a SA-regulated *PR* genes constitutively overexpressed.

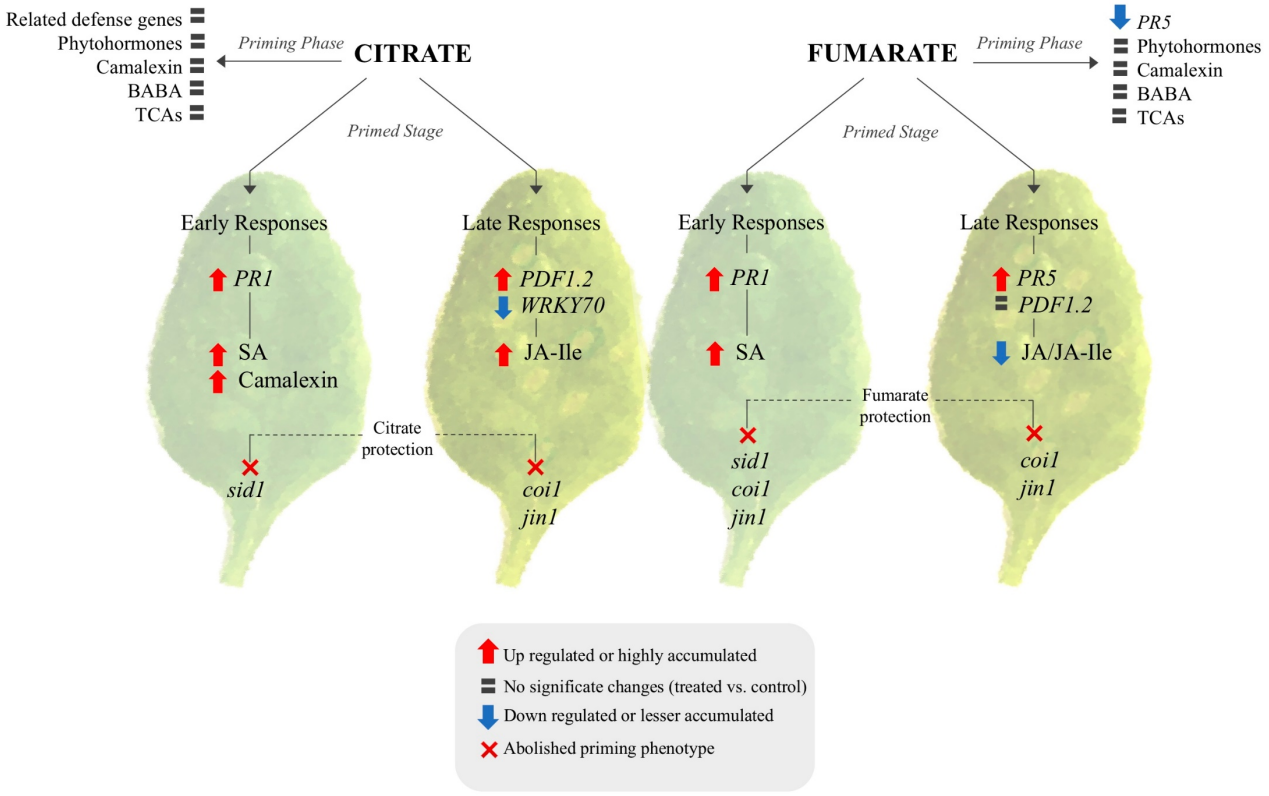


Figure 8. Signaling pathway proposed for citrate and fumarate induce priming in Arabidopsis against *Pst* DC3000. The scheme shows transcriptional and metabolomic changes induced by both TCAs during the priming phase and the primed stage (early and late responses of primed plants against bacterial infection) as well as phytohormone mutants in which citrate and fumarate were impaired in mounting priming. TCA-induced priming can be characterized in two branches: citrate and fumarate specific priming. In both cases the priming stage and priming phase exhibit distinct transcriptomic and metabolomics patterns. Citrate acts over *PR1*, SA and camalexin and is dependent on *sid1* for early defenses responses. Late defenses responses relay on JA pathway. Fumarate on the other hand relay in SA and JA signaling during early and late responses.

In addition to hormone-related genes, actual levels of the phytohormones SA, JA and JA-Ile were quantified during the different priming phases induced by citrate and fumarate. As a general pattern, neither fumarate nor citrate induced phytohormone changes during the primed phase, as expected in light of the observed gene expression pattern (**Figure 8**). However, under *Pst* DC3000 infection, fumarate and citrate induced the accumulation of SA at early time point (24hpi). Subsequently, JA and JA-Ile were down-regulated by fumarate at later time point whereas JA-Ile was upregulated by citrate. The hormone-dependent priming action of TCAs was further corroborated when monitoring TCA-induced priming in phytohormone mutants or Arabidopsis. For instance, the SA-impaired mutant *sid1* was insensitive to priming by citrate, and fumarate at early time point post infection, indicating an incomplete or inefficient priming mechanisms (**Figure**

7). Similarly, in the JA mutant *jin1*, the primed phenotype induced by citrate and fumarate was abolished in all of the analyzed time points. This mutant is known to constitutively express higher *PR1* levels and to accumulate elevated levels of SA upon *Pst* DC3000 (Laurie-Berry et al. 2006). This suggests that in this specific genotype, priming by TCA does not exceed the already present constitutive resistance to bacteria by high endogenous levels of SA. Furthermore, the *coi1* mutant was able to mount a priming response by fumarate at 24hpi, however the phenotype was reversed at 72hpi where elevated bacterial growth was observed compared to control plants. In the same genotype, citrate-induced priming was not effective anymore at 72hpi, indicating that that JA signaling is crucial in mounting a citrate-triggered priming especially during the necrotrophic growth stage of *Pst* DC3000. Recently, JA treatment was demonstrated to have a protective effect against nematode infection in tomato plants, by upregulating amongst others organic acids like citrate and fumarate up to 19% (Bali et al. 2018). Despite the complexity of jasmonate-signaling pathways (Laurie-Berry et al. 2006), this new study reinforces the connection of citrate and JA pathway. However, the link between plant hormones and TCA-triggered priming is likely a multilayered one. For instance, fumarate treatment induced a depletion in JA/JA-Ile contents at later time points of *Pst* DC3000 infection, while *jin1* and *coi1* mutants were impaired to induce priming by this carboxylate. This suggests that not only the hormonal pathways are important during TCA-induced priming, but also the crosstalk between JA and SA. It is well known that JA extensively interacts with other plant hormone signaling pathways. Under *Pst* DC3000 infection, Arabidopsis plants produce both SA and jasmonates to modulate their defense responses (Block et al. 2005). This crosstalk is intriguingly complex and usually dose and time-specific (Kazan et al. 2008). Altogether, the data presented here suggest that predominantly SA rather than JA pathways are important for fumarate and citrate induced priming at early infection stages. Especially at later time points, JA seems to be required for a proper citrate-induced priming response (**Figure 8**). Notably, it has been recently demonstrated that plant defense induction by the avirulent *Pst* avrRpm1 strain leads to a reorganization of primary metabolism in systemic leaves characterized by a strong increment of fumarate and malate contents and a depletion of genes associated with JA and ethylene signaling (Schwachtje et al. 2018). Future work is required to better understand the connection between fumarate and JA signaling.

Another aspect of antibacterial defense is the induction of phyoalexins such as camalexin. Its accumulation is known to inhibit the growth of virulent *Pseudomonas syringae*. The natural variation of camalexin induction is well known to correlate with the resistance phenotype observed

in a natural Arabidopsis population against *Pst* DC3000 (Zhang et al. 2014b). The data obtained here depict an induction of camalexin during *Pst* DC3000 infection development in Col-0 genotype as previously shown (Qiu et al. 2008). Importantly, citrate treatment greatly potentiated camalexin accumulation at early time point post infection, which was not observed in fumarate-induced priming. This finding follows along the general observation here that both TCAs prime plant defense on a distinct signaling pathway (**Figure 8**), which might depend on the status of both host and pathogen cells. This was confirmed when testing a generally necrotrophic pathogen. There, citrate but not fumarate was found to protect against *Plectosphaerella cucumerina* BMM (**Figure S4**). The distinct mode of action of fumarate and citrate could be explained by their involvement in further plant defense strategies. For example, malate and fumarate (5 to 20 mM) treatments were previously demonstrated to reduce stomatal aperture in detached leaves of tomato plants after 2h of incubation (Araujo et al. 2011). This might explain partially the resistance phenotype induced by fumarate in our study. While fumarate treatment was indeed able to induce resistance against *Pst* DC3000, the triggered molecular as well as the metabolic pattern was distinct from citrate. Further studies are required to decipher additional signaling cascades in TCA-induced priming against bacteria. These signaling cascades could involve additional defense layers such as pattern-triggered immunity (PTI) or effector-triggered immunity (ETI).

A plausible scenario of TCA-induced priming could not only involve the potentiation of defense layers, but also a positive feedback loop where TCA treatment itself upregulates intracellular levels of TCA flux. Data presented here demonstrate that endogenous TCA levels did not show any significant changes following treatment with citrate and fumarate, respectively at the time of inoculation with bacteria. This observation might suggest that at 48hpt both exogenously applied carboxylic acids had been catabolized by plants as expected due to the nature of both molecules as providers of carbon skeletons for a large range of biosynthetic process in plant tissues. Daloso et al. (2015) showed a rapid uptake and degradation of TCA intermediates by plants where redistribution of the isotope ^{13}C after 15 mM ^{13}C-malate feed leaves were found in fumarate, succinate and malate intermediates already after 4 hours of incubation. Even though intracellular levels of TCA were not significantly altered in the TCA-induced priming phase, it cannot be ruled out that citrate or fumarate exert a role as extracellular signaling molecule. For instance, citrate was previously shown to be the most abundant metabolite accumulated in the apoplast fluid after *Pseudomonas* infection; citrate levels increased from 300 µM to 1 mM at 4hpi (O'Leary et al. 2016). Moreover, Anderson et al. (2014) demonstrated a dose-dependent signaling effect of citrate on

Pst DC3000 gene expression. Apoplastic citrate levels at physiological concentrations of 50 to 200 µM strongly promoted the expression of the T3SS-secreted effector protein α-AvrPto but completely inhibited its expression at 1 mM. This indicates an intriguing dose-dependent role of citrate as signal modulator of plant effector expression.

In summary, we show here that carboxylic acids can act as priming agents in Arabidopsis in response to *Pst* DC3000. They act by potentiating plant immunity, as no direct bacterial toxicity was observed. The capacity of these compounds to act as priming agents is supported by the finding that they do not induce defense genes during the priming phase, however they potentiate defense gene expression in the primed state in a specific way. SA and JA pathways are eminent in TCA-induced priming, however citrate and fumarate are likely acting over distinct signaling cascades. Further studies will help uncover the promising priming potential of TCAs, as well as TCA-mediated priming against different types of stress.

5. Supplementary Data

Supplementary Figure 1: In Arabidopsis, the TCAs citrate and fumarate confer resistance to *Pst* DC3000.

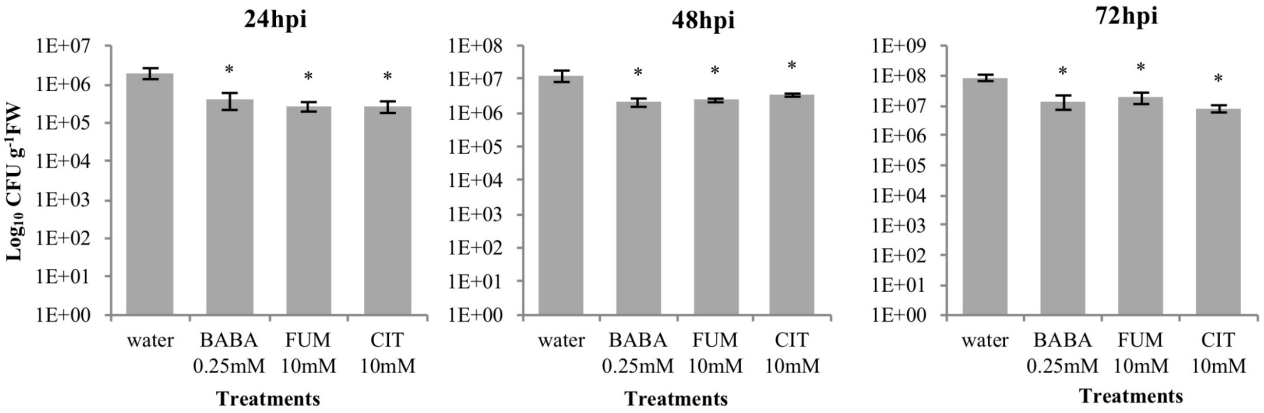


Figure S1. Disease rate in colony forming units (cfu) per gram of fresh material at 24, 48 and 72hpi of Arabidopsis plants soil drenched with fumarate 10 mM, citrate 10 mM, water and BABA 0.25 mM as a controls 2 days before dip-inoculation with *Pst* DC3000 (10^6cfu mL^{-1}). Data represent the mean ± SEM (*n* = 8 biological replicates). The experiments were repeated three times and a representative replicate is shown. Asterisks indicate significant differences: **P* ≤0.05; (treatments vs. water) as determined by *t*-test.

Supplementary Figure 2: Oxoglutarate, malate and succinate levels upon citrate and fumarate treatment in the priming phase.

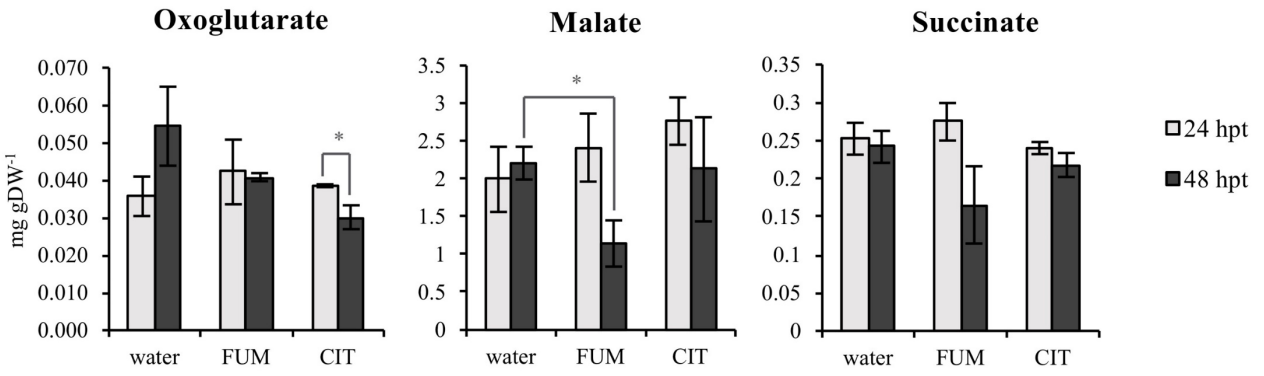


Figure S2. Arabidopsis plants were collected 24 and 48h after soil drench with 10 mM citrate and 10 mM fumarate or water as a control. TCAs levels are expressed in mg gDW^{-1}. Data represent the mean ± SEM (*n* = 3 biological replicates) of one from three independent experiments. Asterisks indicate significant differences (treatments vs. control or 24hpt vs. 48hpt) as determined by *t*-test: **P* ≤0.05.

Supplementary Figure 3: Basal levels of camalexin in Arabidopsis plants.

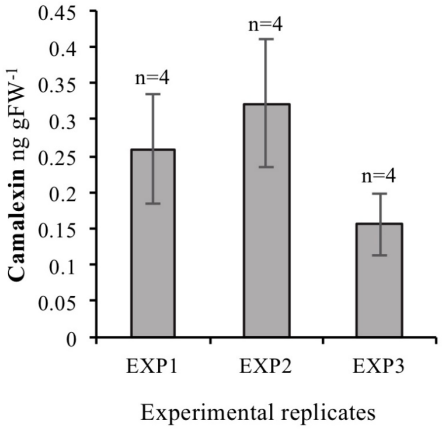


Figure S3. Samples consist of four to five-week-old Arabidopsis Col-0 leaves. Data represent the mean ± SE (*n* = 4 biological replicates). These experiments were repeated on three independent occasions (EXP1, EXP2, EXP3).

Supplementary Figure 4: Citrate but not fumarate was found to protect Arabidopsis plants against *Plectosphaerella cucumerina* BMM.

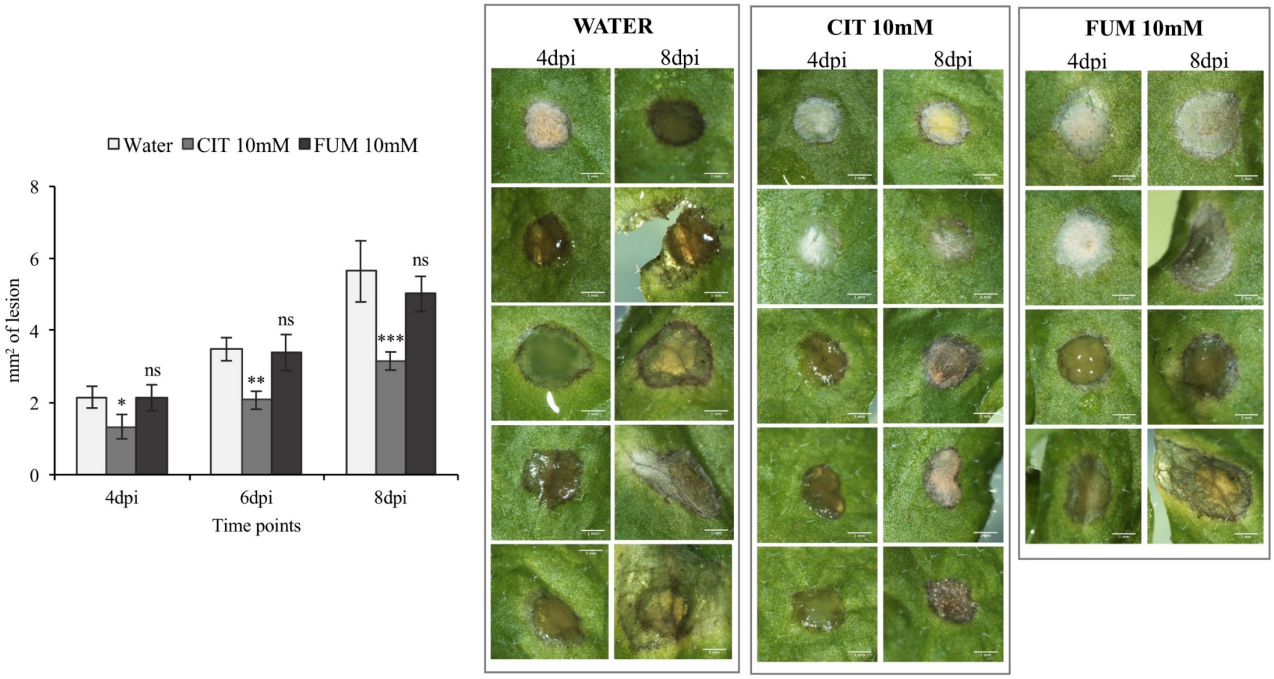


Figure S4. Lesion diameter in mm^2 at 4dpi, 6dpi and 8dpi on Arabidopsis plants soil drenched with water, 10 mM citrate and 10 mM fumarate 2 days before drop inoculation with *Plectosphaerella cucumerina* BMM ($5x10^6$ spores mL^{-1}). Photos from leaves were taken 4dpi and 8pi. Data represent the mean ± SE (*n* = 24 biological replicates). Asterisks indicate significant differences: **P* ≤0.05; ** *P* ≤0.01; *** *P* ≤0.001 (treatments vs. water) as determined by *t*-test.

Supplementary Table 1: Primer sequences and primer efficiency calculated with the help of a dilution curve.

Name	Sequence	Efficiency
AtPR5_fw	GAC TGT GGC GGT CTA AGA TGT	0.99
AtPR5_rev	TGA ATT CAG CCA GAG TGA CG	0.99
AtWRKY70_fw	GGG TTC TCA GGA TCT CAT GG	0.99
AtWRKY70_rev	TCA TTT TCG CTA AAC TCG AAA TC	0.99
AtPDF1.2_fw	GCA ATG GTG GAA GCA CAG A	0.97
AtPDF1.2_rev	GCA AAC CCC TGA CCA TGT	0.97
AtPR1_fw	TGA TCC TCG TGG GAA TTA TGT	0.99
AtPR1_rev	TGC ATG ATC ACA TCA TTA CTT CAT	0.99
AtLOX2_fw	CTT ACC CGC GGA TCT CAT C	0.97
AtLOX2_rev	ACT CCA TGT TCT GCG GTC TT	0.97
At_ACTIN_fw	TGG GAT GAA CCA GAA GGA TG	0.99
At_ACTIN_rev	AAG AAT ACC TCT CTT GGA TTG TGC	0.99
At_PAD3_fw	ACA AGA ACA GGG CAA GGA AA	0.99
At_PAD3_rev	GAT CAG GGG TAA GAG GAC GA	0.99
At_SAND_fw	TGG TAG CCA CAC CGA ATT TA	0.9
At_SAND_rev	GTT AGC CGA TGC AAC CTC AT	0.9

6. References

Anderson, J. et al. (2014) Decreased abundance of type III secretion system-inducing signals in Arabidopsis *mkp1* enhances resistance against *Pseudomonas syringae*. Proc. Natl. Acad. Sci.111, 6846-6851

Araújo, W. et al. (2011) Antisense inhibition of the iron-sulphur subunit of succinate dehydrogenase enhances photosynthesis and growth in tomato via an organic acid–mediated effect on stomatal aperture. Plant Cell. 23, 600-627

Bali, S. et al. (2018) Jasmonic acid-induced tolerance to root-knot nematodes in tomato plants through altered photosynthetic and antioxidative defense mechanisms. Protoplasma. 255, 471-484

Balmer, A. et al. (2015) The 'prime-ome': towards a holistic approach to priming. Trends Plant Sci. 20, 443-452

Balmer, D. et al. (2013) Induced resistance in maize is based on organ-specific defense responses. Plant J. 74, 213-225

Block, A. et al. (2005) Coronatine and salicylic acid: the battle between Arabidopsis and Pseudomonas for phytohormone control. Mol. Plant Pathol. 6, 79-83

Bolton, M. D. (2009) Primary metabolism and plant defense-fuel for the fire. Mol. Plant-Microbe Interact. 22, 487-497

Bolton, M. D. et al. (2008) Lr34-mediated leaf rust resistance in wheat: transcript profiling reveals a high energetic demand supported by transient recruitment of multiple metabolic pathways. Mol. Plant Microbe Interact. 21, 1515-1527

Brooks, D. M. et al. (2005) The *Pseudomonas syringae* phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defenses in *Arabidopsis thaliana*. Mol. Plant Pathol. 6, 629-639

Chen, F. et al. (2017) Combined metabonomic and quantitative RT-PCR analyses revealed metabolic reprogramming associated with *Fusarium graminearum* resistance in transgenic *Arabidopsis thaliana*. Front. Plant Sci. 8, 2177

Conrath, U. et al. (2002) Priming in plant–pathogen interactions. Trends Plant Sci. 7, 210-216

Daloso, D. et al. (2015) Thioredoxin, a master regulator of the tricarboxylic acid cycle in plant mitochondria. Proc. Natl. Acad. Sci. 112, 1392-1400

Fernie, A. et al. (2004) Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. Curr. Opin. Plant Biol. 7, 254-261

Finkemeier, I. et al. (2013) Transcriptomic analysis of the role of carboxylic acids in metabolite signaling in Arabidopsis leaves. Plant Physiol. 162, 239-253

Flors, V. et al. (2008) Interplay between JA, SA and ABA signaling during basal and induced resistance against *Pseudomonas syringae* and *Alternaria brassicicola*. Plant J. 54, 81-92

Glauser, G. et al. (2014) Hormone Profiling. In: Methods in Molecular Biology, Arabidopsis Protocols, Sanchez-Serrano and Salinas Eds, Humana Press, Springer, volume 1062, 597-608

Heck, S. et al. (2003) Genetic evidence that expression of NahG modifies defense pathways independent of salicylic acid biosynthesis in the Arabidopsis–*Pseudomonas syringae* pv. tomato interaction. Plant J. 36, 342-352

Hodges, M. (2002) Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. J. Experimental Bot. 53, 905-916

Jung, H. et al. (2009) Priming in systemic plant immunity. Science. 324, 89-91

Kim, J. M. et al. (2017) Acetate-mediated novel survival strategy against drought in plants. Nature plants. 3, 17097

King, E. O. et al. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44, 301-307

Laurie-Berry, N. et al. (2006) The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. Mol. Plant-Microbe Interact. 19, 789-800

Less, H. et al. (2011) Coordinated gene networks regulating Arabidopsis plant metabolism in response to various stresses and nutritional cues. Plant Cell. 23, 1264-1271

Lorenzo, O. et al. (2004) JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell. 16, 1938-1950

Martinoia, E., Rentsch, D. (1994) Malate compartmentation-responses to a complex metabolism. Annu. Rev. Plant Biol. 45, 447-467

Mhamdi, A. et al. (2010) Cytosolic NADP-dependent isocitrate dehydrogenase contributes to redox homeostasis and the regulation of pathogen responses in Arabidopsis leaves. Plant Cell Environ. 33, 1112-1123

Miflin, B. J., Habash, D. Z. (2002) The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. J. Exp. Bot. 53, 979-987

McCammon, M. et al. (2003) Global transcription analysis of Krebs tricarboxylic acid cycle mutants reveal an alternating pattern of gene expression and effects on hypoxic and oxidative genes. Mol. Biol. Cell. 14, 958-972

Müller, C. et al. (2001) Influence of malate and 2-oxoglutarate on the NIA transcript level and nitrate reductase activity in tobacco leaves. Plant Cell Environ. 24, 191-203

Návarová, H. et al. (2012) Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. Plant Cell. 24, 5123-5141

Nawrath, C., Métraux, J. P. (1999) Salicylic acid induction–deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell. 11, 1393-1404

Nomura, H. et al. (2012). Chloroplast-mediated activation of plant immune signaling in Arabidopsis. Nature Communications. 3, 926

O'Leary, B. M. et al. (2016) Early changes in apoplast composition associated with defense and disease in interactions between *Phaseolus vulgaris* and the halo blight pathogen *Pseudomonas syringae* pv. phaseolicola. Plant Cell Environ. 39, 2172-2184

Park, H. et al. (2015) Pathogen associated molecular pattern (PAMP)-triggered immunity is compromised under C-limited growth. Mol. Cells. 38, 40-50

Pastor, V. et al. (2013a) Primed plants do not forget. Envir. Exp. Bot. 94, 46-56

Pastor, V et al. (2014) Preparing to fight back: generation and storage of priming compounds. Front. Plant Sci. 5, 295

Qiu, J. et al. (2008) Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO J. 27, 2214-2221

Rate, D. N. et al. (1999) The gain-of-function Arabidopsis *acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. Plant Cell. 11, 1695-1708

Rojas, C. et al. (2014) Regulation of primary plant metabolism during plant-pathogen interactions and its contribution to plant defense. Front. Plant Sci. 5, 17

Scheideler, M. et al. (2002). Monitoring the switch from housekeeping to pathogen defense metabolism in *Arabidopsis thaliana* using cDNA arrays. J. Biol. Chem. 277, 10555-10561

Schwachtje, J. et al. (2018) Primed primary metabolism in systemic leaves: a functional systems analysis. Sci. Rep. 8, 216

Shelp, B. et al. (1999) Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4, 446-452

Slaughter, A. et al. (2011) Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. Plant Physiol. 158, 835–843

Stael, S. et al. (2011) Arabidopsis calcium-binding mitochondrial carrier proteins as potential facilitators of mitochondrial ATP-import and plastid SAM-import. FEBS letters. 24, 3935-3940

Staswick, P. E., Tiryaki, I. (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell. 16, 2117-2127

Sweetlove, L. et al. (2010) Not just a circle: flux modes in the plant TCA cycle. Trends Plant Sci. 15, 462-470

Sweetlove, L. et al. (2002) The impact of oxidative stress on Arabidopsis mitochondria. Plant J. 32, 891-904

Thevenet, D. et al. (2017) The priming molecule β-aminobutyric acid is naturally present in plants and is induced by stress. New Phytol. 213, 552-559

Thines, B. et al. (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signaling. Nature. 448, 661

Tretter, L., Adam-Vizi, V. (2000) Inhibition of Krebs cycle enzymes by hydrogen peroxide: a key role of α-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. J. NeuroSci. 20, 8972-8979

Van Aken, O. et al. (2016) Mitochondrial and chloroplast stress responses are modulated in distinct touch and chemical inhibition phases in Arabidopsis. Plant Physiol. 00273

Wagner, S. et al. (2018) Mitochondrial Energy signaling and its role in the low-oxygen stress response of plants. Plant Physiol. 176, 1156-1170

Wellen, K. et al. (2009) ATP-citrate lyase links cellular metabolism to histone acetylation. Science. 324, 1076-1080

Yang, M. et al. (2012) The emerging role of fumarate as an oncometabolite. Front. Oncol. 2, 85

Yi, S. Y. et al. (2014) The activated SA and JA signaling pathways have an influence on *flg22-*

triggered oxidative burst and callose deposition. PLoS One. 9, e88951

Zhang, B. et al. (2014a) The mitochondrial outer membrane AAA ATPase AtOM66 affects cell death and pathogen resistance in *Arabidopsis thaliana*. Plant J. 80, 709-727

Zhang, N. et al. (2014b) Constitutive camalexin production and environmental stress response variation in Arabidopsis populations from the Iberian Peninsula. Plant Sci. 225, 77-85

Zhou, N. et al. (1998) PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell. 10, 1021-1030

Zimmerli, L. et al. (2001) β-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus *Botrytis cinerea*. Plant Physiol. 126, 517-523

Chapter III- **Prime time for plant immunity**

Deciphering the kinetics and proteomic signature of BABA treatment in Arabidopsis

Balmer, A., Heazlewood, J.L., & Mauch-Mani, B. (2018). Deciphering the kinetics and proteomic signature of BABA treatment in Arabidopsis. *Manuscript in preparation*.

Keywords
β-Aminobutyric acid, BABA, BABA uptake, proteomics, priming, priming signals

Deciphering the kinetics and proteomic signature of BABA treatment in Arabidopsis

1. Introduction

During evolution, plants developed the capacity to defend themselves against challenging situations such as the exposure to numerous pathogenic microorganisms, pests or changing environmental conditions. Having no specialized immune cells, plants heavily rely on the potency of inducible defense systems of each single cell. Inducible defense is usually associated with extremely high energy costs, making it of significant importance for plants to harbor a cellular signaling cascade that prepares for a future attack without exhausting precious nutrients. Therefore, plants employ an intricate defense mechanism where cells are potentiated and prepared for future biotic and abiotic challenges. This inducible physiological state called "priming" is characterized by a stronger and more efficient deployment of defense response with minimal fitness cost under unchallenged conditions (Conrath et al. 2002). Priming can be triggered by a pre-exposition to microorganisms, effectors or by natural or synthetic agents (Martinez-Medina et al. 2016). Once in contact with a priming elicitor, an initial physiological state is generated, called "priming phase". The priming phase encompasses the time-lapse between the primary perception of the priming stimuli and the exposure to a future stress.

To date, a broad spectrum of priming inducers have been described (reviewed in Balmer et al. 2015). Amongst the chemical priming stimuli, the non-proteinogenic amino acid β-aminobutyric acid (BABA) has been commonly accepted to be the gold standard of priming compounds, due to its very broad range of action (Cohen 2002). Recent studies demonstrated that this amino acid – previously only known as synthetic compound – is naturally synthesized by several plant species, and its induction plays a significant role within the plant immune system (Thevenet et al. 2016; Baccelli and Mauch-Mani, 2017). As an inducer of priming, BABA can be applied via foliar spray, seed soakage and soil drench, the latter being the most common way of application (Bengtsson et al. 2014a; Worrall et al. 2012). The specific concentration necessary to obtain the desired priming effect depends on a given plant species and the nature of the stress to be defied. In potato plants, 10 mM of BABA was shown to have a protective effect against *Phytophthora infestans* (Bengtsson et al. 2014a). Meanwhile, in Arabidopsis the range between 0.15 mM to 0.25 mM is sufficient to

induce priming against *Plectosphaerella cucumerina* or *Pseudomonas syringae* pv. tomato DC3000 (Ton et al. 2004; Tsai et al. 2011).

The molecular and biochemical processes occurring during BABA-induced priming are still elusive, although it is widely accepted that significant changes in signaling cascades must occur (Bruce et al. 2007). In recent years attempts to elucidate aspects of priming using 'omics approaches have been employed to better understand the signaling dynamics underlying the potentiated immune system (Conrath, 2011). From a molecular point of view, priming defenses were speculated to be the result of epigenetic changes such as DNA methylation, histone modifications or chromatin remodeling (Slaughter et al. 2012; Jaskiewicz et al. 2011). These epigenetic alterations might modulate priming responses at the transcriptomic level. Using metabolomic approaches, it has been furthermore demonstrated that early responses during the BABA-induced priming phase includes a faster induction of reactive oxygen species (ROS), callose accumulation, production of plant defense hormones as well as the rapid accumulation of primary and secondary metabolites (Pastor et al. 2013; Bengtsson et al. 2014b; Pastor et al. 2014). Knowledge at the proteomic level around the adaptation to the primed state is scarce. For instance, the mitogen-activated protein kinases MPK3 and MPK6 were found to act as important signaling proteins during the priming phase in Arabidopsis (Beckers et al. 2009). However, few studies investigated proteomic changes after exposure to BABA and different stresses. In potato leaves, BABA treatment at 10 mM induced significant changes in the abundance of 91 proteins in the apoplastic secretome (Bengtsson et al. 2014b). Amongst the secreted proteins, several pathogenesis-related (PR) proteins like an acid class II 1,3-beta glucanase (PR2) and a Thaumatin-like protein (PR5) as well as a cell wall-peroxidases were found to be highly induced following BABA treatment (Bengtsson et al. 2014b). In contrast, when 2 mM BABA was applied to potato plants, only minor changes in protein levels occurred in treated leaves. Within the group of altered proteins, 18 were involved in primary metabolism including the redox regulated enzymes glyceraldehyde 3-phosphate dehydrogenase, carbonic anhydrase and fructose-bisphosphate aldolase (Arasimowicz-Jelonek et al. 2013). In a similar study, BABA was used to evaluate priming against salt stress in *Malus pumila* (Macarisin et al. 2009). At a concentration of 0.5 mM, BABA treatment only marginally modified the proteomic profile in plants during a mild drought stress. However, under severe dehydration stress, BABA-primed plants revealed drastic changes in the proteomic profiles compared to water or ABA treated plants (Macarisin et al. 2009). These studies highlight the complexity of BABA-induced priming, being dependent on plant species, applied concentration, as well as stress severity.

Importantly, previous BABA-specific proteomic surveys were analyzed during the post-challenge primed state i.e. when a second stimulus (abiotic or abiotic stressor) has been applied to the test system. Proteomic studies focusing on the priming phase are few – despite being the cardinal phase where priming signals are generated and stored in preparation for an upcoming challenge. Although BABA has been studied for decades, it is still not understood how plants sense and translate BABA-modulated priming signals is still not understood. Only a single study has explored the chemical behavior of BABA in plants, 25 years ago Cohen and Gisi (1994) reported that depending on the method of application, BABA could be transported bi-directionally from the roots to the leaves and vice versa. It was also suggested that BABA does not undergo any chemical modification in plants, and that BABA is stable in the plant tissues. Recent advances in chromatographic techniques now allow the accurate quantification of BABA levels in plant tissues (Thevenet at al. 2017). This newly developed protocol opens the door to an extensive characterization of chemical kinetics of BABA in different plant tissues. However, major questions still remain, including the kinetics of BABA uptake and metabolism in plants, the response to BABA in both leaf and root tissue, and what proteins are actually being activated by BABA in plants.

In this study, we aim to decipher the kinetics of exogenously applied BABA in both leaves and roots of *Arabidopsis thaliana*. Despite being the preferred target tissue of BABA treatment for priming, roots have so far been neglected from studies describing BABA-induced signaling. Moreover, besides the chemical kinetics of BABA, we performed a high-coverage proteomic analysis of the tissue-specific priming phase upon BABA treatment. By comparing uptake and stability of BABA, we show distinct chemical patterns in leaves and roots. This in-depth proteomic analysis of the priming phase depicts the differences between different plant organs and time points after BABA treatment. These data will pave the way for further investigations of putative signaling cascades in BABA-mediated priming, as well as help to uncover the role of the roots in priming responses.

2. Material and methods

2.1 Plant growth conditions and chemical treatment

Arabidopsis thaliana (L.) Heynh. accession Columbia-0 (Col-0) seeds were germinated in 33 mL hydrated Jiffy pellets maintained at 21°C day/19°C night, with 9 hours of light (120 µE m^{-2} s^{-1}) and 60% of relative humidity. One week after germination seedlings were individually transferred to either Jiffy pellets or a hydroponic culture system (Araponics: www.araponics.com) and kept under the same conditions until chemical treatments. The nutrient solution containing ½-strength Hoagland was replaced weekly. Four- to five-week old Col-0 plants were soil-drenched with BABA (SIGMA-ALDRICH A44207, racemate), deuterium-labeled BABA ($BABAd_3$, synthetized by Thevenet et al. 2017) or water (control). The applied BABA concentrations differed depending on the specific assays (described below). The experiments were conducted with three independent biological replicates.

2.2 Extraction and liquid chromatography-tandem mass spectrometry analysis of BABA

Endogenous BABA content from leaves and roots were analyzed using an Ultimate 3000 RSLC from Dionex interfaced with a 4000 QTRAP. Liquid chromatography (LC) separation was accomplished using an Acquity UPLC BEH HILIC column (100 mm 9 2.1 mm, 1.7 lm, Waters) as previously described (Thevent et al. 2017, Balmer et al. 2018, in press). To extract BABA, treated plant material was harvested, flash-frozen and ground in liquid nitrogen. The extraction protocol was conducted as previously described (Thevenet et al. 2017).

2.3 Extraction of apoplastic content from Arabidopsis leaves

Apoplastic fluid was extracted from Arabidopsis leaves by an infiltration-centrifugation method as described by Lohaus et al. (2001), with minor modifications. Leaves of 4 to 5 week-old plants that were soil drench with a nutrient solution containing 1.5 µM of BABA for 24 hours were cuted at the petiole. Leaves were repeatedly rinsed in Milli-Q water to wash off surface contaminants and dried on sterile filter paper. The samples (10 leaves per biological replicat) were then infiltrated with 40 mL Milli-Q water using a 50 mL syringe. The procedure was repeated until the leaves were fully infiltrated (dark green colored leaf section). The leaf surface was then again dried on sterile

filter paper. The leaves were rolled in parafilm and placed in a 5 mL syringe and then in a 15 mL Falcon tube. Assembled in this way, the samples were centrifuged at 1000g for 5 minutes at 4°C. The flow-through containing the apoplastic fluid and the leaves were collected and stored at -80°C for further analysis. Malate dehydrogenase (MDH) activity in the apoplastic fluid was taken as an indication of cytoplasmic contamination. Measurement of MDH activity was carried out as described by Jenner et al. (2001).

2.4 Protein extraction

Four week-old Arabidopsis plants were root dipped in a nutrient solution containing 250 µM BABA, control samples were also exposed to a nutrient solution (without BABA). Six and 48 hours post treatment, leaves and roots were collected, flash-frozen and ground in liquid nitrogen. Proteins were isolated from 100 mg ground tissue using the trichloroacetic acid (TCA)-acetone extraction method as previously reported by Wu et al. (2014). The extracted protein pellets were re-suspended in 100 µL of buffer containing 6 M urea, 3 mM EDTA and 20 mM Tris-HCL pH 8.3. Insoluble components were removed by centrifugation and the supernatant was retained for analysis. The protein concentration was determined according to Bradford (1976) with BSA (Bovine Serum Albumin) as a standard. A total of 2 µg protein were reduced by adding TCEP (Tris (2-carboxyethyl) phosphine) to a final concentration of 12.5 mM and incubating for 1h at room temperature. Subsequently, IAA (Iodoacetamide) was added to a final concentration of 25 mM and samples were incubated for 1h at room temperature in the dark. Proteins were then diluted using 20 mM Tris-HCL (pH 8.3) to reduce the urea concentration to less than 1 M. Proteins were digested with 0.2 µg of trypsin overnight at 37 °C. The peptide solution obtained was acidified by adding 0.01% of formic acid. Samples were desalted on a C18 solid phase extraction column as described by the manufacturer (The Nest Group, Southborough, MA, USA). Two µL of each sample were subject to a LC-MS/MS analysis using an Orbitrap Fusion™ Lumos™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific) fitted with a nanoflow HPLC (Ultimate 3000 RSLC, Dionex). The nano-LC system was equipped with an Acclaim Pepmap nano-trap column (Dionex – C18, 100 Å, 75 µm × 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex – C18, 100 Å, 75 µm × 50 cm). The samples were loaded on the trap column with an isocratic flow of 5 µL/min of 3% CH_3CN containing 0.1% formic acid for 5 min before it was switched in-line with the analytical column. The eluents used for the LC were 0.1% (v/v) formic acid (solvent A) and 100% acetonitrile/0.1% formic acid (v/v) (solvent B). The gradient applied was 3 % B to 20 % B for 95 min, 20 % B to 40 % B in 10 min, 40

% B to 80 % B in 5 min and maintained at 80 % B for the final 5 min before equilibration for 10 min at 3 % B. The instrument was operated in positive ion mode at a resolution of 120,000 in full scan mode using data-dependent acquisition (DDA). The MS2 was operated in HCD mode with a resolution of 30,000, AGC target of 50,000 and Activation Q of 0.25 for ions above 50,000 with a charge state between 3 and 8.

2.5 Database search and quantitative proteomic data analysis

Raw data were converted to MGF file format using ProteoWizard (Kessner et al. 2008). The Mascot 2.3.0 software (Matrix Science, Boston, MA, USA) was used to search MGF files against an Arabidopsis database (TAIR10) (www.arabidopsis.org). Search parameters for Mascot were a fragment ion mass tolerance of 0.2 Da and a parent ion tolerance of 5.0 ppm, and a maximum of one missed cleavage was allowed. The carbamidomethylation of cysteines (C) was specified as a fixed modification and oxidation of methionine residues (M) was set as variable modification. Scaffold (version Scaffold_4.8.1, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Protein quantification was calculated by using the Precursor Ion Intensity quantification method. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold local fold discovery rate (FDR) algorithm. Protein identifications were approved if they could be established at greater than 99.0% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. In Scaffold software, a Student's *t*-test was used to identify statistically significant differences between treatment and control group of each given tissue and time point (3 independent biological replicates per condition; each biological replicate is a pool of 6 plants). Proteins which had at least a two-fold difference with respect to the mean ratio, as well as a *P*-value ≤ 0.05, were considered to be significantly altered between the sample groups. Using the same application software, MS/MS data were normalized and 0.5 was set as minimum value to replace missing values in order to increase the confidence of changes between treatments. Proteins were allocated to functional classes using Gene Ontology (GO) terms from gene associations (TAIR) (downloaded Jul 25, 2017) (Ashburner et al. 2000) and visualized using the online Classification SuperViewer Tool BAR (Provart and Zhu, 2003; http://bar.utoronto.ca).

2.6 Resistance assays and pathogen growth conditions

Hyaloperonospora arabidopsidis strain NOCO (compatible interaction with Col-0), was maintained on *NahG* or Col-0 plants. *H. arabidopsidis* infection assays were performed as described by Ton et al. 2005. Wild-type (Col-0) and mutant plants (**Supplemental Table 1**) of two- to three-week old seedlings were inoculated with *H. arabidopsidis* (5×10^4 conidiospores mL^{-1}) two days after treatment with 150 µM of BABA. The *H. arabidopsidis* colonization of leaves was determined microscopically at 7 dpi using the trypan-blue staining method, as previously described (Luna et al. 2014). The stock numbers for Arabidopsis seeds used in this study are outlined in **Table S1** and were obtained from the European Arabidopsis Stock Centre NASC (http://arabidopsis.info).

2.7 Statistical analysis

Variances of BABA levels between different sample groups including malate dehydrogenase activity assays were validated using a Student's *t*-test; a *P* value <0.05 was considered significant. For statistically significant differences in class distribution of pathogen development between wild-type (Col-0) and mutant Arabidopsis plants, a Fisher's exact test was applied (*P* value <0.05; was considered significant). All statistical analyses were performed using GraphPad software (www.graphpad.com).

3. Results

3.1 BABA is rapidly taken up by roots and is more stable in leaves

To date, no accurate data of BABA uptake and kinetics in plant tissue are available due to the lack of a sensitive method to separately quantify the three isomers AABA (alpha aminobutyric acid) BABA and GABA (gamma aminobutyric acid). Here, we made use of a recently developed protocol (Thevenet et al. 2014) to monitor BABA uptake. Four week-old Col-0 plants growing in a hydroponic system were treated with deuterized BABA ($BABAd_3$), which was added to the nutrient solution at 10 µM, 50 µM, 100 µM and 250 µM. Nutrient solution without $BABAd_3$ was used as control. Plants were kept in these conditions until samples from leaves and roots were collected at 1, 6, 48 and 72 hours of incubation (hpi).

As show in **Figure 1A and B**, $BABAd_3$ uptake occurs rapidly and independently of a given concentration. This rapid uptake was even more predominant in roots, where after one hour of incubation the level of $BABAd_3$ reached 10 µg gFW^{-1} at the lowest concentration (10 µM). The early presence of BABA in leaves suggest rapid transportation from roots to leaves (**Figure 1B**). We further tested the sensitivity of BABA uptake by applying 1.5 µM $BABAd_3$, a concentration much lower than normally used in bioassays (Balmer et al. 2014). The uptake pattern was similar in these experimental conditions, as show in **Figure S1**, where after 1 hour of incubation $BABAd_3$ levels reach 1200 ng gFW^{-1} in roots and 10 ng gFW^{-1} in leaves. The difference in BABA levels in leaves and roots is likely a result of roots being in direct contact with the compound, whereas $BABAd_3$ detected in leaves is systemically transported from the roots to the aboveground tissues. The uptake speed reaches a plateau phase after 48 hours of incubation post treatment in leaves as well in roots for all the tested concentrations. Interestingly, after 72 hours of incubation, $BABAd_3$ levels start to decrease in root tissue. On the other hand, in leaves the levels of $BABAd_3$ remain stable between 48 and 72 hours after incubation. These results suggest that $BABAd_3$ is rapidly incorporated into roots to a certain saturation point while being continually transported to leaves. Additionally, our data suggest that this amino acid is catabolized faster in roots tissues than in leaves. Given the higher stability of BABA in leaves, we monitored BABA levels during an extended period. Plants were incubated in 250 µM of $BABAd_3$ for 24h, and during a period of 20 days, samples were taken every two days for $BABAd_3$ quantification. The results show that BABA levels in leaves were decreasing after 4 days post incubation and that this reduction is consistent over 20 days (**Figure S2**). This indicates a saturation of BABA uptake, as well as a gradual degradation of

this amino acid in plant tissue. As with 250 µM, an excess of $BABAd_3$ was applied to the nutrient solution, it can be excluded that the saturation phenotype is due to the absence of $BABAd_3$ molecules in the treatment.

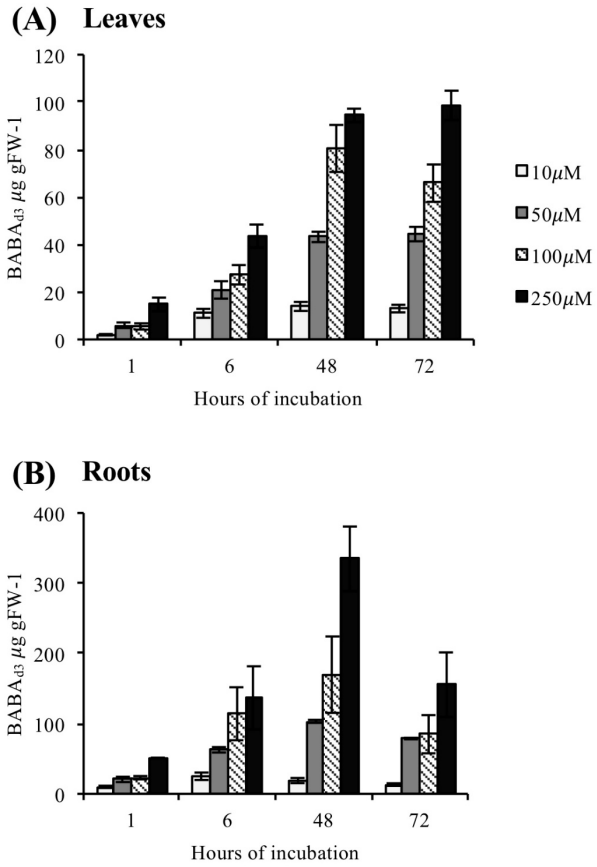


Figure 1. BABA uptake in Arabidopsis leaves and roots. Four week-old Col-0 plants were treated with $BABAd_3$ by adding to the nutrient solution at 10 µM, 50 µM, 100 µM and 250 µM as a final concentration. Nutrient solution without $BABAd_3$ was used as control. Plants were kept in these conditions until samples from leaves and roots were collected at 1, 6, 48 and 72 hours post treatments (hours of incubation). (A). $BABAd_3$ in leaves (µg gFW^{-1}). (B) $BABAd_3$ in roots (µg gFW^{-1}). Data represent the mean ± SE (n = 4 biological replicates). The experiment was conducted twice with similar results.

To examine the catabolism of BABA after uptake into the plant tissue, levels of $BABAd_3$ were measured in leaves and roots of Col-0 plants maintained in a hydroponic system for 24 hours in 1.5 µM of $BABAd_3$ (**Figure 2**) (T0'a). After plants were rinsed and transferred to a BABA-free nutrient solution, samples were collected at 1, 2, 3 and 6 days after the initial $BABAd_3$ incubation.

As show in **Figure 2**, BABA levels decreased faster in roots, starting already at 1dpi. At later time points (6 dpi) only 15% of incorporated BABA was still detectable in roots. On the other hand, in leaves, BABA levels remained stable during all the analyzed time points with a subtle tendency to

decrease at 6dpi. The depleted levels of $BABAd_3$ in roots may be due to a degradation or to a re-mobilization of the amino acid reservoir in roots towards the leaves.

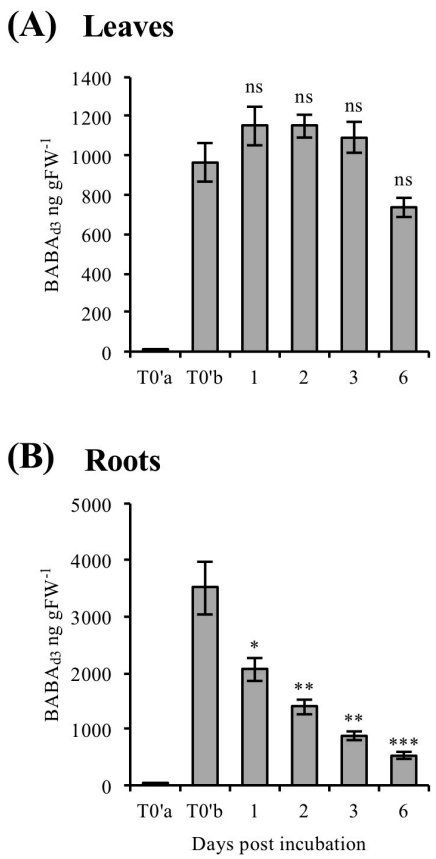


Figure 2. Catabolism and/or metabolization of BABA after uptake into leaves and roots. levels of $BABAd_3$ were measured in leaves and roots of four weeks-old Col-0 plants maintained in a hydroponic system for 24 hours enriched with 1.5 µM of $BABAd_3$ (T0'a). After that, roots were rinsed shortly with water and transferred to a $BABAd_3$-free nutrient solution and samples were collected at 0 (T0'b),1, 2, 3 and 6 days after the initial BABA incubation. (A) $BABAd_3$ in leaves (ng gFW^{-1}). (B) $BABAd_3$ in roots (ng gFW^{-1}). Data represent the mean ± SE (n = 6 biological replicates) of a representative experiment that was repeated three times with similar results. ns indicate no statistically significant differences and asterisks indicate significant differences as determined by a Student t-test: *P < 0.05; **P < 0.01; ***P < 0.001.

3.2 In leaves, $BABAd_3$ is mainly located in the intracellular compartment and only trace amounts are found in the apoplastic fluid.

In order to test whether incorporated BABA is loaded into cells, $BABAd_3$ was quantified both from leaf tissue and apoplastic fluid. After an incubation period of 24h in 1.5 µM $BABAd_3$, the apoplastic fluid was isolated by an infiltration-centrifugation technique. A low $BABAd_3$ concentration was chosen to avoid oversaturation of the tissue. $BABAd_3$ was mainly detected in the intracellular compartment (**Figure 3**. IE: intracellular extract), however small amounts of the amino acid were also detected in the apoplastic fluid (**Figure 3**. AF: Apoplastic fluid). In order to exclude cytoplasmic contamination in the apoplastic samples, a malate dehydrogenase (MDH) activity assay was used to measure both cell compartments. As show in **Figure S3**, MDH activity was detected only in the intracellular extract but not in the apoplastic fluid. The trace concentrations detected in the apoplast suggests that $BABAd_3$ is transported via the apoplastic flow.

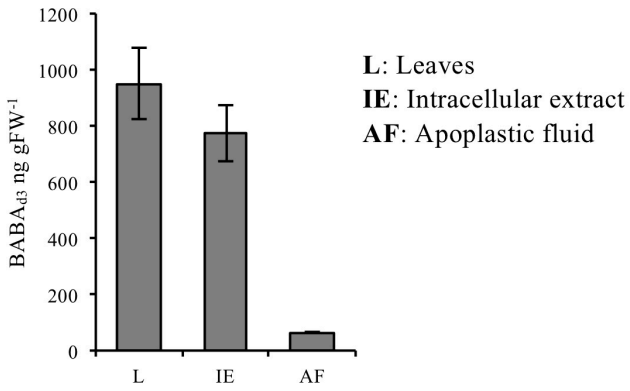


Figure 3. Quantification of BABA levels in leaf tissues and in the apoplastic fluid of Arabidopsis plants. After a root incubation during a period of 24h in 1.5 µM $BABAd_3$, $BABAd_3$ levels were measured in the apoplastic fluid (AF), the intracellular extract (IE) and in the entire leaves (L; or leaves without apoplastic extraction) of Arabidopsis plants. The values represent the mean ± SE (n = 3 biological replicates of a pool of 10 leaves for replicate) of a representative experiment that was repeated twice with similar results.

3.3 Temporal and spatial proteomic profiling of BABA-treated plants

Following the kinetics of BABA uptake, 6h and 48h post treatment were chosen for an in-depth proteomic survey. Plants were soaked in 250 µM of BABA; this concentration was selected since it is commonly used to induce priming in Arabidopsis plants against several biotic and abiotic stressors (Zimmerli et al. 2008; Ton et al. 2005). The BABA-induced proteomic changes in Arabidopsis leaves at different time points were examined by LC-MS/MS and a total of 2433 unique proteins in leaf samples (in all analyzed conditions) and 3859 unique proteins in root samples (in all analyzed conditions) were identified. Comparison between control and treated samples was performed using a "fold change by category" were the three replicates belonging to control or treated samples were pooled and a P <0.05 as described in Dong et al. (2016). The quantitative profiles for each experimental condition were represented in a Venn diagram (**Figure 4A**) and classified in Volcano Plots where the protein distribution was represented as fold change versus P values (**Figure S4B**). As a result, when comparing mock versus BABA treated samples, 578 proteins were identified as differentially accumulated proteins (DAPs) in leaves at 6 hours post incubation (hpi) and 345 DAPs at 48hpi in the same tissue (**Figure 4A**); in roots a total of 581 proteins at 6hpi and 522 at 48hpi were differently regulated. At 6hpi, 170 proteins were exclusively induced by BABA in the leaf tissue; whereas 130 BABA-specific altered proteins were observed in roots. At 48hpi, the number of BABA-specific leaf proteins was 68, whereas 317 proteins were found specific for the BABA-treated sample group in leaves (**Figure 4A**).

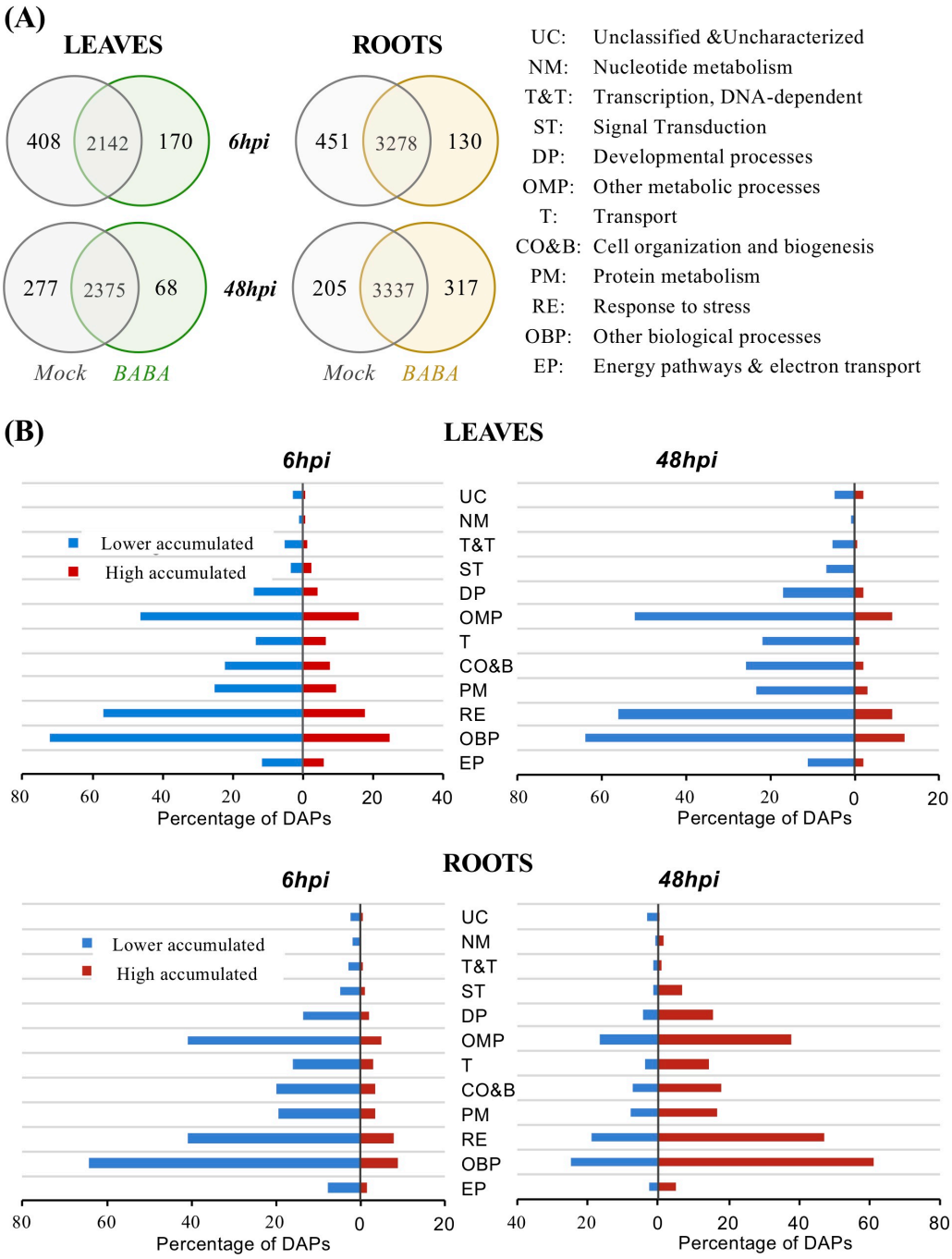


Figure 4. Distribution and functional classification of BABA and mock responsive proteins in leaves and roots of Arabidopsis plants. **(A)** The quantitative profiles of proteins that were differentially accumulated (DAPs) for each experimental condition were represented in a Venn diagram. (**B)** Functional classification of DAPs from each experimental condition via the GO and TAIR databases. The percentage of DAPs that showed lower or high accumulation is shown for each functional category. The abbreviation for each category is indicated in B. hpi, hours post incubation.

Based on the Gene Ontology (GO) annotation and using the classification SuperViewer Tool BAR (Provart and Zhu, 2003), DAPs from each experimental condition were classified according to their biological functions. The percentage of DAPs that showed increased or decreased accumulation is shown for each functional category (**Figure 4B**). In general, BABA treatment predominantly led to

a lower-accumulation of proteins amongst all GO classifications examined. This was observed for all time-points and tissues except at 48hpi in roots, were the number of BABA-high accumulated proteins was found to be greater compared to the group of lower-accumulated proteins. Nonetheless, in leaves, at 6hpi the percentage of DAPs which were accumulated after BABA treatment was higher compared to 48hpi. Particular categories were over-represented in the early time-point including signal transduction, transport, cell organization and biogenesis, responses to stress and energy pathways and electron transport. On the other hand, in root tissues most DAPs induced by BABA showed increased regulation at 48hpi compared to 6hpi. At 6hpi, the majority of classified proteins were increased in mock-treated samples, similar to the leaf tissue. At 48h however, high accumulated proteins in BABA-treated samples were more dominant, especially proteins classified in stress response pathways (**Figure 4B**).

3.5 Characterization of most significantly altered proteins in leaf tissue

After generating a broad picture of the proteome and analyzing the changes in GO classes, we aimed to manually filter the data in order to characterize in detail the most prominently changed proteins in a given tissue. We focused mainly on proteins linked to stress and defense responses, to identify important factors for BABA-triggered priming for biotic stress. Based on the fold change calculated by Scaffold as described in material and methods, a $\log_2$ classification using SuperViewer Tool was calculated. DAPs showing the most dramatic changes in leaves of BABA-treated plants were extracted and grouped (**Table 1A**).

Looking at 6hpi, the most high-accumulated protein was found to be the allene oxide synthase AOS, a protein crucial for jasmonic acid synthesis. Secondly ranked came out AtHOL (HARMLESS TO OZONE LAYER 1), which is involved in glucosinolate metabolism and defense against pathogens (Natagoshi et al. 2009). Intriguingly, multiple proteins linked to the cellular respiration and oxidative stress were highly up-regulated at 6dpi, including cytochrome C1, an NADH dehydrogenase, or a NADPH-dependent oxidoreductase. We also observed an upregulation of PPC2, a phosphoenolpyruvat carboxylase (PEPC) and the MSRB3 (methionine sulfoxide reductase B3) which play protective roles in the cellular response to oxidative stress by reducing oxidized methionine back to methionine (Kwon et al. 2007). This protein is not only known to be important in carbon and nitrogen balance (Shi et al. 2015), but also a central enzyme in synthesis of oxalacetic acid (Krome et al. 2007), which in turn stimulates the citric acid cycle. PEPCs are well described to be induced by treatment with immune stimulators such as BTH or by fungal infection. Moreover,

BABA treatment highly induced the accumulation of lipid-transfer protein (LTP) LP4 at 6pdi in leaves. LTPs are proteins that bind and transport phospholipids, having important roles in biotic and abiotic stress (McLaughlin et al. 2015). At this early time-point, protein trafficking seemed to also be positively induced by BABA, as the transmembrane emp24 domain-containing protein p24 involved in vesicular protein trafficking (Langhans et al. 2008) and the PGM enzyme (plastidic phosphoglucomutase) which participates in the carbon flux (Periappuram et al. 2000) were high accumulated. Additionally, a member of the leucine–rich repeat (LRR) protein family having a significant role in plant defenses (Reviewed by Jones and Jones 1997) showed also a high accumulation triggered by BABA treatment. On the other hand, at 6hpi, proteins showing lower accumulation after BABA treatments were the ERD14 (early response to dehydration) protein and multiple salicylic acid (SA)-related proteins like the glutathione S-transferase 6 and 7 (GSTF6, GSTF7), as well as the translation elongation factor EF1B and the PNP-A (Plant natriuretic peptide A). Additionally, Succinyl-CoA ligase, an enzyme that is part of the tricarboxylic acid cycle (TCA) and is known to be responsive to oxidative stress (Sweetlove et al. 2002) was also less accumulated upon BABA treatment.

Subsequently, we took a closer look at proteins high accumulated in leaves upon BABA treatment at a later time-point. At 48hpi and inverse pattern was observed when comparing maximal protein abundance (log_2) values to early time point (6hpi). At 6hpi log_2 fold changes of most altered proteins reached up to 25, whereas at 48hpi the highest value was 4. At 48hpi the most abundant protein induced by BABA was MSRB2 (methionine sulfoxide reductase B2). Thionin, a small protein with antimicrobial activity that is locally and systemically activated after infection with a necrotizing pathogen or exogenous application of methyl jasmonate (Vignutelli et al. 1998) was also found at elevated levels upon BABA treatment, similarly to OZI1 which is known to accumulate in response to reactive oxygen species (Sharma 1995). Again a member of the leucine–rich repeat (LRR) family was found to be induced, as well as CDS1 and CDS2 (for superoxide dismutase Cu-Zn1) both enzymes crucial in detoxifing superoxide radicals and proteins with eminent roles in dealing with reactive oxygen stress (Sunkar et al. 2006).

A set of multiple proteins showing lower accumulation in response to BABA at 48hpi were also revealed. For instance, a thaumatin-related protein involved in host defense (Liu et al. 2010) and PBA1 a proteasome subunit -mediated cellular response leading to hypersensitive cell death and resistance to avirulent bacterial pathogens (Hatsugai et al. 2009) were present at higher levels in

mock samples. Additionally, several proteins exerting a chaperone activity were also less abundant after BABA treatment or in some cases even no detectable. Amongst them, the two mitochondrial heat shock proteins the HSP70-1 and HSC70-2, CCS (a copper chaperone) and BIP1 (a heat shock protein) that shows the lower levels quantified. Notably, we identified a group of 4 SA-related proteins which were exclusively present in leaves from water treated plants, such as WHY2 (a member of the whirly family of proteins), SSI2 protein (for suppressor of SA insensitive 2), SAR4 protein (for SAR DEFICIENT 4) and the serine protease (Kunitz trypsin) inhibitor AtKTI1. Also, the IAR3, JR3 (jasmonoyl-L-amino acid hydrolase) was particularly less abundant in leaves after BABA treatment compared to mock-treated plants.

3.6 Characterization of most significantly altered proteins in roots

The vast majority of identified DAPs showed lower accumulation levels in roots exposed for 6 hours to 250 µM BABA (**Table 1B**). Only two stress response-related proteins showed a slight increase upon BABA treatment, namely KIN2 that is known to be induced by cold stress, abscisic acid (ABA) and water stress (Wang et al. 1995) and a chitinase famly protein. On the other hand, amongst the proteins showing massively lowered levels after BABA exposure were 3 members of the family of aquaporin proteins PIP1-5 (Probable aquaporin PIP1-5), PIP1-1 (Aquaporin PIP1-1) and PIP1B (Plasma membrane intrinsic protein 1B). Aquaporins facilitate water uptake and mediate the regulation of root hydraulic conductivity in response to a large variety of environmental stresses (Boursiac et al. 2005).

Table 1A. Details of the differentially accumulated proteins showing the most dramatic changes in leaves of Arabidopsis plants after roots - dipping treatments with 250 µM of BABA.

(A)	Protein name	Accesion no.	No. Unique peptide seq. Matches	Seq. cov (%)	Subcellular local.	More/less abundance (Log2) upon BABA treatment			
						6h leaves	48h leaves	6h roots	48h roots
Leaves 6hpt	AOS, CYP74A, DDE2 | allene oxide synthase	AT5G42650.1	10	28	ch,m	25*	0	1	2
	HOL1 | HARMLESS TO OZONE LAYER 1	AT2G43910.1	4	30	pm	24*	-1	0	0
	Cytochrome C1 family	AT3G27240.1	6	33	v,m,pm	24*	-21	0	1
	NADH dehydrogenase [ubiquinone] flavoprotein 1	AT5G08530.1	9	33	m	24*	-21	-2	0
	LP4.4 | lipid-transfer protein	AT5G55450.1	4	71	ecr	24*	2	nd	nd
	AER | NADPH-dependent oxidoreductase 2-alkenal reductase	AT5G16970.1	4	25	n,c	24*	0	nd	nd
	MSRB3| methionine sulfoxide reductase B3	AT4G04800.1	8	56	n,er	23*	-2	nd	0
	emp24/gp25L/p24 family/	AT1G21900.1	4	9.7	er,g	21*	1	1	nd
	AtPGMP, PGM1, STF1 | phosphoglucomutase	AT5G51820.1	8	20	ch,apo,cy	5*	-22**	nd	nd
	AtPPC2| phosphoenolpyruvate carboxylase 2	AT2G42600.2	24	33	ch,cy,apo,pm,pl	5	1	-1	1
	Leucine-rich repeat (LRR) family protein	AT1G49750.1	11	26	ch,pm	3	1	nd	nd
	ERD14 | Dehydrin family protein	AT1G76180.1	16	68	ch,cy,pm	-3	-3	2	-2
	GSTF6, GSTF3, GST1, ERD1 | glutathione S-transferase 6	AT1G02930.1	12	53	cy,cw,m,v,c,pl	-3	-2	1	2
	GSTF7, GST11, GSTF8, | glutathione S-transferase 7	AT1G02920.1	5	61	cy,n,v	-3	0	1	2
	Translation elongation factor EF1B/ribosomal protein S6 family protein	AT5G19510.1	6	55	cy,apo,v	-3	-1	0	-1
	PNP-A | plant natriuretic peptide A	AT2G18660.1	6	49	cw,apo,ecr	-4	1	nd	nd
	Succinyl-CoA ligase, alpha subunit	AT5G08300.1	5	23	m,cw	-5	-22**	0	0
Leaves 48hpt	MSRB2 | methionine sulfoxide reductase B 2	AT4G21860.3	12	45	ch	26*	4	nd	0
	Plant thionin	AT1G66100.1	3	38	ecr	23*	3	nd	nd
	OZI1 | Stress-induced protein	AT4G00860.1	4	60	m,pe	21*	2	4	2
	Leucine-rich repeat (LRR) family protein	AT5G21090.1	5	39	pm	21*	2	0	1
	CSD1 | Superoxide dismutase [Cu-Zn] 1	AT1g08830.1	8	60	cy,n	-1	2	1	2
	CSD2 | Superoxide dismutase [Cu-Zn] 2	AT2g28190.1	12	67	ch	-1	2	2	1
	Pathogenesis-related thaumatin superfamily protein	AT1G18250.2	3	28		19*	-1	22*	0
	HSP70-1 | mitochondrial heat shock protein 70-1	AT4G37910.1	7	24	cw,m,v	-1	-5	0	0
	PBA1 | Proteasome subunit beta type-6	AT4G31300.1	6	34	n,c	-2	-7	2	-1
	WHY2 | WHIRLY 2	AT1G71260.1	3	27	m	0	-22**	0	0
	SSI2, FAB2 | suppressor of SA insensitive 2, fatty acid biosynthesis 2	AT2G43710.2	7	26	ch,p	2	-22**	-1	1
	HSP70-10, HSC70-2 | mitochondrial heat shock protein	AT5G09590.1	10	25	m,cw,ch	-2	-22**	0	0
	CCS | copper chaperone for superoxide dismutase	AT1G12520.1	4	20	ch,cy	-2	-22**	0	-2
	SARD4 | Protein SAR DEFICIENT 4	AT5G52810.1	6	42	ch	-1	-23**	-3	-3
	KTI1 | kunitz trypsin inhibitor 1	AT1G73260.1	4	33	m	-2	-23**	1	-1
	IAR3, JR3 | jasmonoyl-L-amino acid hydrolase	AT1G51760.1	9	41	er,pm	-1	-24**	-2	-1
	BIP1 | heat shock protein 70 (Hsp 70) family protein	AT5G28540.1	3	33	n,er	-27**	-27**	1	0

Table 1B. Details of the differentially accumulated proteins showing the most dramatic changes in roots of Arabidopsis plants after root dipping treatments with 250 µM of BABA.

(B)	Protein name	Accesion no.	No. Unique peptide seq. Matches	Seq. cov (%)	Subcellular local.	More/less abundance (Log2) upon BABA treatment			
						6h roots	48h roots	6h leaves	48h leaves
Roots 6hpt	KIN2, COR6.6 | stress-induced protein (KIN2) /cold-regulated protein (COR6.6)	AT5G15970.1	7	88	n,ch,pm	3	2	-3	3
	Chitinase family protein	AT2G43590.1	6	31	ecr	2	1	2	0
	PIP1B, TMP-A, ATHH2, PIP1;2 | plasma membrane intrinsic protein 1B	AT2G45960.3	10	35	v,ch,pm	-5	2	4	-1
	PIP1A, PIP1 | plasma membrane intrinsic protein 1A , Aquaporin	AT3G61430.2	2	36	v,ch,m,pm	-6	2	nd	nd
	PIP1D, PIP1;5 | plasma membrane intrinsic protein 1;5, Probable aquaporin	AT4G23400.1	3	37	pm	-6	0	nd	nd
	ADF3 | actin depolymerizing factor 3	AT5G59880.1	3	24	c,m,cy,pl,ch	-6	0	nd	nd
	PFP-BETA2 | Phosphofructokinase family protein	AT4G04040.1	3	12	cy,c	-23**	0	nd	nd
	EXL4 | EXORDIUM like 4	AT5G09440.1	4	21	ecr,apo,pl	-23**	-1	nd	nd
	BSK | Brassinosteroid-signaling kinase 7	AT1G63500.1	5	23	pm,pl	-23**	1	nd	nd
	RHD4 | Phosphoinositide phosphatase family protein, ROOT HAIR DEFECTIVE 4	AT3G51460.1	8	22	er,pm	-24**	0	nd	nd
	NAD(P)-linked oxidoreductase superfamily protein	AT1G59960.1	7	37	cy	-24**	0	nd	nd
	PCNA2 | proliferating cell nuclear antigen 2	AT2G29570.1	3	35	n	-25**	0	nd	nd
Roots 48hpt	HSP90-2, HSP81-2, ERD8 | heat shock protein 90-2	AT5G56030.1	3	53	c	0	27*	5	0
	TUB7 | tubulin beta-7 chain	AT2G29550.1	3	54	cy,v,cw,ga,cy,pm	0	27*	nd	nd
	HSC70-2, MED37D | Heat shock protein 70 (Hsp 70) family protein	AT5G02490.1	4	39	c,n	0	26*	0	0
	Coatomer, alpha subunit	AT2G21390.1	22	22	cy,re,m	0	25*	23	22
	GLT1 | NADH-dependent glutamate synthase 1	AT5G53460.1	29	19	ch	0	25*	23	-17
	ACS | acetyl-CoA synthetase	AT5G36880.2	10	14	ch,pe	-1	24*	22	-19
	MPK3 | mitogen-activated protein kinase 3	AT3G45640.1	3	20	n,c	1	24*	nd	nd
	PLP2, PLA2A | Patatin-like protein 2, phospholipase A 2A	AT2G26560.1	3	14	c	21*	22*	nd	nd
	IBI1 | Aspartate-tRNA ligase 2	AT4G31180.2	3	10	cy,er	1	22*	19*	nd
	HST, HCT | Hydroxycinnamoyl-Coenzyme A shikimate	AT5G48930.1	6	20	cy,c,m	0	23*	nd	nd
	ACX1 | acyl-CoA oxidase 1	AT4G16760.1	6	13	pe,pl	-1	6	22	3
	RPN2B | 26S proteasome non-ATPase regulatory subunit 1	AT1G04810.1	5	17	cy,n,ch,pm	-2	5	nd	nd
	AMT1-1 | ammonium transporter 1 member 1	AT4G13510.1	3	8	n,me,pl	-1	4	-2	-2
	RS6 | Raffinose synthase family protein	AT5G20250.4	7	14	cy,ch,pl	-1	4	nd	nd
	MPK6| mitogen-activated protein kinase 6	AT2G43790.1	4	17	n,c	-3	2	nd	nd
	ALDH3H1 | aldehyde dehydrogenase 3H1	AT1G44170.2	6	16	er,ga,v,m,pl,p	0	2	nd	nd
	PAL1, ATPAL1 | PHE ammonia lyase 1	AT2G37040.1	16	35	c	-1	2	nd	nd
	CSDP1 | cold shock domain protein 1	AT4G36020.1	8	40	n,c	-1	-6	-2	-23
	Peroxidase superfamily protein	AT2G37130.2	6	24	ecr	1	-24**	-2	-22
	SHM1, STM, SHMT1 | serine transhydroxymethyltransferase 1	AT4G37930.1	4	20	m,c	0	-24**	-1	-2

Some proteins related to plant development or energy metabolism show a lower accumulation pattern at early time-points in roots upon BABA exposure. For example, ADF3 (Actin-depolymerizing factor 3) which severs actin filaments (F-actin) and binds to actin monomers. ADF3 is known to be induced by the root-knot nematode *Meloidogyne incognita* (Clément et al. 2009). Amongst additional lower accumulated proteins were PFP-BETA2 (Pyrophosphate-fructose 6-phosphate 1-phosphotransferase subunit beta 2) (Lim et al. 2009), RDH4 (root hair defective 4) which regulates the accumulation of phosphatidylinositol-4-phosphate [PI(4)P] on membrane compartments at the tips of growing root hairs (Thole et al. 2008), and a NAD(P)-linked oxidoreductase superfamily protein. This protein is well established to be responsive to salt stress in Arabidopsis roots (Jiang et al. 2007). We also observed DNA regulatory proteins that show less accumulation, namely PCNA2 (Proliferating cell nuclear antigen 2), an auxiliary protein of DNA polymerase delta involved in the control of eukaryotic DNA replication (Anderson et al. 2008). In

addition, we found 2 proteins involved in brassinosteroid regulation, EXL4 (EXORDIUM like 4) which plays a role in a brassinosteroid-mediated growth and development signaling (Schröder et al. 2009), and BSK7, which is a member of the brassinosteroid signaling kinases (BSK) that also regulate growth and development (Sreeramulu et al. 2013).

Looking at an advanced time-point (48hpi) after BABA exposure, we detected several proteins exclusively high accumulated in roots upon BABA treatments. Two molecular chaperones proteins were identified, HSP90-2 (heat shock protein 90-2) and HSC70-2 (heat shock protein 70-2), both are involved in the regulation of plant hormone-regulated stress responses and immune responses triggered by resistance genes (Clément et al. 2011; Huang et al. 2014). Also, some proteins involved in plant development and energy metabolism such as TUBB7 (Tubulin beta-7 chain), GLT1 (NADH-dependent glutamate synthase 1), the cytosolic protein complex coatomer that mediates biosynthetic protein transport and the enzyme ACS enzyme (acetyl-CoA synthetase) which catalyzes the production of acetyl-CoA destinated for lipid synthesis or for energy generation (Behal et al. 2002) were present at higher levels in response to BABA. AMT1-1 (Ammonium transporter 1 member 1) a protein involved in ammonium uptake from soil and transport to shoots (Mayer et al. 2006) and the HCT (Hydroxycinnamoyl-Coenzyme A shikimate transferase), which is involved in flavonoid synthesis were also high accumulated upon BABA treatment (Hoffmann et al. 2004). Furthermore, BABA treatment strongly induced the accumulation of proteins involved in ROS and biotic or abiotic stress responses, a picture similarly seen in our proteomic data from leaves. Examples of these ROS and biotic stress-related proteins are MPK3 and MPK6 (mitogen-activated protein kinase 3 and 6), ALDH3H1(Aldehyde dehydrogenase family 3 member H1), PLP2 (phospholipase A 2A), ACX1(acyl-CoA oxidase 1), PAL1 (Phenylalanine ammonia-lyase 1) and the RS6 (Raffinose synthase family protein). The regulation of endogenous protein balance seems also to be altered upon BABA exposures, as RPN2B (a regulatory subunit of the 26 proteasome which is involved in the ATP-dependent degradation of ubiquitinated proteins) was highly regulated. Interestingly, we also could detect the known BABA-related protein IBI1 (Luna et al. 2014). IBI1 was found here to highly accumulate in roots after 48hours of BABA treatment.

At 48hpi, we detected significantly less BABA-specific lower accumulated proteins in roots compared to leaves. For instance, the chaperone protein CSDP1 (cold shock domain protein 1) which is involved in cold resistance (Park et al. 2009), a Peroxidase, as well as SHM1 protein (Serine hydroxymethyltransferase 1) were less accumulated after BABA treatment (Moreno et al. 2005).

Interestingly, the three-above mentioned proteins were found in lower quantities in BABA-exposed sample in both leaves and roots at early (6hpi) and late stages (48hpi) compared to mock-treated samples.

4. Discussion

Synthetic BABA is a fundamental compound amongst chemical priming inducers for a variety of plant species. It has agronomic potential for stress management in agricultural important crop species (Cohen et al. 2002), especially in the light of the recent discovery describing it as a natural plant compound. However, few *in planta* studies on BABA have been conducted. Using a novel and ultra precise quantification method (Thevenet et al. 2017), we demonstrate that BABA uptake occurs very rapidly in roots, as it is also transported rapidly to the leaves. Total levels of BABA were three times higher in roots than in leaves. This is probably due to the fact that roots were in direct contact with the BABA solution and that vascular transport via the xylem to shoots is less efficient. In both root and shoot tissues, BABA uptake reached a peak of maximal absorption around 48hpi, and was independent of the initially applied concentration. These results are in line with previous studies by Cohen and colleagues (1999b) who described that the protection of BABA in the leaves of grape plants is due to BABA translocation via the vascular system of the plant. Moreover, we observed that BABA mostly accumulates intracellularly, with only small amounts of BABA detected in the apoplastic fluid. This suggests in contrast to previous studies that BABA is not only transported via the vascular system but can also be stored and transported in the apoplast. This could occur along with the transport and storing of other apoplast-typical content such as sugar, ions or phytohormones (Sattelmacher 2001; Sauter et al. 2001).

Using radioactively labelled BABA-C^{13} Cohen and Gisi (1999) showed that BABA was not degraded *in planta*. However, after looking closer at the stability of BABA in roots and leaves, we detected that following the maximal peak of accumulation, BABA levels dropped after 3dpt in roots while in leaves the reduction of BABA levels appeared at 6dpt. Classically, *in planta* stability of BABA has been assessed by testing the capacity of this molecule to induce resistance following an extended period after soil drench. The protective effect of BABA in leaves against *Plasmopara viticola* still persisted after more than 14 days post exposure (Cohen et al. 1999b); however, in this experimental setup the priming capacity, rather than the stability of the molecule itself, could be explored. A more recent study also is in line with our observation of relative instability of BABA (Slaughter et al. 2011). There, plants soil-drenched with BABA accumulated high amounts of BABA in leaves at 48 and 72 hours post incubations while only traces of BABA were found in the same tissues 3 weeks post incubations (Slaughter et al. 2011). Benefitting from the advantages of using a hydroponic growth system, we were able to monitor in detail the levels of BABA at different time

points in both leaves and roots. With this newly reported instability in mind, we hypothesize that BABA is either metabolized/degraded by an enzymatic pathway, or conjugated to larger molecules such as peptides. In bacteria for instance, BABA was described to be part of an antibiotic synthesis pathway (Takaishi et al. 2012). There it is used as substrate within the enzymatic synthesis pathway. A similar mechanism could occur in plants, given the fact how chemically stable BABA is and how convenient its structure would be to form a building block or fragment of other peptides.

With respect to the distinct BABA kinetics in roots compared to leaves, we strongly speculate here for the first time that roots may indeed play a crucial role during BABA perception and signaling. In order to screen for a tissue-specific priming phenotype we analyzed proteomic changes in leaves and roots at an early (6hpi) and late (48hpi) time-point upon BABA exposure. At an early time-point (6hpi), all GO groups were generally found to be down-regulated in BABA-treated roots while interestingly at a later stage the pattern was the opposite in roots, with the more BABA-specific up-regulated proteins. When looking at a late time-point in leaves, a down-regulation similarly to the early time-point was observed.

Nonetheless, generally amongst functional groups, the GO family of stress-response pathways showed the most altered changes (down- or upregulated depending on the time-point and the specific tissue). Notably, only roots at 48hpi with BABA showed an increased abundance of proteins belonging to the stress response GO family – in all other time-points both tissues exhibit a higher number of lower accumulated proteins in response to BABA. This general reduced protein accumulation and a sudden time-point-specific peak of high accumulation may suggest that BABA is perceived as a chemical stress signal by plants. As most of the related defenses proteins were less accumulated after BABA treatment this 'stress-situation' is not costly for plants, supporting the definition of priming which is supposed to be an energy-efficient immune mechanism (Conrath et al. 2002). It remains intriguing though how these primed cells - which exhibit lower levels of defense-related proteins - are then suddenly able to react stronger to a biotic stress once being challenged. An explanation might be the observed proteotype of most induced proteins belonging cellular respiration as well as primary and secondary metabolism pathways and chaperones (**Table 1AB; Figure 5**). For instance, several proteins involved in the electron transport chain as well as a phosphoenolpyruvate dehydrogenase (PEPC) were altered by BABA in leaves and roots. This enzyme feeds into the citrate cycle and stimulates generation of amino acid precursors. In respect to recent metabolomics studies showing an alteration of TCA cycle components upon BABA

(Pastor et al. 2014) and a direct induction of priming by some components of this cycle (Balmer et al. 2018), we propose that the modification of cellular respiration and the stimulation of amino acid precursor production is a pivotal mechanism during BABA-mediated priming. Nevertheless, we also detected an accumulation of some defense-related proteins upon BABA exposure. For instance, the pathogenesis-related (PR) protein thaumatin was found to strongly accumulate in leaves and roots at 6hpi. This result is in line with previous studies on potato plants treated with 10 mM of BABA, where thaumatin was also found induced at 24pht (Bengtsson et al 2014). Moreover, we found two leucine rich repeat (LRR) proteins family members accumulated in leaves after BABA treatment at both early and late stages. Previous studies demonstrated that several LRR proteins were also present in high levels in the secretome of potato leaves two days after being sprayed with 10 mM BABA (Bengstsson et al. 2014). At the transcriptomic level, expression of one member of this family was previously also found to be induced by BABA treatment in Arabidopsis leaves after 24hpi (Tsai et al. 2011). LRR proteins play an eminent role in plant immunity, acting as resistance genes to modulate effector-triggered immunity against pathogens. We also monitored a general lower accumulation of SA-related proteins (**Table 1**) across all time-points and tissues. This indicates an early and complex modulation of plant hormone signaling by BABA, especially considering the lower accumulation of SSI2, a negative regulator of SA accumulation. Again, this could mean a similar orchestration as observed for the stimulation of amino acid precursor synthesis pathways: BABA treatment could be "resetting" cellular defense hormone pathways and in the same time preparing the cells for an efficient upcoming SA synthesis event.

DAPs related to plant respiration and energy metabolism, mitochondria and chloroplast were found to be differentially accumulated after BABA treatment (**Figure 5**). In leaves, specially enzymes involved in oxidative stress were high accumulated. Amongst them, we found NADH coenzymes, methionine sulfoxide reductases, a lipid transfer protein, aldehyde dehydrogenase 3H1 and two cupper/zinc superoxide dismutases (CSD1, CSD2). This was similarly observed at the transcriptomic level in (Tsai et al. 2011), where the gene expression of both cupper/zinc superoxide dismutases (CSD1, CSD2) mentioned above were found to be up-regulated by 200 µM of BABA at 48hpi. Besides proteins related to cellular energy production, we found that at early stage after BABA treatment, roots suffered a general depletion of accumulation of several proteins involved in growth and carbon/nitrogen balance regulation, such as 3 members of the aquaporin family (PIB1A, PIB1B, PIB1D) involved in water flux (Alexandersson et al. 2005). This resembles previous

findings where two members of the aquaporin family were also down-regulated in potato leaves after BABA treatments (Bengstsson et al. 2014). Last but not least, proteins with a chaperone function were mostly less accumulated in leaves and roots upon BABA exposure. Amongst these chaperones, we detected heat shock proteins (HSP70-1, HSP70-10, BIP1), a copper chaperone protein (CCS), one member of dehydrin family protein (ERD14) and a cold shock protein (CSDP1). CSDPI1 was previously also found to be down-regulated on the transcriptomic level in Arabidopsis leaves after BABA treatment (Tsai et al. 2011).

Following a screening of a larger collection of Arabidopsis mutants, few proteins were described to be specificly required to induce priming by BABA as *ibi1* (IBI encodes an aspartyl-tRNA synthetase), *ibs1,2* and *3* (affected in a cyclin-dependent kinase–like protein, a polyphospho-inositide phosphatase and a zeaxanthin epoxidase, respectively), *lecrk-VI.2-1* (L-type lectin receptor kinase-VI.2) and *ald1* (pipecolate-deficient mutant) (Luna et al. 2014; Ton et al. 2005; Singh et al. 2012; Návarová et al. 2012). In this study 30 knock-out mutants selected randomly between the most and less accumulated proteins after BABA treatment were evaluated in regard of their capacity to induce resistance against *H. arabidopsidis* after BABA treatment. As shown in **Figure S5**, no differences in susceptibility between the wild type Col-0 genotype and the mutants were observed. This outcome demonstrated the necessity of larger screening in order to dissect proteins that are crucial for a normal priming induction by BABA in Arabidopsis.

Altogether, our results showcase rapid proteomic modifications upon BABA treatment in Arabidopsis roots and leaves. In line with the here described fast BABA uptake by roots and translocation into leaves, we observed a rapid massive alteration in issue and time-point specific proteomic patterns. A general trend was that most significant changes comparing BABA and mock treatment comprised a lower accumulation of a multitude of proteins belonging to various pathways including cellular respiration, salicylic acid signaling, carbon/nitrogen balance and stress signaling. Considering this consequent depletion of signaling-related proteins over most time-points and tissues, we speculate that BABA triggers a cellular "reset" on the proteomic level. It might hence be suggested that within different plant organs different cells exhibit diverse and maybe even slightly uncoordinated signaling patterns. BABA-induced lower accumulation of stress-signaling related proteins could in turn globally alleviate this uncoordinated signaling vocabulary, thus relaxing the global cellular stress response status including levels of reactive oxygen species, metabolites, and nutrient levels. On the other hand, this goes in hand with the

induction of proteins specifically linked to amino acid precursor synthesis and defense-related proteins such as thaumatins. Considering the above-described resetting of the cellular signaling vocabulary, this concomitant induction of specific defense proteins might strengthen the defense capacity of these cells, thus turning them into newly assigned immune cells. Given the fact that plants do not have specific immune cells, this assignment might be the crucial mode of action of BABA-induced priming against biotic stresses.

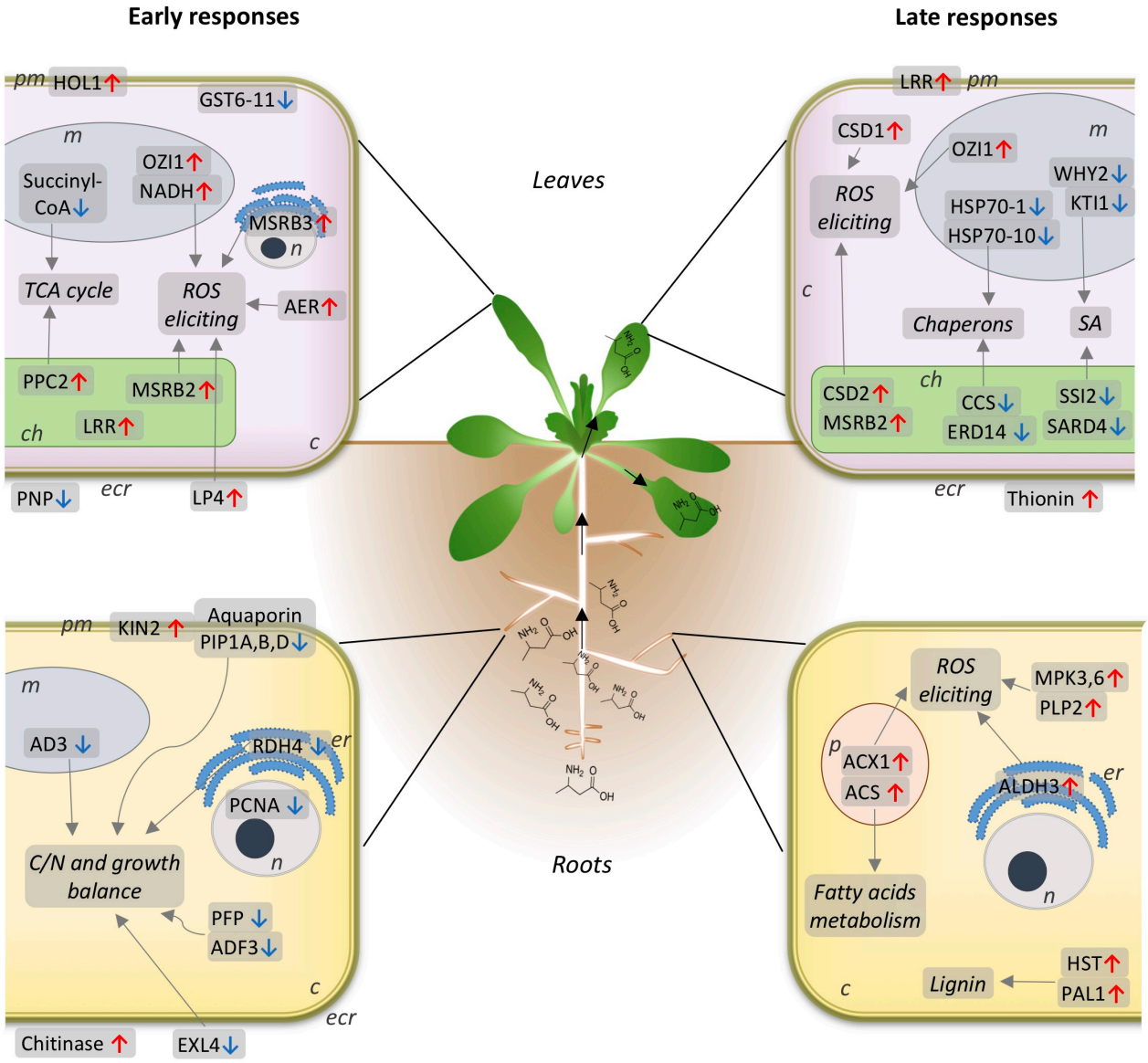


Figure 5. A putative early and late BABA-responsive protein network in leaves and roots of Arabidopsis plants. Up- and down-regulation are marked as red (up) and blue (down) arrows, respectively. Detailed descriptions of subcellular compartments and the full name for each abbreviation are given in the text and in Table 1A and B.

Previous findings based on transcriptomic and metabolomics analysis (Tsai et al. 2011; Pastor et al. 2014) support some of the present proteomic results, however here for the first time we were able to characterize cellular responses to BABA on a wider perspective, with a focus as well on the yet only scarcely explored roots. This study highlights that roots are likely to play a cardinal role in BABA-induced priming. In addition, we propose that during priming not only transcriptional and epigenetic mechanisms are eminent in modulating a stimulated defense response, but also proteomic alterations. Thus, for further priming-related studies, we think analyzing the proteome is essential in understanding priming mechanisms. Additional future proteomic studies, especially looking at post-translational modifications and systems biology approaches, will shed more light into these intriguing priming processes.

5. Supplementary Data

Supplementary Figure 1: BABA uptake in Arabidopsis leaves and roots.

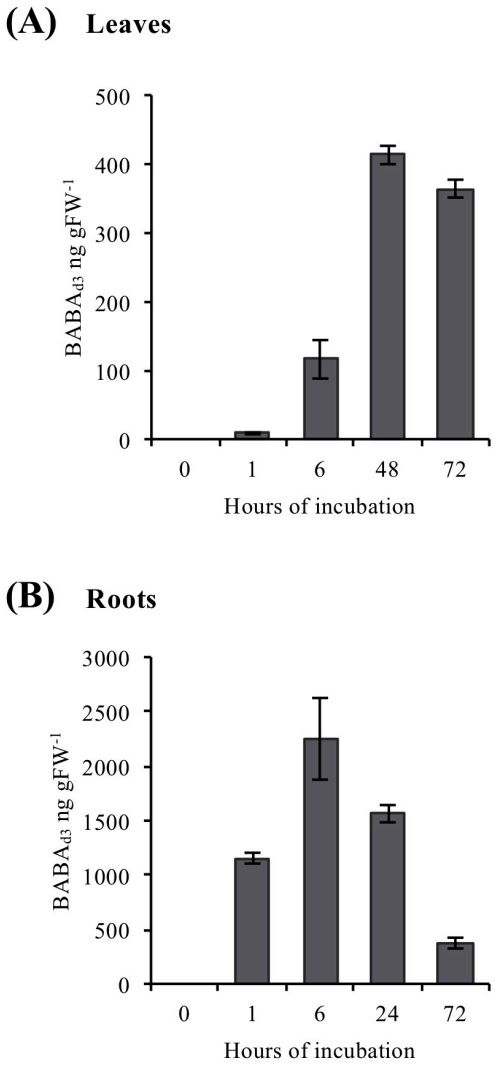


Figure S1. Four weeks-old Col-0 plants were soil treated with $BABAd_3$ by adding to the nutrient solution 1.5 µM as a final concentration. Nutrient solution without $BABAd_3$ was used as control. Plants were kept in these conditions until samples from leaves and roots were collected at 1, 6, 48 and 72 hours post treatments (hours of incubation). A. $BABAd_3$ in leaves (ng gFW^{-1}). B. $BABAd_3$ in roots (ng gFW^{-1}). Data represent the mean ± SE (*n* = 4 biological replicates). The experiment was conducted twice with the same results.

Supplementary Figure 2: Catabolism and/or metabolism of BABA after uptake into leaves.

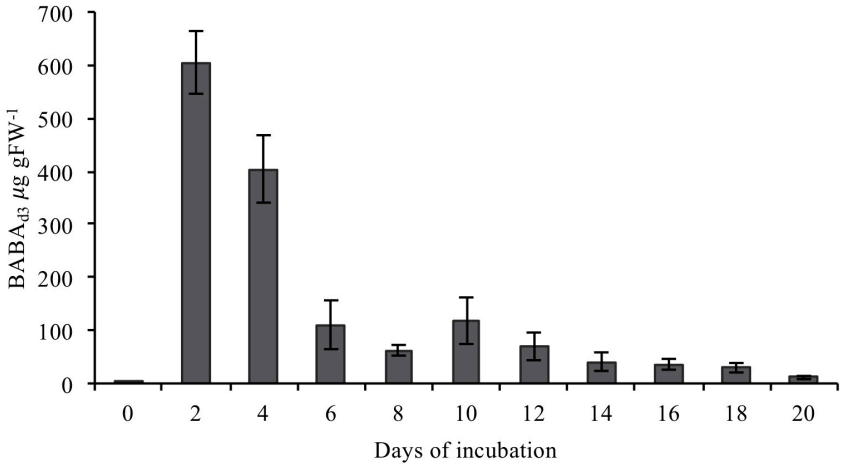


Figure S2. Plants were soil treated with 250 µM of BABAd$_3$ for 24h, and during a following period of 20 days every two days leaves samples were taken and BABAd$_3$ was quantified (µg gFW^{-1}). Data represent the mean ± SE (*n* = 4 biological replicates). The experiment was conducted twice with the same results.

Supplementary Figure 3: Malate dehydrogenase (MDH) activity in the intracellular extract and in the apoplastic fluid of leaves of Arabidopsis plants.

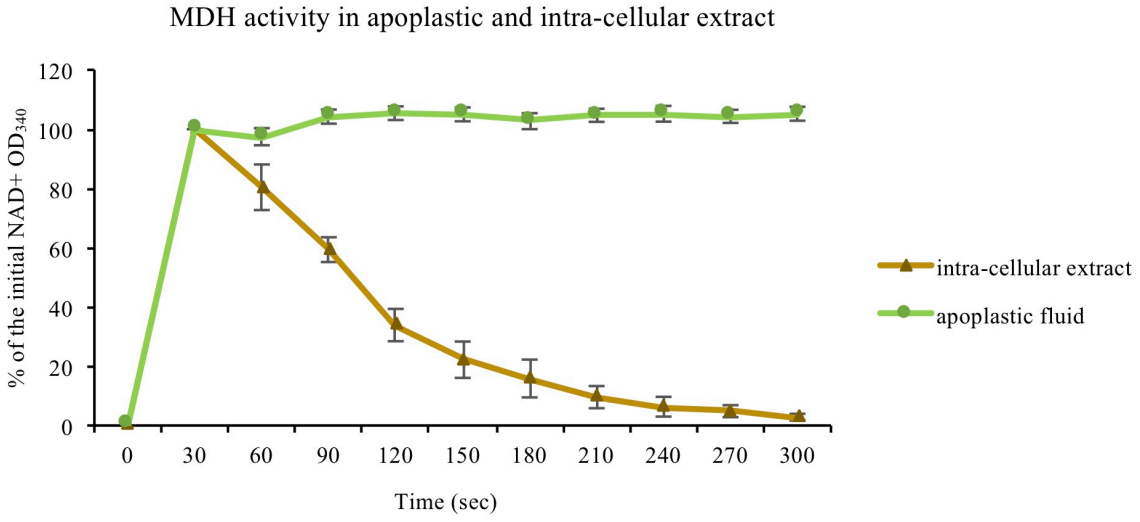


Figure S3. Plants were soil treated with 1.5 µM of BABAd$_3$ for 24h. The values represent the mean ± SE (*n* = 3 biological replicates of a pool of 10 leaves for replicate) of a representative experiment that was repeated twice with similar results.

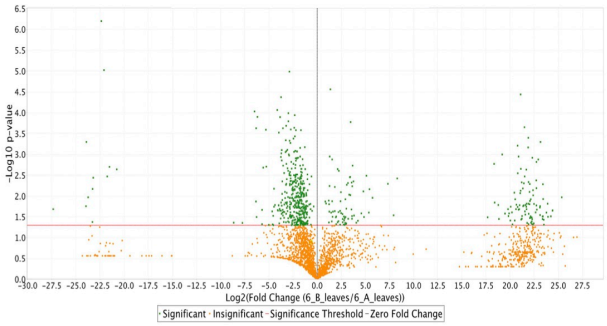
Supplementary Figure 4: Differentially accumulated proteins from A. leaves and B. roots at 6hpt and 48hpt (hours post BABA treatment) were classified in Volcano Plots where the proteins distribution was represented as fold change (treated vs control) versus *P* values.

(A)

Leaves

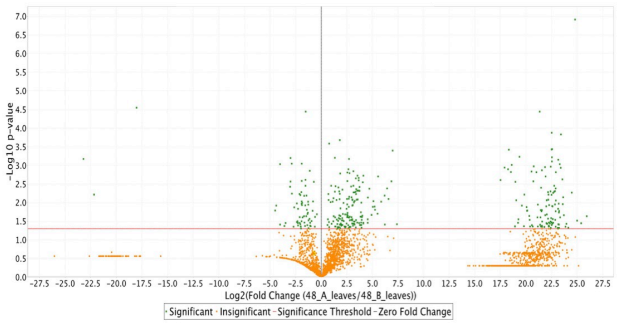
6hpt

t-test, P<0.05 (Treated vs control)



48hpt

t-test, P<0.05 (Treated vs control)

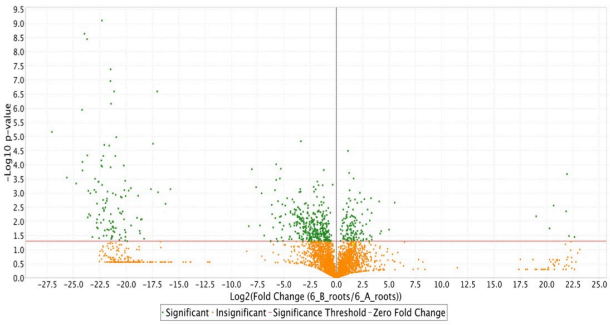


(B)

Roots

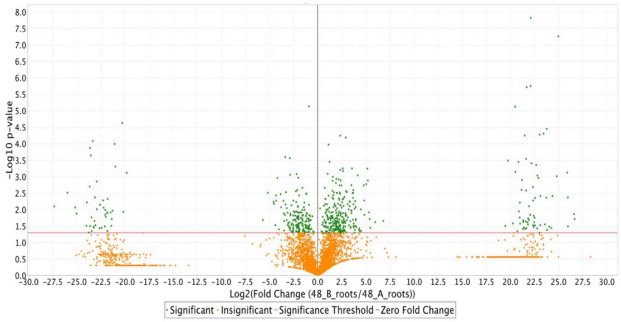
6hpt

t-test, P<0.05 (Treated vs control)



48hpt

t-test, P<0.05 (Treated vs control)



Supplementary Figure 5: Capacity of BABA to induce priming against the Noco strain of *Hyaloperonospora arabidopsidis* BMM in a set of several Arabidopsis knock-out mutants selected randomly between the most and least accumulated proteins after BABA treatment and the wild type Col-0.

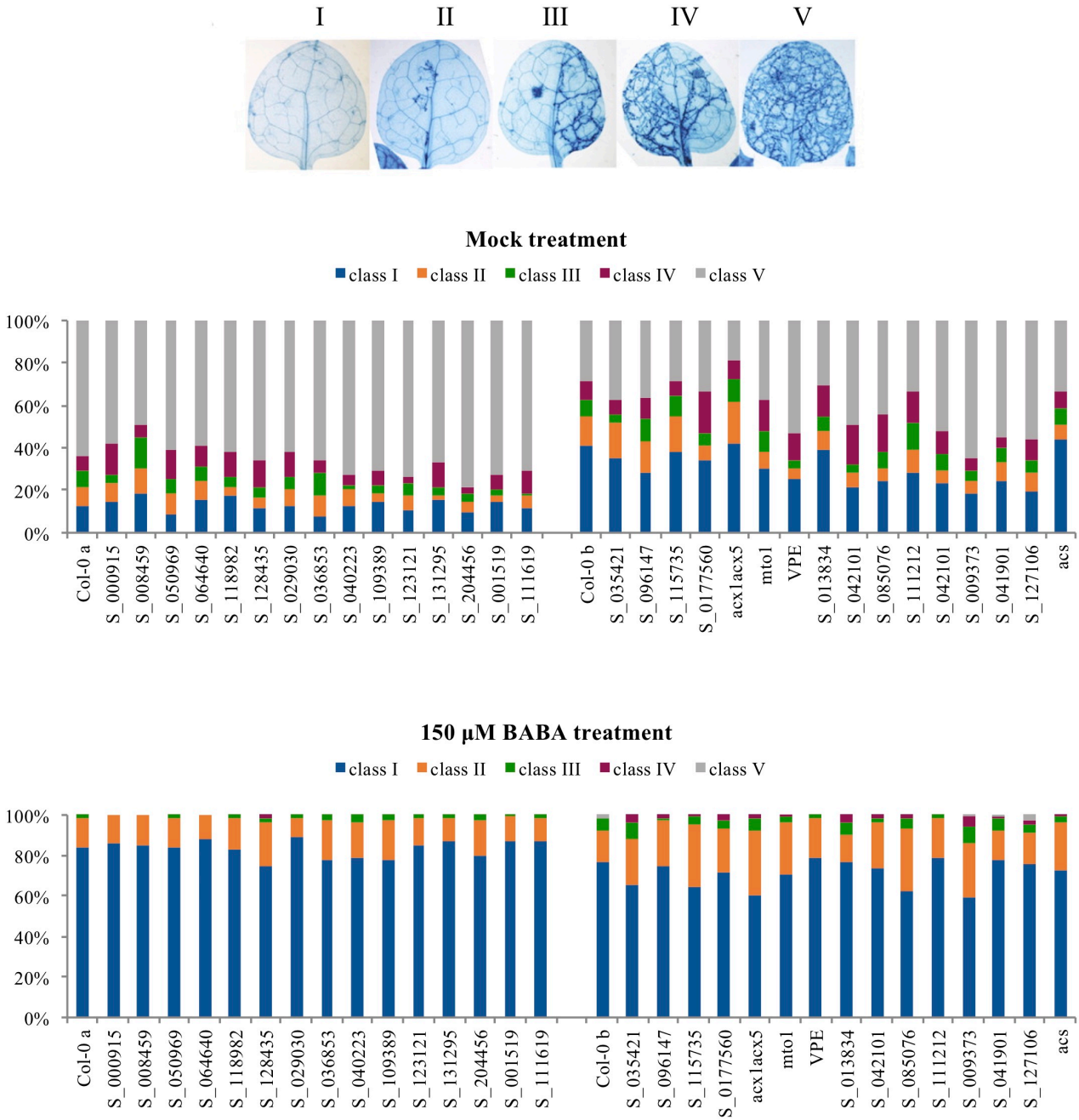


Figure S5. Disease ratings were classified based on the percentage of leaf colonization by mycelium. Class I: 0%; class II: 1-25%; class III: 26 -50%; class IV: 51 -75% and class V: 76 -100%. Disease ratings are shown as the percentage of leaves in each resistance class. A statistical Fisher test was applied to verify if a statistically significant difference in the percentage of leaves in the different classes of mutants compared to Col-0 were observed. Mock treatment: plants were soil drench with water. BABA treatment: plants were soil drench with 150 µM of BABA.

Supplementary Table 1: Accession numbers of seeds obtained from The European Arabidopsis Stock Centre NASC (http://arabidopsis.info).

Gene ID	Description	Stock SALK	
At5g42650.1	AOS, CYP74A, allene oxide synthase, cytochrome P450 74A,	SALK_017756C	N659698
At1g10840.1	TIF3H1, translation initiation factor 3 subunit h1	SALK_127106C	N663932
At4g04800.1	MSRB3, methionine sulfoxide reductase B3	SALK_050969C	N668243
At4g30530.1	GGP1, gamma-glutamyl peptidase 1	SALK_029030C	N672465
At3g16910.1	AAE7, acetate non-utilizing 1, ACN1, acyl-activating enzyme 7	SALK_009373C	N654987
At2g47400.1	CP12, CP12 domain-containing protein	SALK_008459C	N667730
At3g01120.1	CGS, CGS1, cystathionine gamma-synthase, cystathionine gamma-synthase 1	CS196	N196
At2g18660.1	PNP-A, plant natriuretic peptide A	SALK_000951C	N681890
At1g02920.1	GST11, GSTF7, glutathione s-transferase 11, glutathione s-transferase 7	SALK_204456C	N693233
At1g02930.1	early responsive to dehydration 11, ERD11, glutathione s-transferase1;6, GST1, GSTF6	SALK_040223C	N674606
At2g30860.1	GSTF9, glutathione s-transferase phi 9	SALK_001519C	N685023
At4g11290.1	Peroxidase superfamily protein. Response to oxidative stress	SALK_096147C	N655758
At1g08830.1	Copper/zinc superoxide dismutase 1, CSD1, SOD1, superoxide dismutase 1	SALK_109389C	N680525
At2g28190.1	Copper/zinc superoxide dismutase 2, CSD2, SOD2, superoxide dismutase 2	SALK_041901C	N665863
At1g04710.1	KAT1, 3-keto-acyl-coa thiolase 1	SALK_013834C	N665315
At1g51760.1	IAA-alanine resistant 3, IAR3, jasmonic acid responsive 3, JR3	SALK_042101C	N665865
At5g52810.1	SARD4, SAR deficient 4,	SALK_131295C	N676947
At1g73260.1	KTI1, kunitz trypsin inhibitor 1	SALK_128435	N628435
At2g43590.1	Chitinase family protein. Defense response, polysaccharide catabolic process	SALK_118982C	N673299
At1g13930.2	Involved in response to salt stress	SALK_111619C	N681608
At5g56000.1	HSP81.4, heat shock protein 81.4, heat shock protein 90.4	SALK_036835C	N674476
At1g63500.1	BSK7, brassinosteroid-signaling kinase 7	SALK_147216C	N684747
At4g32940.1	GAMMA-VPE, gamma vacuolar processing enzyme	CS67918	N67918
At3g22310.1	RNA HELICASE 9, putative mitochondrial rna helicase 1	SALK_035421C	N656360
At5g46790.1	RCAR12, regulatory components of ABA receptor 12	SALK_054640C	N668293
At5g02490.1	HSP70-2	SALK_085076C	N663161
At5g53460.1	GLT1, NADH-dependent glutamate synthase 1	SALK_115735C	N667102
At5g36880.2	ACS, acetyl-coa synthetase	CS65789	N65789
At4g16760.1	ACX1, acyl-coa oxidase 1	CS66499	N66499
At2g37130.2	Peroxidase superfamily protein	SALK_123121C	N668966
At1g77630.1	LYM3, LYP3, lysin-motif (LYSM) domain protein 3	SALK_111212C	N663666

6. References

Aharon, R. et al. (2003) Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. Plant Cell, 15, 439-447

Alexandersson, E. et al. (2005) Whole gene family expression and drought stress regulation of aquaporins. Plant Mol. Biol. 59, 469-484

Anderson, H. et al. (2008) *Arabidopsis thaliana* Y-family DNA polymerase η catalyses translesion synthesis and interacts functionally with PCNA2. Plant J. 55, 895-908

Arasimowicz-Jelonek, M. et al. (2013) The proteome response of potato leaves to priming agents and S-nitrosoglutathione. Plant Sci. 198, 83-90

Asai, T. et al. (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature, 415, 977

Ashburner, M. et al. (2000) Gene Ontology: tool for the unification of biology. Nature Genet. 25, 25

Bao, F. et al. (2014) Arabidopsis HSP90 protein modulates RPP4-mediated temperature-dependent cell death and defense responses. New Phytol.202, 1320-1334

Balmer, Dirk, et al. (2013) Induced resistance in maize is based on organ-specific defense responses. Plant J. 74, 213-225

Beckers, G. et al. (2009) Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. Plant Cell, 21, 944-953

Behal, R. H. et al. (2002) Role of acetyl-coenzyme A synthetase in leaves of *Arabidopsis thaliana*. Arch. Biochem. Biophys. 402, 259-267

Bengtsson, T. et al. (2014a) Activation of defense responses to *Phytophthora infestans* in potato by BABA. Plant Pathol. 63, 193-202

Bengtsson, T. et al. (2014b) Proteomics and transcriptomics of the BABA-induced resistance response in potato using a novel functional annotation approach. BMC genomics, 15, 315

Boursiac, Y. et al. (2005) Early effects of salinity on water transport in Arabidopsis roots. Molecular and cellular features of aquaporin expression. Plant phys. 139, 790-805

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254

Bruce, T. et al. (2007) Stressful "memories" of plants: evidence and possible mechanisms. Plant Sci.173, 603-608

Clément, M. et al. (2009) Actin-depolymerizing factor2-mediated actin dynamics are essential for root-knot nematode infection of Arabidopsis. Plant Cell, 21, 2963-2979

Clément, M. et al. (2011) The cytosolic/nuclear HSC70 and HSP90 molecular chaperones are important for stomatal closure and modulate abscisic acid-dependent physiological responses in Arabidopsis. Plant phys. 56, 481-1492

Cohen, Y. R. (2002) β-aminobutyric acid-induced resistance against plant pathogens. Plant dis. 86, 448-457

Cohen, Y., and U. Gisi. (1994) Systemic translocation of 14C-DL-3-aminobutyric acid in tomato plants in relation to induced resistance against *Phytophthora infestans*. Physiol. Mol. Plant Path. 45, 441-456

Cohen, Y. et al. (1999b). Local and systemic activity of BABA (DL-3-aminobutyric acid) against *Plasmopara viticola* in grapevines. Eur. J. Plant Pathol.105, 351-361

Conrath, U. et al. (2002) Priming in plant–pathogen interactions. Trends Plant Sci. 7, 210–216

Desveaux, D. et al. (2004) A "Whirly" transcription factor is required for salicylic acid-dependent disease resistance in Arabidopsis. Develop. Cell, 6, 229-240

Desveaux, D. et al. (2005) Whirly transcription factors: defense gene regulation and beyond. Trends Plant Sci. 10, 95-102

Ding, P. et al. (2016) Characterization of a pipecolic acid biosynthesis pathway required for systemic acquired resistance. Plant Cell, tpc-00486

Dong, C. J. et al. (2016) Quantitative Proteomic Profiling of Early and Late Responses to Salicylic Acid in Cucumber Leaves. PloS one, 11, e0161395

Dos Santos, C. V. et al. (2005) The Arabidopsis plastidic methionine sulfoxide reductase B proteins. Sequence and activity characteristics, comparison of the expression with plastidic methionine sulfoxide reductase A, and induction by photooxidative stress. Plant Phys. 138, 909-922

Dudkina, N. V. et al. (2005) Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III. Pro. Natl. Acad. Sci. USA. 102, 3225-3229

Edwards, R. et al. (2000) Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. 5, 193-198

Fromm, S. et al. (2016) Depletion of the "gamma-type carbonic anhydrase-like" subunits of complex I affects central mitochondrial metabolism in *Arabidopsis thaliana*. Biochim. Biophys. Acta 1857, 60-71

Guan, Q. et al. (2013) Heat stress induction of miR398 triggers a regulatory loop that is critical for thermotolerance in Arabidopsis. Plant J. 74, 840-851

Héricourt, F., & Jupin, I. (1999) Molecular cloning and characterization of the *Arabidopsis thaliana* α-subunit of elongation factor 1B. FEBS letters, 464, 148-152

Hoffmann, L. et al. (2004) Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. Plant Cell, 16, 1446-1465

Huang, J. et al. (2010) Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. Plant Physiol. 153, 1526-1538

Huang, S. et al. (2014) HSP 90s are required for NLR immune receptor accumulation in Arabidopsis. Plant J, 79, 427-439

Jaskiewicz, M. et al. (2011) Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. EMBO reports, 12, 50-55

Jenner, H. L. et al. (2001) NAD malic enzyme and the control of carbohydrate metabolism in potato tubers. Plant Physiol.126, 1139-1149

Jiang, Y. et al. (2007) Comparative proteomic analysis of NaCl stress-responsive proteins in Arabidopsis roots. J. Exp. Bot. 58, 3591-3607

Jones, A. M. et al. (2006) Modifications to the Arabidopsis defense proteome occur prior to significant transcriptional change in response to inoculation with *Pseudomonas syringae*. Plant phys. 142, 1603-1620

Jones, D. A.,Jones, J. D. (1997) The role of leucine-rich repeat proteins in plant defenses. In Advances in botanical research, Vol. 24, pp. 89-167. Academic Press

Kessner, D. et al. (2008) ProteoWizard:open source softward for rapid proteomics tools development. Bioinformatics, 24, 2534-2536

Kirch, H. H. et al. (2005) Detailed expression analysis of selected genes of the aldehyde dehydrogenase (*ALDH*) gene superfamily in Arabidopsis thaliana. Plant Mol. Biol. 57, 315-332

Kovacs, D. et al. (2008) Chaperone activity of ERD10 and ERD14, two disordered stress-related plant proteins. Plant phys. 147, 381-390.

Kovtun, Y. et al. (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. 97, 2940-2945

Krome, K. et al. (2007) The effect of benzothiadiazole and fungal extracts of *Cercospora beticola* and *Fusarium graminearum* on phosphoenolpyruvate carboxylase activity in cucumber leaves. JPDP. 114, 250-255

Kwon, S. et al. (2007) Role of the methionine sulfoxide reductase MsrB3 in cold acclimation in Arabidopsis. Plant Cell Physiol. 48, 1713-1723

La Camera, S. et al. (2005) A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. Plant J. 44, 810-825

Lancien, M. et al. (2002) Arabidopsis *glt1-T* mutant defines a role for NADH-GOGAT in the non-photorespiratory ammonium assimilatory pathway. Plant J. 29, 347-358

Langhans, M. et al. (2008) *In vivo* trafficking and localization of p24 proteins in plant cells. Traffic, 9, 770-785

Lieberherr, D. et al. (2003) The rapid induction of glutathione S-transferases AtGSTF2 and AtGSTF6 by avirulent *Pseudomonas syringae* is the result of combined salicylic acid and ethylene signaling. Plant Cell Physiol. 44, 750-757

Li, J. et al. (2008) Kunitz trypsin inhibitor: an antagonist of cell death triggered by phytopathogens and fumonisin b1 in Arabidopsis. Mol. Plant. 1, 482-495

Lim, H. et al. (2009) Altered expression of pyrophosphate: fructose-6-phosphate 1-phosphotransferase affects the growth of transgenic Arabidopsis plants. Mol. Cells. 27, 641-649

Liu, J. et al. (2010) The superfamily of thaumatin-like proteins: its origin, evolution, and expression towards biological function. Plant Cell Rep. 29, 419-436

Lohaus, G. et al. (2001) Is the infiltration-centrifugation technique appropriate for the isolation of apoplastic fluid? A critical evaluation with different plant species. Physiol. Plant. 111, 457-465

Luna, E. et al. (2014) Plant perception of β-aminobutyric acid is mediated by an aspartyl-tRNA synthetase. Nature Chem. Biol. 10, 450

Mano, J. et al. (2005) Protection against photooxidative injury of tobacco leaves by 2-alkenal reductase. Detoxication of lipid peroxide-derived reactive carbonyls. Plant Physiol. 139, 1773-1783

McLaughlin, J. et al. (2015) A lipid transfer protein increases the glutathione content and enhances Arabidopsis resistance to a trichothecene mycotoxin. PloS one, 10, e0130204

Meier, S. et al. (2008) Co-expression and promoter content analyses assign a role in biotic and abiotic stress responses to plant natriuretic peptides. BMC Plant Biol. 8, 24

Moreno, J. et al. (2005) Arabidopsis SHMT1, a serine hydroxyl-methyltransferase that functions in the photorespiratory pathway influences resistance to biotic and abiotic stress. Plant J.41, 451-463

Nagatoshi, Y., Nakamura, T. (2009). Arabidopsis HARMLESS TO OZONE LAYER protein methylates a glucosinolate breakdown product and functions in resistance to *Pseudomonas syringae* pv. maculicola. J. Biol. Chem. 284, 19301-19309

Nandi, A. et al. (2005) Arabidopsis *ssi2*-conferred susceptibility to *Botrytis cinerea* is dependent on *EDS5* and *PAD4*. Mol. Plant Microbe Interac.18, 363-370

Návarová, H. et al. (2012) Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. Plant Cell, tpc-112

Nesvizhskii, A. I. et al. (2003) A statistical model for identifying proteins by tandem mass spectrometry. Anal. Chem. 75, 4646-4658

Noël, L. D. et al. (2007) Interaction between SGT1 and cytosolic/nuclear HSC70 chaperones regulates Arabidopsis immune responses. Plant Cell, 19, 4061-4076

Park, J. H. et al. (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J. 31, 1-12

Park, S. J. et al. (2009) Cold shock domain proteins affect seed germination and growth of *Arabidopsis thaliana* under abiotic stress conditions. Plant Cell Physiol. 50, 869-878

Pastor, V. et al. (2014) Preparing to fight back: generation and storage of priming compounds. Front. Plant Sci. 5, 295

Periappuram, C. et al. (2000) The plastidic phosphoglucomutase from Arabidopsis. A reversible enzyme reaction with an important role in metabolic control. Plant Physiol. 122, 1193-1200

Provart, N., & Zhu, T. (2003) A browser-based functional classification SuperViewer for Arabidopsis genomics. Curr. Comput. Mol. Biol. 2003, 271-272

Rajjou, L. et al. (2006) Proteomic investigation of the effect of salicylic acid on Arabidopsis seed germination and establishment of early defense mechanisms. Plant Physiol. 141, 910-923

Ren, D. et al. (2008) A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. Proc. Natl. Acad. Sci. 105, 5638 - 5643

Sappl, P. G. et al. (2004) Proteomic analysis of glutathione S-transferases of *Arabidopsis thaliana* reveals differential salicylic acid-induced expression of the plant-specific phi and tau classes. Plant Mol. Biol. 54, 205-219

Sauter, A. et al. (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. J. Exp. Bot. 52, 1991-1997

Sattelmacher, B. (2001) The apoplast and its significance for plant mineral nutrition. New Phyto. 149, 167-192

Schilmiller, A. et al. (2007) Functional diversification of acyl-coenzyme A oxidases in jasmonic acid biosynthesis and action. Plant Physiol. 143, 812-824

Sharma, Y. K., Davis, K. R. (1995) Isolation of a novel Arabidopsis ozone-induced cDNA by differential display. Plant Mol. Biol. 29, 91-98

Shi, J. et al. (2015) Phosphoenolpyruvate carboxylase in Arabidopsis leaves plays a crucial role in carbon and nitrogen metabolism. Plant Physiol. 167, 671-681

Singh, P. et al. (2012) The lectin receptor kinase-VI. 2 is required for priming and positively regulates Arabidopsis pattern-triggered immunity. Plant Cell, tpc-112

Slaughter, A. et al. (2011) Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. Plant Physiol. pp-111

Snustad, D. P. et al. (1992) The small genome of Arabidopsis contains at least nine expressed beta-tubulin genes. Plant Cell, 4, 549-556

Sreeramulu, S. et al. (2013) BSKs are partially redundant positive regulators of brassinosteroid signaling in Arabidopsis. Plant J. 74, 905-919

Srivastava, R. et al. (2013) BINDING PROTEIN is a master regulator of the endoplasmic reticulum stress sensor/transducer bZIP28 in Arabidopsis. Plant Cell, 25, 1416-1429

Sunkar, R. et al. (2003) Overexpression of a stress-inducible aldehyde dehydrogenase gene from

Arabidopsis thaliana in transgenic plants improves stress tolerance. Plant J. 35, 452-464

Sunkar, R. et al. (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell, 18, 2051-2065

Sweetlove, L. et al. (2002) The impact of oxidative stress on Arabidopsis mitochondria. Plant J. 32, 891-904

Takahashi, F. et al. (2007) The mitogen-activated protein kinase cascade MKK3–MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. Plant Cell, 19, 805-818

Takaishi, M. et al. (2012) A unique pathway for the 3-aminobutyrate starter unit from L-glutamate through β-glutamate during biosynthesis of the 24-membered macrolactam antibiotic, incednine. Org. Lett.14, 4591-4593

Tanou, G. et al. (2012) Priming against environmental challenges and proteomics in plants: update and agricultural perspectives. Frontiers in plant science, 3, 216

Thevenet, D. et al. (2017) The priming molecule β-aminobutyric acid is naturally present in plants and is induced by stress. New Phytol. 213, 552-559

Thole, J. et al. (2008) Root hair defective4 encodes a phosphatidylinositol-4-phosphate phosphatase required for proper root hair development in *Arabidopsis thaliana*. Plant Cell, 20, 381-395

Ton, J., Mauch-Mani, B. (2004) β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. 38, 119-130

Ton, J. et al. (2005) Dissecting the β-aminobutyric acid–induced priming phenomenon in Arabidopsis. Plant Cell, 17, 987-999

Tsai, C. et al. (2011) Priming for enhanced defense responses by specific inhibition of the Arabidopsis response to coronatine. Plant J. 65, 469-479

Vignutelli, A. et al. (1998) Systemic and local induction of an Arabidopsis thionin gene by wounding and pathogens. Plant J. 14, 285-295

Wang, H. et al. (1995) Promoters from *kin1* and *cor6.6*, two homologous *Arabidopsis thaliana* genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum and dehydration. Plant Mol. Biol. 28, 605-617

Wang, W. et al. (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9, 244-252

Wagner, U. et al. (2002) Probing the diversity of the Arabidopsis glutathione S-transferase gene family. Plant Mol. Biol. 49, 515-532

Widemann, E. et al. (2013) The amidohydrolases IAR3 and ILL6 contribute to jasmonoyl-isoleucine hormone turnover and generate 12-hydroxyjasmonic acid upon wounding in Arabidopsis leaves. J. Biol. Chem. 288, 31701-31714

Worrall, D. et al. (2012) Treating seeds with activators of plant defense generates long-lasting priming of resistance to pests and pathogens. New Phytol. 193, 770–778

Wu, X. et al. (2014) Universal sample preparation method integrating trichloroacetic acid/acetone precipitation with phenol extraction for crop proteomic analysis. Nat. Protoc. 9, 362

Wu, C-C. et al. (2009) L-Glutamine inhibits beta-aminobutyric acid-induced stress resistance and priming in Arabidopsis. J. Exp. Bot. 61, 995-1002

Zimmerli, L. et al. (2008) The xenobiotic β-aminobutyric acid enhances Arabidopsis thermotolerance. Plant J. 53, 144-156

Chapter IV- **BABA in Arabidopsis: a physiological approach**

Accumulation patterns of endogenous β-aminobutyric acid during plant development and defense in *Arabidopsis thaliana*

adapted from:

Balmer, A., Mauch-Mani, B., Baccelli, I. (2018). Accumulation patterns of endogenous β-aminobutyric acid during plant development and defense in *Arabidopsis thaliana*. Accepted.

Thevenet, D., Pastor, V., Baccelli, I., Balmer, A., Vallat, A., Neier, R., Glauser G., & Mauch-Mani, B. (2017). The priming molecule β-aminobutyric acid is naturally present in plants and is induced by stress. New Phytologist, 213(2), 552-559.

Keywords

BABA, β-aminobutyric acid, priming, defense, pathogen infection, senescence, *cpr5*, age-related resistance

Accumulation patterns of endogenous β-aminobutyric acid during plant development and defense in *Arabidopsis thaliana*

1. Introduction

The non-proteinogenic β-aminobutyric acid (BABA) has been often described in respect to its important role as inducer of defense priming in plants (Conrath et al. 2015; Mauch-Mani et al. 2017). The application of BABA protects a large range of plant species including *Arabidopsis thaliana* against multiple virulent pathogens, insect herbivores, nematodes, and abiotic stresses, by potentiating specific plant resistance mechanisms (Balmer et al. 2014; Baccelli and Mauch-Mani, 2016). From a chemical point of view, this molecule belongs to the group of non-proteinogenic amino acids and contains two functional groups: a carboxyl group at the first carbon atom, and an amino group at the third carbon atom. Depending on the position of the amino group in relation to the carboxyl group, BABA has two isomers: α-aminobutyric acid (AABA) and γ-aminobutyric acid (GABA), which both show no or only limited resistance-inducing capacity when applied to plants (Cohen, 2002; Šašek et al. 2012).

Up to only recently BABA was mostly described as a plant-active xenobiotic molecule. However, an analytical method developed in our laboratory has finally allowed separation and simultaneous quantification of aminobutyric acid isomers in plant tissues, leading to the exciting discovery of BABA as a novel endogenous plant metabolite (Thevenet et al. 2017). This latest finding not only provides additional significance to the previous discovery of a perception mechanism for BABA in Arabidopsis (Luna et al. 2014), but demands now a reorientation of the way this molecule is perceived. BABA is produced by several plant species including monocots, dicots, and the moss *Physcomitrella patens* (Thevenet et al. 2017). BABA is constitutively present at a low basal level in both leaves and roots, and can be induced in both organs after salt stress (Thevenet et al. 2017). However, it is not known whether BABA levels remain constant during the whole plant life cycle under normal conditions, and whether other plant parts and organs can produce the same amount of BABA found in leaves or roots.

Salt stress, submergence, treatment with defense elicitors, and pathogens with distinct lifestyles have been all found to induce BABA accumulation in Arabidopsis (Thevenet et al. 2017; Baccelli et

al. 2017). Importantly, endogenous BABA levels were found to be tightly controlled by the plant's immune system and rise specifically during plant defense reactions (Baccelli et al. 2017), providing clear evidence that plants can synthesize BABA and modulate its levels when exposed to stress, in the likely attempt to prime their defensive machinery. Since the biosynthetic pathway of BABA is still unknown, quantitative data on the accumulation of this molecule in plants represent nowadays the only way to understand more about the role that this molecule may play in plants, and accordingly to orientate future research directions. For instance, while synthetic BABA has been reported to be transported to several plant organs (Cohen et al. 1999), the capacity of endogenous BABA to act as a mobile signal is still unknown. Mobile molecules are important players in plant defense reactions, such as the case of systemic acquired resistance (SAR), which is an immune response induced in distal plant parts following a locally restricted primary infection (Fu and Dong, 2013).

During SAR, various metabolites are generated in locally infected tissues and some of those are transported to other plant parts. Methyl salicylate, azelaic acid, or glycerol-3-phosphate (Park et al. 2007; Jung et al. 2009; Chanda et al. 2011) are perceived in the systemic tissues and lead to the activation of protection against a broad spectrum of mostly biotrophic pathogens. Likewise, molecules without signaling role such as proline were described to be specifically translocated over a long distance between plant organs under stress conditions (Lehmann et al. 2010). In this study, we aimed at investigating the accumulation of BABA in different Arabidopsis tissues and organs during normal plant development and aging, and at establishing whether BABA is systemically accumulated in the plant during bacterial or fungal infection.

2. Materials and methods

2.1 Plant material and growth conditions

***Arabidopsis thaliana*:** ecotype Columbia (Col-0), Landsberg erecta (LER) and Wassilewskija (Ws), *cpr5-2* mutant, and the following Arabidopsis T-DNA insertion lines; *pop2* (N6385), *aap6* (SALK_013231), *prot1* (SALK_030711), *gat1* (SALK_031983), *aap3* (SALK_148822) were germinated in 33 mL hydrated Jiffy-7 peat pellets maintained at 21°C day/19°C night, with 9 hours of light (120 µE m^{-2} s^{-1}) and 60% of relative humidity. One week after germination, seedlings were individually transferred to either Jiffy-7 peat pellets or a hydroponic culture system (Araponics: www.araponics.com), and kept in the same conditions until the experiments were run. The nutrient solution containing half-strength Hoagland was replaced weekly.

Physcomitrella patens: Protonema was cultured in 9 cm Petri dishes containing solid ammonium tartrate culture medium overlaid with a cellophane disk (W.E. Cannings, Bristol, UK), at 26 °C in discontinuous 16 h light (50 to 80 µmol m^{-2} s^{-1}).

Maize (*Zea mays* ssp. variety Delprim) and **Teosinte** (*Z. mays* ssp. Mexicana)**:** Seeds of maize and teosinte were sown in plastic pots (150 ml volume) with a 60:40 soil:sand mixture (Ricoter Aussaaterde). The plants were grown in a climate chamber with 12 h light (350 µmol/m^2/sec1 light), 12 h dark, at 25 °C and 60% relative humidity.

Wheat (*Triticum spp.* var. Campala "Spring")**:** Seeds were germinated and grown in potting soil with a 60:40 soil:sand mixture (Ricoter Aussaaterde), in a growth chamber at 15 °C with a 12 h light (350 µmol m^{-2} s^{-1}) and 12 h darkness cycle and 60% relative humidity.

Chinese cabbage (*Brassica rapa*)**:** Plants were grown in potting soil with a 60:40 soil:sand mixture (Ricoter Aussaaterde), in a climate chamber with 12 h light (200 µmol m^{-2} s^{-1}) and 12 h darkness, at 25 °C and 60% relative humidity.

2.2 Assays for tissues specific-accumulation of BABA

In order to study the accumulation of BABA in different plant organs during growth, BABA levels were analyzed: a) in roots and leaves of 4 week-old Arabidopsis plants; b) in leaves of 7 week-old plants; c) in stems, cauline leaves, pollinated and un-pollinated flowers; d) in siliques and in seeds. The organ-specific accumulation of BABA was also analyzed in mutants of amino acid transporters, such as *aap3, aap6, gat1*, *prot1*, and the GABA-transaminase knock-out mutant *pop2*, which shows elevated levels of GABA (Fischer et al. 1995; Fischer et al. 2002; Breitkreuz et al. 1999; Palanivelu et al. 2003).

Additionally, the levels of BABA were measured in leaves from 7 week-old Arabidopsis Col-0 plants in which the flowering process was interrupted by repeatedly cutting the floral stems during a period of two weeks. Leaves, stems and flowers of plants kept under the same growing conditions and having a normal flowering process were used as a control.

2.3 Salt stress essays

Salt stress assays were performed as plants soil- drenched with 200 mM NaCl, or water as a control and samples were taken 48 hours post treatment. In Arabidopsis ecotypes essays were performed with 4-5 weeks- old plants. Cellophane disks with seven days cultures of *Physcomitrella patens* were transferred to a new Petri dish containing medium enriched with 200 mM of NaCl or water as a control. In maize, teosinte and wheat, salt stress assays were performed with 3 weeks- old plants as described above. For chinese cabbage the salt stress assays were performed with 4 weeks- old plants.

2.4 Plant infections

Arabidopsis Col-0 plants at the age of 5 weeks were used to analyze the systemic accumulation of BABA after local infection with bacterial of fungal pathogens. To investigate the systemic accumulation in the aboveground plant parts (leaf-to-leaf accumulation), three leaves per plant were infected and both locally infected leaves and corresponding systemic leaves were analyzed. Infections were performed by placing 6-µl drops of a spore suspension ($5x10^6$ spores mL^{-1}) of *Plectosphaerella cucumerina* BMM in half strength (12 g L^{-1}) Potato Dextrose Broth (PDB, Sigma-Aldrich) on the adaxial leaf surface (4-5 drops per leaf), or by pressure-infiltrating the abaxial leaf surface with a suspension 10^6 colony-forming units (CFU) mL^{-1} in 10 mM $MgCl_2$ of *Pseudomonas*

syringae pv. tomato (*Pst*) DC3000 *avrRpt2*. Plants were then incubated in 100% relative humidity (RH) conditions for the first 6h (*Pst* DC3000 *avrRpt2* infections) or for the whole period (*P. cucumerina* infections). As a control (mock), different plants were treated on three leaves with 6-µl drops of half strength PDB (*P. cucumerina* control) or were infiltrated with 10 mM $MgCl_2$ (*Pst* control), and both locally treated and corresponding systemic leaves were collected. After 24, 48, and 72 h post infection (hpi), local leaves and systemic leaves were collected and immediately frozen in liquid nitrogen. In order to investigate the leaf-to-root systemic accumulation, all fully developed leaves from five-week-old Arabidopsis plants were drop inoculated with *P. cucumerina* BMM in half strength PDB (Sigma-Aldrich) and incubated as previously described. Treatments with half strength PDB were performed as a control (mock). After 48 and 72 hpi, *P. cucumerina*-infected and mock-treated leaves were collected along with their respective root tissues, and immediately frozen in liquid nitrogen for further analysis as described below.

2.5 Essays for BABA production by bacteria

To compare the capacity of BABA production by the bacterial strain of *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 *avrRpt2*, a bacteria culture was made from a fresh stock on King's B (King et al. 1954) agar enriched with 100 µg/mL of rifampicin. After three days of growth in the dark at 25°C, a single colony of *Pst* DC3000 *avrRpt2* was picked and transferred to 50 mL of three different bacteria liquid culture media: King's medium B enriched with 100 µg/mL of rifampicin, King's medium B with 100 mM of sodium chloride (NaCl) or King's medium B without antibiotic (as a control). Liquid bacterial cultures were kept overnight at 28°C, under continuous shaking at 150rpm. Liquid cultures with an optical density at a wavelength of 600nm (OD_{600}) of 1 were used for BABA extraction. Different growth media were used in order to test the capacity of *Pst* DC3000 *avrRpt2* to induce BABA under stressful conditions.

2.6 Senescence induction

Leaves were numbered in order of appearance until the plants were 5 weeks old. Subsequently, the 6^{th} up to the10^{th} leaf were detached and transferred to 9-mm-diameter Petri dishes containing a double-layer of Whatman filter paper humidified with 15 mL of distilled water. The petri dishes were sealed with Parafilm tape and kept at 21°C day/19°C night and exposed to 9 hours of light (120 µE m^{-2} s^{-1}) as a control or were incubated in the dark by wrapping with double-layer aluminum foil to induce senescence. Samples were collected after 2 and 7 days of incubation.

2.7 Hydrogen peroxide treatments

The effect of hydrogen peroxide on the accumulation of BABA was tested on 5 week-old Col-0 plants by infiltrating 6 leaves per plant with a solution of H_2O_2 (30% w/w, Sigma-Aldrich) diluted in sterile distilled water at the final concentration of 50 and 500 µM, respectively. Pressure infiltrations were performed on the abaxial (lower) leaf surface. As a control, leaves were infiltrated with sterile distilled water. Plants were incubated in 100% RH for the first 6 h. Infiltrated leaves were collected after 6, 24, and 48h and immediately frozen in liquid nitrogen for BABA extraction.

2.8 Quantification of endogenous BABA levels

The content of endogenous BABA present in plant tissues and bacteria was quantified by using an Ultimate 3000 RSLC from Dionex interfaced with a 4000 QTRAP, and the liquid chromatography (LC) separation was accomplished on an Acquity UPLC BEH HILIC column (100 mm 9 2.1 mm, 1.7 lm, Waters) as previously described (Thevenet et al. 2017, Balmer et al. 2018). The extraction of endogenous BABA from plant material was performed in formic acid 0.1% containing a deuterium-labelled internal standard, according to the protocol described in Thevenet et al. (2017). BABA extraction from bacterial cells was performed from an overnight liquid culture (OD_{600}=1) after centrifugation at 3000 rpm for 10 minutes and double washing in sterile water. Bacteria were then re-suspended in formic acid 0.1% containing the internal standard, and the suspension was sonicated three times for 1 minute. Bacterial extracts were then purified by solid phase extraction, according to the protocol used for plant extracts (Thevenet et al. 2017; Baccelli et al. 2017).

2.9 Statistical analysis

Variances of BABA levels between different sample groups were analyzed by a *t*-test or ANOVA. All statistical analyses were performed by using GraphPad software version 5 (www.graphpad.com).

3. Results

3.1 Plant-produced BABA shows tissue specific-accumulation in Arabidopsis

Mobile amino acids such as proline show organ-specific accumulation in plants, not only under stress conditions but also during normal development, for instance in those organs that undergo dehydration as part of their development (Girousse et al. 1996; Schmidt et al. 2007). In order to investigate this possibility for endogenous BABA, we quantified BABA levels in both roots and leaves of 4 week- old plants, in leaves of 7 week- old plants, in stems, in cauline leaves, in pollinated and un-pollinated flowers, as well as in siliques and seeds. The organ-specific accumulation of BABA was also evaluated in different mutants for amino acid transporters, such as *aap6* and *aap3* (amino acid permeases 6 and 3), *prot1* (proline transporter 1) and *gat1* (GABA transporter 1), as well as in the GABA-transaminase knock-out mutant *pop2* (pollen-pistil incompatibility 2). These mutants were selected in order to explore the possibility that endogenous BABA is transported by amino acid transporters with multiple affinity for molecules showing similarity to BABA in their chemical structure, such as GABA, AABA, glycine betaine, and β-alanine.

As presented in **Figure 1**, BABA showed a tissue-specific accumulation in Arabidopsis. In all the genotypes analyzed, high levels of BABA were measured in pollinated and un-pollinated flowers, in siliques, and in seeds, whereas lower levels were detected in roots, leaves, stems, and cauline leaves. In addition, the amino acid transporter mutants *aap6, app3, prot1*, and *gat1*, showed BABA levels similar to the wild-type (Col-0) in the various plant tissues (**Figure 1**). A similar accumulation pattern also occurred in the *pop2* mutant (**Supplementary Figure S1**). Interestingly, the BABA content found in leaves from older plants that are starting the flowering process (7 week-old) is higher than in leaves from younger plants (4 week-old) (**Figure 1**). However, when the floral stem was impeded to develop because repeatedly cut and removed from Col-0 plants, leaves did not accumulate more BABA than that found in leaves from flowering plants (**Supplementary Figure S2**).

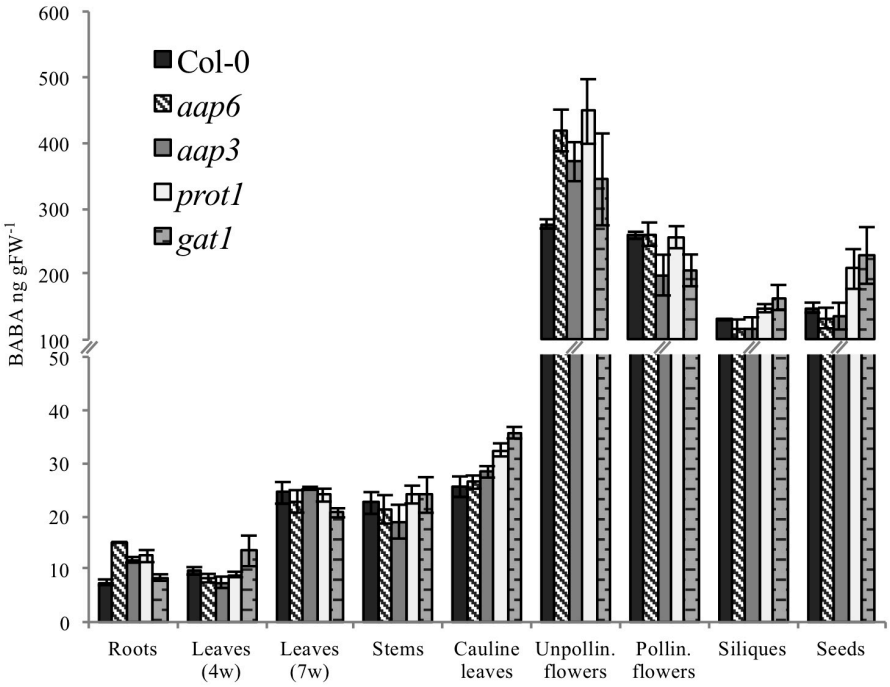


Figure 1. BABA content in different plant tissues. BABA levels were analyzed in Col-0 Arabidopsis plants (wild-type) and in the amino acid transporter mutants *aap6, aap3, prot1*, and *gat1*. Samples were collected from roots, young plant leaves (4 weeks old, 4w), mature plant leaves (7 weeks old, 7w), stems, cauline leaves, unpollinated flowers (Unpolli. flowers), pollinated flowers (Pollin. flowers), siliques, and seeds. BABA levels represent the mean of three independent replicates ± SEM. Each replicate was made of a pool of samples collected from 6 plants.

3.2 Presence and accumulation of BABA in leaves and roots from various plant species

To determine whether the occurrence of BABA was specific to Arabidopsis Col-0 plants or more widespread in the plant kingdom, we screened various plant species and different Arabidopsis ecotypes. The plants tested included monocots, dicots and a bryophyte. These healthy unstressed plants contained 5–15 ng g^{-1} FW of BABA in leaves and 10–37 ng g^{-1} FW of BABA in roots (**Figure 2**). Depending on the plant species, salt stress induced the accumulation of BABA in leaf tissue to various extents. After 48 h of salt stress, Arabidopsis Ler, *Zea mays* and *Zea mays* ssp. mexicana showed the highest values of BABA accumulation (up to 20-fold) in leaves. By contrast, *Triticum aestivum* and *Physcomytrella patens* displayed the lowest (**Figure 2A**). In general, BABA accumulated to a lesser extent in roots than in leaves after exposure to salt stress (**Figure 2 A,B**).

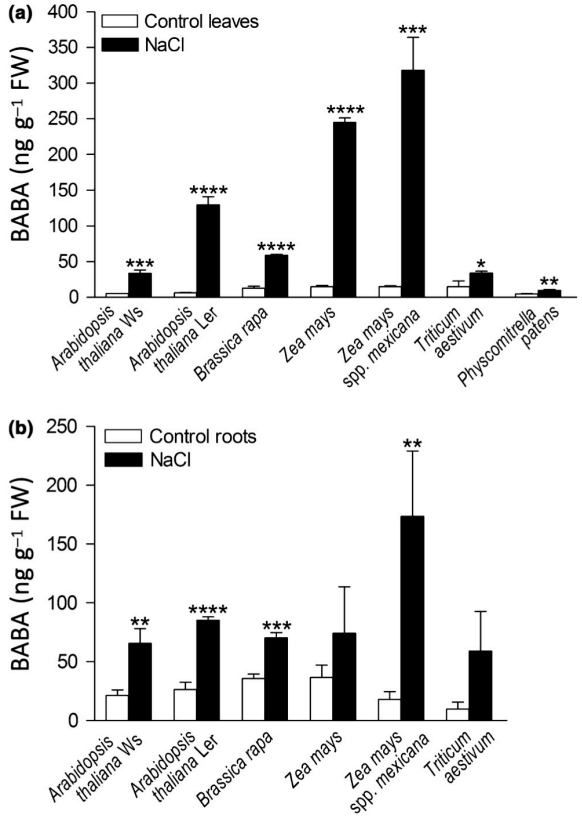


Figure 2. Quantification of BABA in different plant species. Induction of BABA in leaf (A) and root (B) tissues 48 h after soil-drench with 200 mM NaCl, or water as a control. FW, fresh weight. Data represent the mean and standard deviation (n = 3 biological replicates). All experiments were repeated twice. Asterisks indicate significant differences as determined by a Student t-test: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****P < 0.0001.

3.3 BABA is highly accumulated during leaf senescence and in cpr5-2 plants

Since 7 week-old plants showed a higher BABA content than 4 week-old plants (**Figure 1**), the accumulation of BABA during normal development and aging was monitored weekly over 9 weeks of growth (the end of the plant life cycle). As shown in **Figure 3**, BABA levels did not statistically vary during the first 6 weeks of growth, but started to increase after 7 weeks of growth, corresponding to the onset of the floral transition (**Figure 3**). After 8 weeks of growth the BABA content increased again, reaching the plateau, with a value almost double than that found in 1- to 6-week-old leaves (**Figure 3**). In our experimental conditions, symptoms associated to leaf senescence, such as leaf yellowing, appeared around 8 to 9 weeks after germination (data not shown). Therefore, BABA levels were quantified after artificially dark-induced senescence in detached Arabidopsis leaves. As shown in **Figure 3B**, a pronounced BABA accumulation was readily observed in the leaves kept in the dark for 2 days. After 7 days of dark incubation, when

clear symptoms of senescence (pronounced leaf yellowing) were visible (**Figure 3C**), BABA levels reached about 300 ng gFW^{-1}, as compared to about 60 ng gFW^{-1} of BABA found under light conditions (**Figure 3B**). However, a moderate increase in BABA levels over time was also detectable under control (light) conditions (**Figure 3B**), probably because of the long incubation to which detached leaves were subjected. These results collectively suggested that BABA levels were strongly influenced by the process of senescence, adding further information to the factors that can regulate the accumulation of this molecule. Since the immune system is also a regulator of BABA accumulation (Baccelli et al. 2017), we then analyzed the recessive Arabidopsis mutant *cpr5-2* (constitutive expressor of *PR* genes 5), which combines together an increased defense phenotype with an accelerated leaf senescence phenotype (Bowling et al. 1997; Jing et al. 2007), with the expectation of finding a mutant with high constitutive levels of BABA. As shown in **Figure 4A**, *cpr5-2* plants produced higher levels of BABA than Col-0 plants (**Figure 4A**) after 4, 5, and 6 weeks of growth. Since the process of leaf senescence has been reported to be associated to a state of high-cellular oxidative stress in *cpr5* plants (Jing et al. 2007), we then tested whether higher BABA levels could be due to an altered ROS balance. However, infiltration of Col-0 leaves with 50 or 500 µM hydrogen peroxide did not cause any significant variation in BABA content during 48 h of treatment (**Figure 4B**).

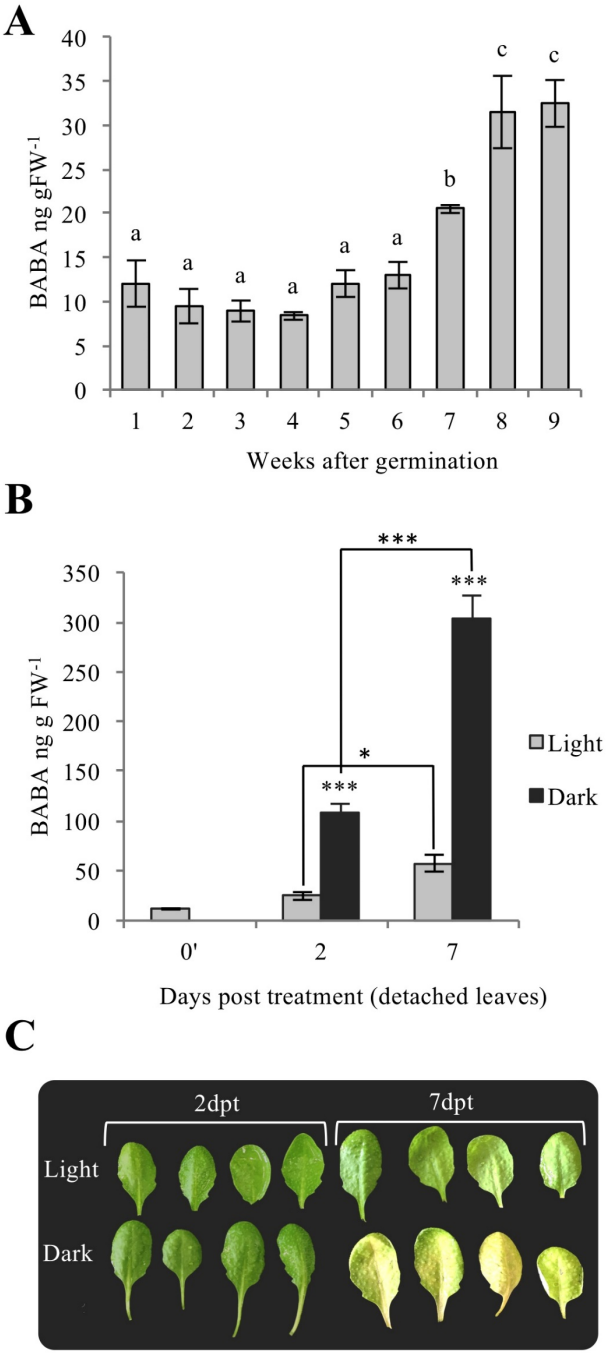


Figure 3. BABA accumulation during natural and induced senescence in Arabidopsis leaves. **A**. BABA levels in leaves from plants 1 to 9 weeks old. Values are mean ± SEM (*n* = 3 biological replicates). Data were analyzed by using One-way ANOVA ($P \leq 0.05$) and significant differences are indicated by the letters. **B**. BABA levels were measured in Arabidopsis detached leaves (5 week-old) placed in Petri dishes and exposed to 9 hours of light (120 µE m^{-2} s^{-1}) as a control, or incubated in the dark to induce senescence. Samples were collected after 0, 2, and 7 days of incubation. Values are mean ± SEM (*n* = 6 biological replicates, each replicate is a pool of 12 leaves). Significant differences between light (control) and dark treatments, as well as between time points, were obtained by *t*-test. Asterisks indicate significant differences at $*P \leq 0.05$; *** $P \leq 0.001$. The experiment was repeated twice with similar results. **C**. Representative pictures from B showing Arabidopsis leaves incubated under the experimental conditions used to induce senescence (dark) or incubated as a control (light). dpt, days post treatment.

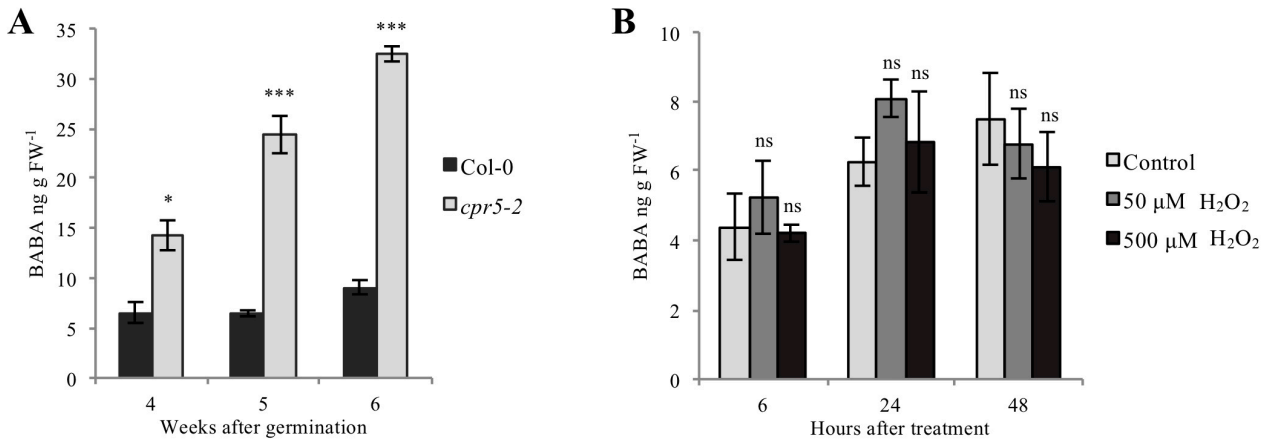


Figure 4. BABA levels in *cpr5-2* Arabidopsis plants as compared to the wild type (**A**), and in Col-0 plants after hydrogen peroxide (H_2O_2) infiltration (**B**). **A**. BABA levels in leaves from Col-0 (wild type) and *cpr5-2* mutants, after 4, 5, and 6 weeks of growth (Col-0 and *cpr5-2* plants were grown together and sampled at the same time). Data represent the mean ± SEM (*n* = 3 biological replicates). **B**. Leaves from 5-week-old Col-0 plants infiltrated with H_2O_2 at two different concentrations: 50µM and 500 µM. Control, leaves infiltrated with sterile distilled water. Significant differences were determined by *t*-test (Col-0 vs. *cpr5-2*; treated vs. control) and shown as follow: *$P \leq 0.05$; *** $P \leq 0.001$ as. Values are the mean ± SEM (*n* = 3 biological replicates). ns, no significant difference.

3.4 Endogenous BABA accumulates locally after infection with bacterial and fungal pathogens

In order to test whether BABA accumulated throughout the plant after local infection with different plant pathogens, BABA levels were determined in both locally infected leaves and systemic non-infected leaves. As shown in **Figure 5**, neither the infection with the hemibiotrophic bacterium *Pst* DC3000 *avrRpt2* nor the infection with the necrotrophic fungus *P. cucumerina* were able to induce accumulation of BABA in distal systemic leaves. In fact, while already after 24 hpi with *Pst* DC3000 *avrRpt2* a high accumulation of BABA was detectable in locally infected leaves, no BABA increase was detectable in the systemic leaves (**Figure 5A**). Non-infected systemic leaves did not show any variation in the BABA content even after 48 and 72 hpi (**Figure 5A**). In order to exclude a possible contribution of *Pst*-produced BABA in the amount detectable in the leaves after infection, we quantified the amount of BABA produced *in vitro* by *Pst* DC3000 *avrRpt2* cells. As shown in **Supplementary Figure S3**, the amount of BABA detectable in the bacterial cell extracts was negligible when compared to the levels detected in infected leaves. In addition, similarly to the infection with *Pst* DC3000 *avrRpt2*, the infection with *P. cucumerina* did not induce any accumulation of BABA in systemic non-infected leaves, while the content of BABA significantly increased in locally infected leaves (**Figure 5B**). Finally, *P. cucumerina*-infected plants were used to

check whether endogenous BABA accumulated systemically in roots, rather than leaves, upon foliar infection. However, no increase of BABA was detectable in the roots, while again BABA levels significantly increased in infected leaves (**Figure 6**).

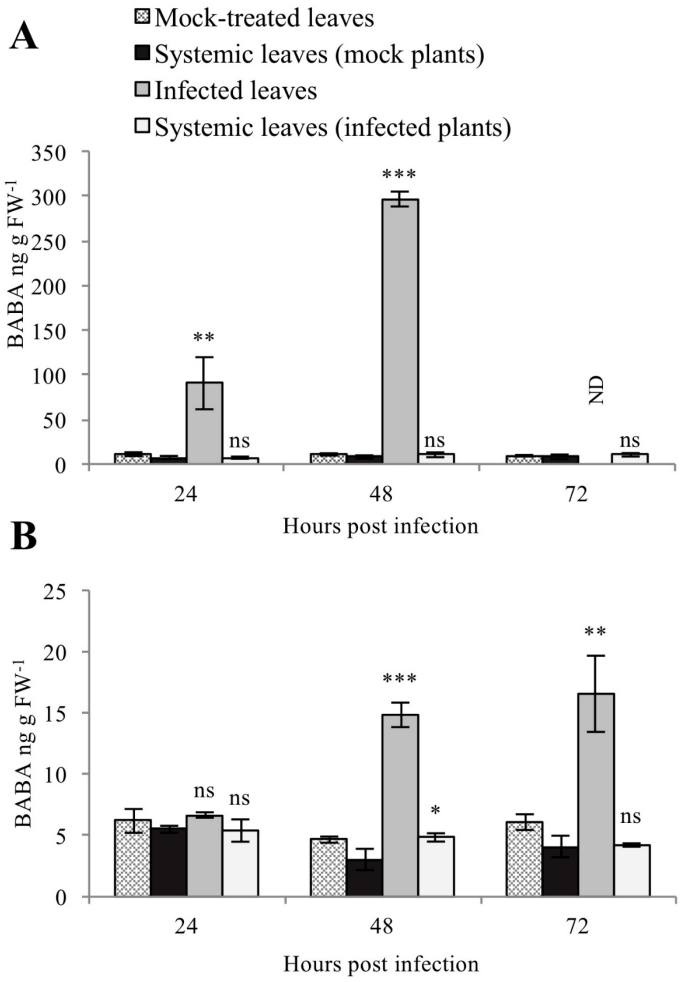


Figure 5. BABA accumulation in locally infected leaves and systemic non-infected leaves after bacterial or fungal infection. **A**. BABA levels in Arabidopsis plants after infection with avirulent *Pseudomonas syringae* pv. *tomato* DC3000 *avrRpt2*. Infections were performed locally by infiltrating three leaves per plant (infected leaves) with 10^6 colony-forming units (CFU) mL^{-1}. In mock-treated plants, leaves were infiltrated locally with 10 mM $MgCl_2$ (mock-treated leaves). Corresponding systemic (non-infected) leaves (systemic leaves) were collected and analyzed from both infected plants and mock-treated plants. After 72 h of infection, locally infected leaves were not analyzed (not determined, ND) for BABA accumulation after 72 h of infection because of the extensive necrosis covering the whole leaf surface. **B**. BABA levels in Arabidopsis plants after infection with virulent *Plectosphaerella cucumerina* BMM. Infections were performed locally by placing 6-µl drops of a spore suspension ($5x10^6$ spores mL^{-1}). In mock-treated plants, leaves were treated by placing 6-µl drops of half strength PDB (mock-treated leaves). Corresponding systemic (non-infected) leaves (systemic leaves) were collected and analyzed from both infected plants and mock-treated plants. Data represent the mean ± SD (n = 3 biological replicates). The experiments were repeated with similar results. Asterisks indicate significant differences: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (mock vs. infected) as determined by t-test; ns, no significant difference.

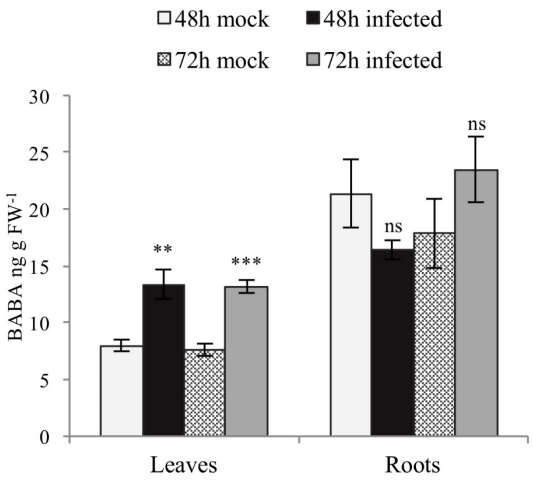


Figure 6. BABA accumulation in leaves and roots after foliar infection with *Plectosphaerella cucumerina* BMM. Leaves from 5-week-old Arabidopsis plants growing in a hydroponic culture system were drop inoculated with a spore suspension ($5x10^6$ spores mL^{-1}) of *P. cucumerina* BMM in half strength PDB. Mock-treated leaves were drop inoculated with half strength PDB. BABA levels were measured in *P. cucumerina*-infected and mock-treated leaves, along with their respective root tissues, after 48 and 72 hours post infection. Data were analyzed by *t*-test. Asterisks indicate significant differences: *$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ (mock vs. infected). ns, no significant difference. Values are the mean ± SEM. Similar results were obtained in three independent experiments.

4. Discussion

The study of plant-produced (endogenous) BABA is a very recent topic of research (Thevenet al. 2017; Baccelli et al. 2017) if we consider that this amino acid has been known for almost 60 years for its effects on plants (Cohen et al. 2016). Data about endogenous BABA are obviously still limited when compared to the large amount of data existing on the exogenous application of BABA, and in particular on its ability to induce resistance through priming in plants (for reviews, see Cohen et al. 2016; Baccelli and Mauch-Mani et al. 2016). For instance, synthetic BABA is known to be a very mobile molecule when applied exogenously to plants, being transported both upwards and downwards by the receiving plant (Cohen et al. 1999), while no information existed yet on the systemic behavior of endogenous BABA after stress. Data obtained up to date on endogenous BABA indicate that the production of BABA is ubiquitous in plants, and its accumulation after stress is tightly controlled by the defensive system of the plant, possibly because endogenous BABA is involved in the induction of natural priming (Baccelli et al. 2017; Thevenet al. 2017).

One of the issues we aimed at investigating in the present work was therefore the capacity of endogenous BABA to be transported, or accumulated, systemically after pathogen infection. Inducible defenses can be activated, or primed, in distal organs of the plant after local challenge by a pathogen (Vlot et al. 2008). However, our results obtained with *P. cucumerina* and *Pst* DC3000 *avrRpt2* showed clearly that BABA levels rose in the plant in an exclusively localized manner (Figure 4 and 5). Leaf-to-leaf and leaf-to-root transport of (free) BABA in the infected plant, or boosted synthesis of BABA in distal non-infected plant parts, could be excluded by the absence of variation in the BABA levels detectable in non-infected leaves and roots. Nevertheless, we cannot exclude that endogenous BABA was transported and accumulated as a conjugate thereby eluding our detection method. It is known, in fact, that plant amino acids form conjugates with plant hormones, as known for amide-linked tryptophan conjugates of indole-3-acetic acid (IAA-Trp), or isoleucine conjugates with jasmonic acid (JA-Ile) (Staswick, 2009). Conjugation may play various roles, from inactivating excess hormone (IAA-Trp) to hormone activation (JA-Ile), but the efforts made in our laboratory to discover possible BABA conjugates have failed so far.

In the present paper we also analyzed the levels of BABA during normal plant development and aging. BABA was measured in different Arabidopsis plant tissues, in wild-type plants and amino-

acid transporter mutants. The analyses revealed that BABA was not present at the same level in the different plant tissues (**Figure 1**). Reproductive organs exhibited the highest level of BABA, while leaves from younger plants the lowest (**Figure 1** and **2**). The levels of BABA did not increase in leaves when the flowering organ was impeded to develop (**Figure S2**), suggesting that the higher content of BABA present in the flowers was not stemming from the leaves, but was rather produced in situ. The analyses performed on the different tissues also allowed to exclude the transport of BABA by the amino acid transporters GAT1, PROT1, AAP3, and AAP6, since no differences in the BABA level were found as compared to wild-type (**Figure 1**). It was also possible to exclude that BABA can originate from its **γ** isomer, since the mutant *pop2*, which produces elevated constitutive levels of GABA (Palanivelu et al. 2003), did not show higher levels of BABA (**Figure S1**).

Besides the localized and organ-specific accumulation patterns reported above, it was found that BABA accumulated in an age-dependent manner. BABA was more abundant in leaves from aged plants than in leaves from younger plants. The increase of BABA naturally started after 7 weeks of growth (coinciding with the onset of the floral transition), and increased again after 8 weeks (when some senescence symptom started to appear) (**Figure 3A**). As revealed by artificially inducing senescence to relatively young leaves (5 week-old) (**Figure 3B**), the increase of BABA could be triggered by senescence-associated processes. It is worth mentioning here that older Arabidopsis plants are known to be more resistant to the infection with biotrophic and hemibiotrophic pathogens, because of the still unclarified phenomenon referred to as age-related resistance (ARR) (Carella et al. 2015). ARR is clearly detectable when the growth in planta of virulent *Pst* DC3000 is compared between 4- and 7-week-old plants (Carella et al. 2015). Remarkably, our analyses precisely showed that a significant BABA rise occurred at 7 weeks (**Figure 3A**). ARR is associated to high SA levels, possibly because ARR-competent Arabidopsis plants can alleviate coronatine-mediated suppression of SA accumulation by virulent *Pst* (Carella et al. 2015). Intriguingly, the application of BABA to Arabidopsis plants has been reported to prime for enhanced resistance to *Pst* by specifically inhibiting the coronatine-dependent suppression of SA-dependent defenses (Tsai et al. 2011). These observations highlight an interesting link between ARR and the endogenous BABA content.

In the *cpr5* mutant with its constitutively primed state (Kohler et al. 2002), the BABA content was higher in than Col-0 plants (**Figure 4**). This difference was observed at early developmental stages (4 weeks-old plants), and became more prominent during more advanced stages (5- and 6-week-old plants). Notably, the mutant *cpr5* – also called *hys1*, for hypersenescence1 – has also been

reported to exhibit an early senescence phenotype associated to a high-cellular oxidative stress (Yoshida et al. 2002; Jing et al. 2007). Our analyses, however, led us to exclude that BABA could be induced by a high leaf ROS content (**Figure 4**).

In summary, data obtained in the present study show that endogenous BABA levels not only rise after environmental stresses (biotic and abiotic), but also during the normal aging of the plant because of senescence-associated processes. We also found that reproductive organs possess a high content of BABA. This evidence is particularly intriguing when considering the possible role in defense played by endogenous BABA. In fact, the application of synthetic BABA was found to cause transgenerational effects in the descendants of the receiving plants, by enhancing their pathogen resistance (Slaughter et al. 2012). Finally, we show that endogenous BABA is exclusively accumulated within the infected leaf and is not transported or accumulated (as the free form) in distal non-infected plant parts. Although this feature was unexpected, and is in apparent contrast with the previously known mobility of synthetic BABA, it does not preclude an involvement of endogenous BABA in plant defense as suggested by previous results (Baccelli et al. 2017). In view of the data obtained on endogenous BABA in this paper and previously, and taking also into account the priming ability of synthetic BABA for which a large body of literature exists, we propose here a comprehensive model for the role of endogenous and synthetic BABA, in order to explain the priming potency of BABA in plants (**Figure 7**). Our hypothesis contemplates that the local accumulation of endogenous BABA can lead to the generation of a different still unknown mobile priming signal (**Figure 7A**), or alternatively is needed for the establishment of a still unknown local defense response (**Figure 7B**). In this latter case, synthetic BABA would trigger defense priming in an indirect way, by "mimicking" a widespread defense response.

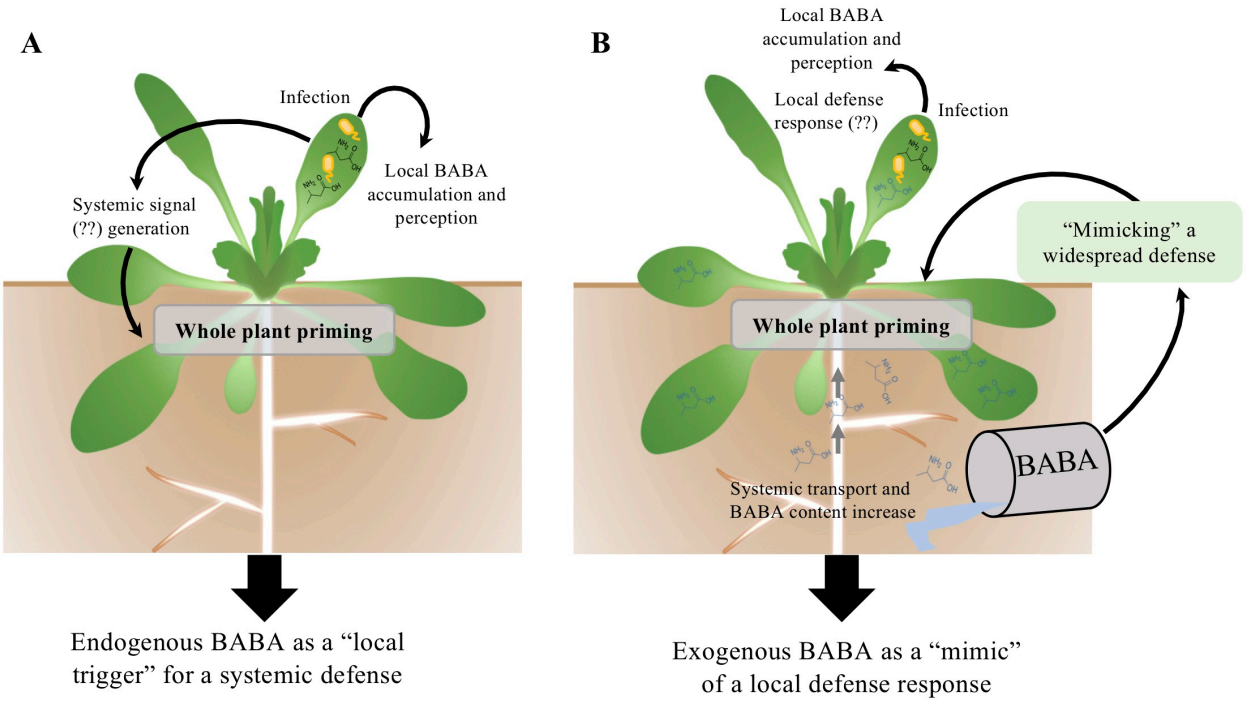


Figure 7. Mechanisms proposed to explain the priming potency of BABA in plants. In **A**, pathogen infection leads to the local accumulation of BABA (as found in the present paper), which is in turn perceived through *ibi1*, or a different and more specific receptor, and leads to the generation of a different (unknown) mobile priming signaling. **B**, the local accumulation of BABA is needed for the establishment of a (unknown) local defense response, thus explaining previous results linking BABA to pathogen defense (Baccelli et al. 2017). In A, the application of synthetic BABA could prime plants by increasing the endogenous BABA content thereby boosting the generation of the unknown priming signal; in B, synthetic BABA, given its mobility, could prime plants indirectly by mimicking the unknown defense response throughout the plant.

1. Supplementary Data

Supplementary Figure 1: BABA content in different tissues in the mutant *pop2*. BABA levels were also analyzed in Col-0 plants (wild-type).

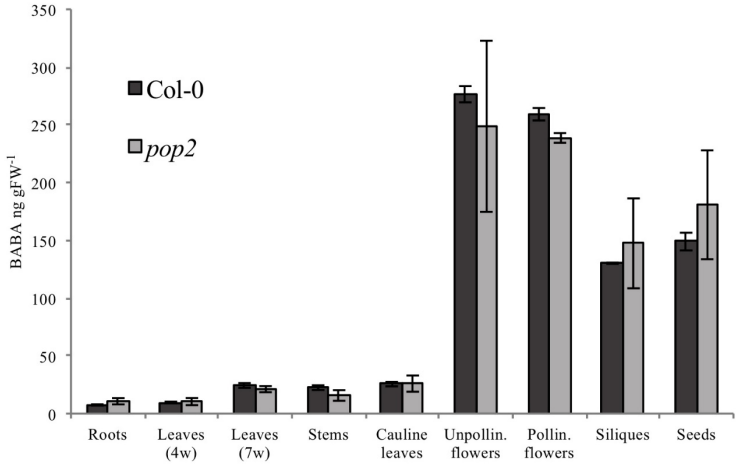


Figure S1. Samples were collected from roots, young plant leaves (4 weeks old, 4w), mature plant leaves (7 weeks old, 7w), stems, cauline leaves, unpollinated flowers (Unpolli. flowers), pollinated flowers (Pollin. flowers), siliques, and seeds. BABA levels represent the mean of three independent replicates ± SEM. Each replicate was made of a pool of samples collected from 6 plants.

Supplementary Figure 2: BABA levels in found in leaves from plants where the flowering process was repeatedly interrupted by cutting the floral stem, as well as in stems and flowers.

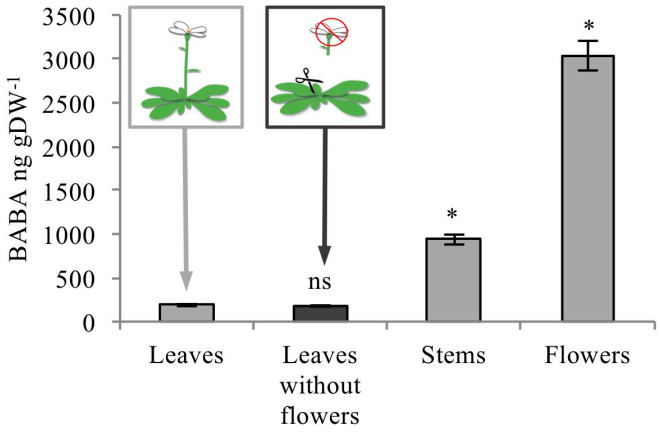
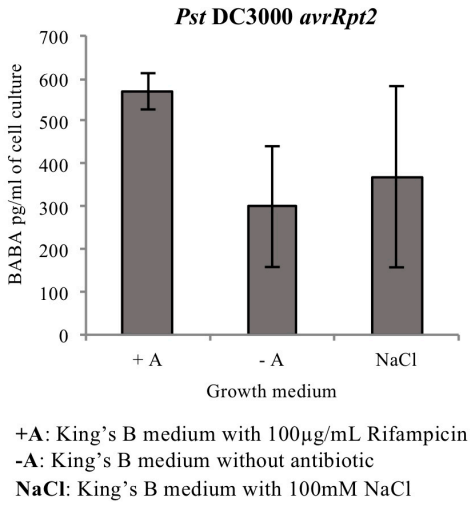


Figure S2. Data represent the mean ± SEM (n = 3 biological replicates, each replicate is a pool of 6 plants). Significant differences were determined by t-test by comparing to "leaves" samples, and shown as *=P ≤0.05. ns, no significant difference.

Supplementary Figure 3: BABA content in *Pseudomonas syringae* pv. tomato DC3000 *avrRpt2* cells.



+A: King's B medium with 100µg/mL Rifampicin
-A: King's B medium without antibiotic
NaCl: King's B medium with 100mM NaCl

Figure S3. The levels of BABA were measured after an overnight growth (OD_{600}=1). Bacteria were grown in King's B medium enriched with 100 µg/mL of rifampicin (+A), without antibiotics (-A) or supplemented with 100 mM NaCl (NaCl). Date are the mean ± SEM (*n* = 4 biological replicates). This experiment was repeated twice with similar results.

2. References

Baccelli, I., Mauch-Mani, B. (2016) Beta-aminobutyric acid priming of plant defense: the role of ABA and other hormones. Plant Mol. Biol. 91, 703-711

Baccelli, I. et al. (2017) The accumulation of β-aminobutyric acid is controlled by the plant's immune system. Planta, 246, 791-796

Balmer, A. et al. (2015) The 'prime-ome': towards a holistic approach to priming. Trends Plant Sci. 20, 443-452

Balmer, A. et al. (2018) Tricarboxylates induce defense priming against bacteria in *Arabidopsis thaliana*. Front. Plant Sci. 9, 1221

Bowling, S.A. et al. (1997) The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell, 9, 1573-1584

Breitkreuz, K. et al. (1999) Identification and characterization of GABA, proline and quaternary ammonium compound transporters from *Arabidopsis thaliana*. FEBS letters, 450, 280-284

Buchanan-Wollaston, V. et al. (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J. 42, 567-585

Cameron, R. K., Zaton, K. (2004) Intercellular salicylic acid accumulation is important for age-related resistance in Arabidopsis to *Pseudomonas syringae*. Physiol. Mol. Plant Pathol. 65, 197-209

Carella, P. et al. (2015) Some things get better with age: differences in salicylic acid accumulation and defense signaling in young and mature Arabidopsis. Front. Plant Sci. 5, 775

Chanda, B. et al. (2011) Glycerol-3-phosphate is a critical mobile inducer of systemic immunity in plants. Nature Genet. 43, 421

Clarke, J. D. et al. (2000) Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in Arabidopsis. Plant Cell, 12, 2175-2190

Cohen, Y. et al. (2016) BABA-induced resistance: milestones along a 55-year journey. Phytoparasitica, 44, 513-538

Cohen, Y. (2002) β-aminobutyric acid-induced resistance against plant pathogens. Plant Dis. 86, 448-457

Cohen, Y. et al. (1999) Local and systemic activity of BABA (DL-3-aminobutyric acid) against *Plasmopara viticola* in grapevines. Eur. J. Plant Pathol. 105, 351-361

Conrath, U. et al. (2015) Priming for enhanced defense. Annu. Rev. Phytopathol. 53, 97–119

Fischer, W. et al. (1995) Substrate specificity and expression profile of amino acid transporters (AAPs) in Arabidopsis. J. Biol. Chem. 270, 16315-16320

Fischer, W. et al. (2002) Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. Plant J. 29, 717-731

Fu, Z.Q., Dong, X. (2013) Systemic acquired resistance: turning local infection into global defense. Annu. Rev. Plant Biol. 64, 839-863

Girousse, C. et al. (1996) Water deficit-induced changes in concentrations in proline and some other amino acids in the phloem sap of alfalfa. Plant Physiol.111, 109-113

He, Y. et al. (2002) Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. Plant Physiol.128, 876-884

Jing, H.C., et al. (2007) Arabidopsis *CPR5* is a senescence-regulatory gene with pleiotropic functions as predicted by the evolutionary theory of senescence. J. Exp. Bot. 58, 3885-3894

Jung, H.W. et al. (2009) Priming in systemic plant immunity. Science, 324, 89-91

King, E. et al. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. Transl. Res. 44, 301-307

Kohler, A. et al. (2002) Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPR1/NIM1* gene in Arabidopsis. Plant Physiol. 128, 1046-1056

Lehmann, S. et al. (2010) *In planta* function of compatible solute transporters of the AtProT family. J. Exp. Bot. 62, 787-796

Lim, P. et al. (2007) Leaf senescence. Annu. Rev. Plant Biol. 58, 115-136

Luna, E. et al. (2014) Plant perception of β-aminobutyric acid is mediated by an aspartyl-tRNA synthetase. Nat. Chem. Biol.10, 450

Ma, X., et al. (2016) Dual and opposing roles of xanthine dehydrogenase in defense-associated reactive oxygen species metabolism in Arabidopsis. Plant Cell. tpc.15.00880

Mauch-Mani, B. et al. (2017) Defense priming: an adaptive part of induced resistance. Annu. Rev. Plant Biol. 68, 485-512

Morris, K. et al. (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J. 23, 677-685

Palanivelu, R. et al. (2003) Pollen tube growth and guidance is regulated by *POP2*, an Arabidopsis gene that controls GABA levels. Cell, 114, 47-59

Park, S.W. et al. (2007) Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science, 318, 113-116

Rivero, R.M. et al. (2007) Delayed leaf senescence induces extreme drought tolerance in a flowering plant. Proc. Natl. Acad. Sci.104, 19631-19636

Šašek, V. et al. (2012) β-aminobutyric acid protects *Brassica napus* plants from infection by *Leptosphaeria maculans*. Resistance induction or a direct antifungal effect?. Eur. J. Plant Pathol. 133, 279-289

Schmidt, R. et al. (2007) The amino acid permease *AAP8* is important for early seed development in *Arabidopsis thaliana*. Planta, 226, 805-813

Staswick, P. (2009) Plant hormone conjugation: a signal decision. Plant Signal. Behav. 4, 757-759

Steyn, W. et al. (2002) Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytol.155, 349-361

Tsai, C. et al. (2011) Priming for enhanced defense responses by specific inhibition of the Arabidopsis response to coronatine. Plant J. 65, 469-479

Thevenet, D. et al. (2017) The priming molecule β-aminobutyric acid is naturally present in plants and is induced by stress. New Phytol. 213, 552-559

Vlot, A. et al. (2008) Systemic acquired resistance: the elusive signal (s). Curr. Opin. Plant Biol.11, 436-442

Wu, C. et al. (2009) L-Glutamine inhibits beta-aminobutyric acid-induced stress resistance and priming in Arabidopsis. J. Exp. Bot. 61, 995-1002

Yoshida, S. et al. (2001) Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in *Arabidopsis thaliana*. Plant Cell Physiol. 42, 170-178

Yoshida, S. et al. (2002) Identification of a novel gene *HYS1/CPR5* that has a repressive role in the induction of leaf senescence and pathogen-defence responses in *Arabidopsis thaliana*. Plant J. 29, 427-437

Zhao, H. et al. (2010) A pleiotropic phenotype is associated with altered endogenous hormone balance in the developmentally stunted mutant (*dsm1*). J. Plant Biol. 53, 79-87

Chapter V- **General conclusions and perspectives**

General conclusions

The results presented herein widen our understanding of the priming phenomenon in *Arabidopsis thaliana* and proposes TCAs as new promising priming agents. Further conclusions are summarized in the following sections and in a more detailed approach in the proposed signaling pathways presented for each chapter (Chapter II, page 65; Chapter III, page 101; Chapter IV, page 133).

Citrate and Fumarate render plants more resistant

The results presented in this study show that both molecules have the capacity to induce priming in Arabidopsis against *Pst* DC3000 by potentiating plant immunity over distinct signaling cascades. These data corroborate the fact that citrate and fumarate act as modifiers of transcriptional signaling during the post-challenge primed stage. Intriguingly, both carboxylates did not induce any significant changes during the priming phase neither at the molecular or metabolic level nor phenotypically, suggesting that they act as classical priming agents thus not causing metabolic costs for primed plants. This finding renders them extremely attractive to be used as a priming agents in a more applied scale, given the fact that these small molecules are naturally present and quite abundant in plants.

Notably, whereas we only just begin to understand the role of TCA flux intermediates in priming of immune responses in plants, these molecules are thoroughly explored for their decisive role in mammalian immune responses. Perturbations in TCA flux intermediates is tightly linked to cancer pathogenesis (Sajani et al. 2017), and mutations in TCA flux enzymes (e.g. fumarase and succinate dehydrogenase (SDH)) is widely believed to be a major driver for causing the accumulation of so-called oncometabolites (Sciacovelli and Frezza, 2016). This evidently all highlights the eminent role of mitochondria as the "powerhouses" of cellular immune signaling (Mills et al. 2017). For instance, citrate is known to be a key regulator of inflammatory and antibacterial host responses in mammalian cells (Mills et al. 2017). Citrate is converted to itaconate, which then inhibits the SDH enzyme to block inflammatory responses, as well as alleviates bacterial-induced glyoxylate shunt. Moreover, citrate generates acetyl-CoA and oxaloacetate in mammalian cells, both needed for reactive oxygen species production. It remains elusive if similar mechanisms are playing a role during plant priming responses, but the knowledge described above might serve as inspiration to explore new areas in plant priming research.

Synthetic vs. endogenous BABA: complexity of kinetics

This study demonstrates that synthetic BABA has a high mobility between organs as well as being rapidly taken up by roots. On the other hand, endogenous BABA was induced only locally and not systemically from leaves to leaves or leaves to roots and *vice versa*. In conclusion, the local accumulation and perception of endogenous BABA in leaves during pathogen infection induced the generation of a yet unknown signal that could activate a full plant priming stage. On the other hand, synthetic BABA is systemically transported throughout the entire plant thereby mimicking a widespread defense inducing a full priming stage. The reason for the difference between the mobility of both natural and synthetic BABA needs to be further investigated. It could be speculated that endogenous BABA moves as a chemical conjugate form, which might not be detectable with the currently available chemical analysis method, or special uptake mechanisms are activated during application of synthetic BABA which promotes systemic movement. These results especially underline the need of distinct biological models and approaches for further elucidation of endogenous BABA signaling, which must be seen in a different light compared to mechanistic assays based on application of synthetic BABA.

Another relevant aspect presented in this thesis is the implication of synthetic as well as endogenous BABA during aging processes in Arabidopsis. The results demonstrate that both synthetic as well as endogenous BABA are implicated during senescence and putatively during age-related pathogen resistance. Nonetheless, further studies are required to elucidate the intricate signaling cascades which are modulated by both endogenous and synthetic BABA. The link of endogenous BABA to senescence might have a wider meaning especially in aging-related processes during different biological situations, e.g. oxidative stress, reproductive development, fruit formation and so one, which can be independent of plant priming responses and remain yet to be discovered.

Omics during the priming phase: quest for the bigger picture

In this thesis, the proteomic profile of the priming phase induced by BABA in Arabidopsis leaves and roots was discussed. Cellular responses to BABA on the yet scarcely explored roots are presented. Notably, after BABA treatment the GO family of stress-responses pathways showed the most altered changes. However, a general trend was that the most significant changes comparing mock and BABA treatment in both tissues comprised a reduction of protein abundances belonging to various pathways including cellular respiration, salicylic acid signaling, carbon/nitrogen balance and stress singling in response to BABA. This pictures as general reprogramming of the proteome,

and not the specific regulation of distinct pathways, resembling a "diversification" rather than a "focalization" strategy to be ready to combat possible biotic and abiotic stresses. To date most priming-related omics studies focused on the identification of putative priming signals in the form of overexpressed enzymes, receptors or transcription factors. This approach might be an elusive quest, if - as the data presented here suggest - a general resetting of the proteome is occurring, rather than the modulation of specific pathways. Moreover, as shown here, there is the additional layer of tissue-specificity, developmental conditions and genetic background, which make BABA-induced omics-alterations rather difficult to dissect. Intriguingly, BABA-induced changes in -omics were so far mainly examined in the light of priming for plant immunity, although with the here described link to senescence, it will be interesting to widen this concept towards BABA and possible developmental priming. For instance, proteomic reprogramming involving heat shock proteins was observed in pollen formation (Chaturvedi et al. 2016), which ultimately revealed developmental stages that were primed for the following reproductive phase. It might be speculated that BABA could play a dual role in immune priming as well as developmental priming, or that this molecule is even a decisive factor responsible for linking both cellular mechanisms.

Perspectives

Capacity of citrate and fumarate to protect plants against additional biotic and abiotic stressors

We observed here that citrate and fumarate were able to protect Arabidopsis against the virulent bacteria *Pst* DC3000 and both TCA components displayed a distinct mode of action. Additionally, when using the neucrotrophic fungus *P. cucumerina* we observed that only citrate was able to protect Arabidopsis against fungal infection. Moreover, citrate has been described to increase plant tolerance to aluminum toxicity by the formation of stable complexes with Al^{3+} that are not toxic to plants or that cannot enter the roots (Barone et al. 2008). It will be intriguing to test whether both carboxylates are active as priming inducers against other biotic stresses, herbivores or abiotic stress such as salt or drought. It also remains elusive which others defense pathways might be involved during priming induced by citrate or fumarate against *Pst* DC3000 or *P. cucumerina*, for example, callose deposition, ROS accumulation, secondary metabolites or even MAMP-triggered immunity.

Even more intriguing than the link to classical priming-related immune mechanisms would be the question of deeper understanding of TCA flux, intermediates or mitochondria in general during

plant immune priming. This goes further than the analysis single defense pathways, but to understand the cell's system biology during priming and primed phases. In mammalian cells, TCA intermediates are known to be involved in the regulation of histone and DNA methylation, as shown for succinate which exerts feedback inhibition of Ten-eleven translocation (TET) family of 5mC (DNA methylation at the 5 position of cytosine) hydroxylases (Mills et al. 2017), or fumarate which interferes with histone demethylation during *Candida albicans* infection. It remains elusive but highly intriguing whether TCA intermediates exert a similar role in the epigenome of plants. In general, the findings presented here highly encourage to pursue further research of plant immune priming to be focused towards TCA biochemistry and the role of mitochondrial signaling. In fact, recent immunology studies on T cell activation demonstrated the importance of "mitochondrial priming" during immune reponses (Geltink et al. 2017). Thereby, long-lasting immune memory is only achieved in T cells containing the receptor CD28, which promotes expression of a mitochondrial fatty acid oxidation enzyme. Future research might uncover analogous roles of plant mitochondria during defense priming.

Moreover, this study demonstrated that citrate and fumarate induced different changes in treated plants. Analyzing the capacity of additional TCA components to protect plants against biotic and abiotic stressors would help to enhance our knowledge on primary metabolism and priming defenses. If TCA intermediates would indeed exhibit broad priming activity against pathogens on both dicots and monocots, one could imagine to specifically target this pathway for crop enhancement. This could be done by either screening for natural compounds with similar chemical properties, or by applying TCA intermediates in field applications alone or as additive. Considering the efficient production of most TCA intermediates by fermentation (Liu et al. 2018), this could be a much cheaper alternative compared to synthetic compounds such as BABA that are produced via more complex synthetic routes. Future research should focus on identifying ideal pathogen/host combinations that might allow to harness the priming potential of citrate and fumarate and other TCA intermediates.

Signal dissection of synthetic and endogenous BABA and senescence

In our results, synthetic BABA was rapidly taken up by roots and transported to leaves. After incorporation, the levels of synthetic BABA substantially decreased after 2 days in root tissues and started to decrease after 6 days in leaves tissues. However, it is not known if synthetic BABA could be transformed into other compounds in plant tissues. In bacteria, BABA was described to be part of the synthesis pathways of bigger molecules such as the antibiotic incednine (Takaishi et al. 2012). In order to better understand the translocation or mobility and the catabolism of this molecule in

plant tissues it will be required to perform extensive biochemical studies using radioactively labeled version of BABA like C^{14}BABA.

It will be also of great interest to mine public databases in order to identify similar examples where mobile and catabolic difference are found between synthetic and endogenous molecules. This might help us to better understand the discrepancies. Moreover, considering that we are far from understanding signaling or biosynthesis pathways of BABA, a delighting and promising approach would be the use of plant reporter lines which are sensitive to synthetic or endogenous BABA molecules, and which can be used to dissect long-distance and local signaling in a non-disruptive manner. Such an approach was recently successfully applied to dissect calcium-based long-distance herbivore defense in Arabidopsis (Toyota et al. 2018). One could imagine to utilize designed synthetic BABA receptors (e.g. synthetic IBI1) optimized for bioluminescence resonance energy transfer (BRET), which would alter their conformation upon BABA binding and emit a BRET signal that can be detected using cooled charge-coupled (CCD) camera devices. Given the low endogenous level of BABA, this approach might not be promising, but not completely impossible considering the high dynamic range of modern CCD cameras and new high-signal BRET assays such as nanoBRET (Promega).

Understanding the cellular kinetics would also allow to elucidate the role of BABA during senescence. Using synthetic BABA as a model, the next close step is to analyze a set of senescence-associated gene such as *SAG12, SAG13* or *SAG21* (James et al. 2018; Chang et al. 2015) during natural senescence but also after synthetic BABA treatment. In addition, it will be intriguing to focus on specific BABA accumulation patterns on a cellular level during different developmental stages. Pollen development could provide a useful system, where defined cell types can be dissected and analyzed. Enhanced analytical methods such as single-cell mass spectrometry might one day help to define specific BABA patterns, once it is possible to detect low-molecular weight molecules with a limited intracellular abundance.

Nevertheless, results shown here suggest the use of BABA for senescence-related applications, such as fruit storage or enhanced flowering. For instance, BABA was shown to have a protective effect on sweet cherries during storage (Wang et al. 2015), linked to cell wall modifications. Hence future work should be directed towards studying particular aging-related biological systems, including approaches to counteract BABA-induced senescence.

Unknown networks of BABA regulation

In our preliminary results the proteomic profile induced by BABA in root tissues of Arabidopsis plants suggests that this organ could play an essential role during priming signaling. Considering this observation, it is suggested that additional *omics* analysis like a metabolome or a transcriptome could give a clearer picture of the role of this organ during the priming induced by BABA. As discussed above, BABA-induced changes are characterized by intricate network/system changes, thus only the combination of multiple biological conditions and datasets will help to understand the function of BABA during priming. General shotgun metabolomics and proteomics seem to be rather limited in helping answer these questions, although they are laying a basis for future research. It will be of great interest to focus next on posttranslational modifications induced by BABA, hence to generate methylation, phosphorylation or ubiquitination profiles. Given the dynamic nature of priming responses, more targeted omics approaches will further construct the yet unknown network of BABA signaling in plants.

References

Sajnani, K. et al. (2017) Genetic alterations in Krebs cycle and its impact on cancer pathogenesis. Biochimie, 135, 164-172.

Sciacovelli, M., Frezza, C. (2016) Oncometabolites: Unconventional triggers of oncogenic signalling cascades. Free Rad. Biol.Med. 100, 175-18.

Mills, E. et al. (2017) Mitochondria are the powerhouses of immunity. Nat. Immunol. 18, 488.

Chaturvedi, P. et al. (2016) Pollen proteomics: from stress physiology to developmental priming. Plant Reprod. 29, 119-132.

Barone, P. et al. (2008) Bacterial citrate synthase expression and soil aluminum tolerance in transgenic alfalfa. Plant Cell Rep. 27, 893-901.

Geltink, R. et al. (2017) Mitochondrial priming by CD28. Cell, 171, 385-397.

Liu, J. et al. (2018) Biological production of L-malate: recent advances and future prospects. World J. Microbiol. Biotechnol. 34, 6.

Takaishi, M. et al. (2012) A unique pathway for the 3-aminobutyrate starter unit from L-glutamate through β-glutamate during biosynthesis of the 24-membered macrolactam antibiotic, incednine. Org. Lett. 14, 4591-4593.

Toyota, M. et al. (2018) Glutamate triggers long-distance, calcium-based plant defense signaling. Science, 361, 1112-1115.

James, M. et al. (2018) *SAG12*, a major cysteine protease involved in nitrogen mobilization during senescence for seed production in *Arabidopsis thaliana*. Plant Cell Physiol. 59, 2052-2063.

Chang, J. et al. (2015) Early differential gene expression profiling of harvest-induced senescence in detached Arabidopsis plants. Acta Physiol. Plant. 37, 165.

Wang, L. et al. (2015) Effect of β-aminobutyric acid on cell wall modification and senescence in sweet cherry during storage at 20° C. Food Chem.175, 471-477.

Appendix

Acknowledgements

I would like add a few words of appreciation for the following people who have supported me during the course of this project.

First, I would like to thank my thesis supervisor, Prof. Brigitte Mauch-Mani for giving me the opportunity to conduct this project in her team and to give me the chance to develop myself scientifically and as well personally. I want to express my highest appreciation for her encouragement, for being present and supporting my ideas and my growth. I would also like to acknowledge Prof. Daniel Croll, Dr. Victor Flors and Dr. Gaëtan Glauser for taking time to be part of my committee; I want to especially thank Dr. Victor Flors and Dr. Gaëtan Glauser for all the precious advises given to me along this adventure.

I would like to thank Dr. Victoria Pastor (Universitat Jaume I, Spain) for the valuable work we did together and for the scientific as well as personal encouragement given to me. I also want to share my acknowledgements to the members of the NPAC (University of Neuchâtel) for their technical support. During my PhD, I had the pleasure to work with some amazing colleagues, such as Guillaume Gouzerh, Damien Thevenet and Ivan Baccelli, all of them I want to express my gratitude for all the moments we lived through in the laboratory.

Special thanks goes to everybody who supported me through their great friendship, namely Noemie, Sophie, Empar, Patricia, Carla, Marie, Fares, Francesco, Alba, Jonas and Chiara, also a big thanks to the past and present lab members as well as all the kind people from the other labs in Neuchâtel. I leave this university with my mind full of great souvenirs of moments shared with all of you. Many thanks as well my dear "Neuchatel" friends.

I would like to thanks my beloved family for playing a big role in my live and providing me endless support during every path I went through. Special thanks to Jo and Philippe for selflessly having once created a new home for me when I needed it most.

Finally, I have not enough words of "thanks" to the person who supported me in all the aspects during these years, thanks to my husband Dirk for his patience and for spending this experience hand in hand together with me. He was backing me during the ups and downs I felt during the last years of this passionate "roller coaster" called thesis.

Accumulation patterns of endogenous β-aminobutyric acid during plant development and defense in *Arabidopsis thaliana*

Andrea Balmer, Brigitte Mauch-Mani, Ivan Baccelli.

Accepted in Plant Biology. Submission date Oct 2018

We have recently discovered that **β**-aminobutyric acid (BABA), a molecule known for its ability to prime defenses in plants, is a natural plant metabolite. However, the role played by endogenous BABA in plants is currently unknown. In this study we investigated the systemic accumulation of BABA during pathogen infection, the levels of BABA during plant growth and development, and analyzed mutants possibly involved in BABA transport or regulation. BABA levels were quantified by UHPLC-MS according to a previously developed protocol. Systemic accumulation of BABA was determined by analyzing non-infected leaves and roots after localized infections with *Plectosphaerella cucumerina* or *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 *avrRpt2*. The levels of BABA were also quantified in different plant tissues and organs during normal plant growth, and in leaves during senescence. Mutants affecting amino acid transport (*aap6, aap3, prot1*, and *gat1*), **γ**-aminobutyric acid levels (*pop2*), and senescence/defense (*cpr5-2*) were analyzed. BABA was found to accumulate only locally after bacterial or fungal infection, with no detectable increase in non-infected systemic plant parts. In leaves, the BABA content increased during natural and induced senescence. Reproductive organs were found to show the highest levels of BABA, and the mutant *cpr5-2* was found to produce constitutively high levels of BABA. Synthetic BABA is highly mobile in the receiving plant, whereas endogenous BABA appears to be produced and accumulated locally in an organ-specific way. We discuss a possible role for BABA in the age-related resistance, and propose a comprehensive model for endogenous and synthetic BABA.

Tricarboxylates induce defense priming against bacteria in *Arabidopsis thaliana*

Balmer, A., Pastor, V., Glauser, G., & Mauch-Mani, B.

Frontiers in plant science. publication date Oct 2018

Exposure of plants to biotic stress results in an effective induction of numerous defense mechanisms that involve a vast redistribution within both primary and secondary metabolisms. For instance, an alteration of tricarboxylic acid (TCA) levels can accompany the increase of plant resistance stimulated by various synthetic and natural inducers. Moreover, components of the TCA flux may play a role during the set-up of plant defenses. In this study, we show that citrate and fumarate, two major components of the TCA cycle, are able to induce priming in Arabidopsis against the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000. Both citrate and fumarate show no direct antimicrobial effect and therefore enhanced bacterial resistance found in planta is solely based on the induction of the plant defense system. During the priming phase, both TCA intermediates did not induce any changes in transcript abundances of a set of defense genes, and in phytohormones and camalexin levels. However, at early time points of bacterial challenge,

citrate induced a stronger salicylic acid and camalexin accumulation followed later by a boost of the jasmonic acid pathway. On the other hand, adaptations of hormonal pathways in fumarate-treated plants were more complex. While jasmonic acid was not induced, mutants impaired in jasmonic acid perception failed to mount a proper priming response induced by fumarate. Our results suggest that changes in carboxylic acid abundances can enhance Arabidopsis defense through complex signaling pathways. This highlights a promising feature of TCAs as novel defense priming agents and calls for further exploration in other pathosystems and stress situations.

Signs of silence: Small RNAs and antifungal responses in *Arabidopsis thaliana* and *Zea mays*

Balmer, A., De Paoli, E., Si-Ammour, A., Mauch-Mani, B., & Balmer, D.

In book: Plant EngineeringPublisher: INTECH. publication date Nov. 2017

Plant small RNAs (sRNAs) are pivotal regulators of gene expression, which are crucial in maintaining genome integrity and flexibility during development, abiotic and biotic stress responses. Current evidence suggests that sRNAs might be inherent to the sophisticated plant innate immune system battling bacteria. However, the role of sRNAs during antifungal plant defenses is less clear. Therefore, this chapter investigates the sRNA-mediated plant antifungal responses against the hemibiotrophic fungi *Colletotrichum higginsianum* and *Colletotrichum graminicola* in their respective compatible hosts *Arabidopsis thaliana* and *Zea mays*. A phenotypic and metabolomic analysis of *A. thaliana* sRNA mutants in response to *C. higginsianum* infection was performed, showing a hormonal and metabolic imbalance during fungal infection in these plants. To find whether fungal-induced sRNA could directly regulate defense genes in an agricultural important plant model, the expression of maize miRNAs in response to *C. graminicola* leaf and root infections was investigated. The results revealed the tissue-specific local and systemic adaptation of the miRNA transcriptome, where only a few miRNAs were targeting defense pathways. The general picture presented here points towards a role of sRNAs as fine-tuners of genetic and metabolomic defense response layers. This chapter also further discusses the potential of utilizing sRNA-based fungal control strategies.

The priming molecule β-aminobutyric acid is naturally present in plants and is induced by stress

Thevenet, D., Pastor, V., Baccelli, I., Balmer, A., Vallat, A., Neier, R., Glause G., & Mauch-Mani, B.

New Phytologist. publication date Oct 2016

The defense system of a plant can be primed for increased defense, resulting in an augmented stress resistance and/or tolerance. Priming can be triggered by biotic and abiotic stimuli, as well as by chemicals such as **β**-aminobutyric acid (BABA), a nonprotein amino acid considered so far, a xenobiotic. Since the perception mechanism of BABA has been recently identified in *Arabidopsis thaliana,* in the present study we explored the possibility that plants do synthesize BABA. After developing a reliable method to detect and quantify BABA in plant tissues, and unequivocally separate it from its two isomers **α**- and **γ**-aminobutyric acid, we measured BABA levels in stressed and nonstressed *A. thaliana* plants, and in different plant species. We show that BABA is a natural

product of plants and that the endogenous levels of BABA increase rapidly after infection with necrotrophic, biotrophic and hemibiotrophic pathogens, as well as after salt stress and submergence. Our results place the rise in endogenous BABA levels to a point of convergence in plant stress response and provide biological significance to the presence of a receptor in plants. These findings can explain the extremely widespread efficacy of BABA and open the way to unravel the early steps of priming.

The 'prime-ome': Towards a holistic approach to priming

Balmer, A., Pastor, V., Gamir, J., Flors, V., & Mauch-Mani, B.

Trends in Plant Science. publication date Apr 2015

Plants can be primed to respond faster and more strongly to stress and multiple pathways, specific for the encountered challenge, are involved in priming. This adaptability of priming makes it difficult to pinpoint an exact mechanism: the same phenotypic observation might be the consequence of unrelated underlying events. Recently, details of the molecular aspects of establishing a primed state and its transfer to offspring have come to light. Advances in techniques for detection and quantification of elements spanning the fields of transcriptomics, proteomics, and metabolomics, together with adequate bioinformatics tools, will soon allow us to take a holistic approach to plant defense. This review highlights the state of the art of new strategies to study defense priming in plants and provides perspectives towards ‘prime-omics'.

Preparing to fight back: Generation and storage of priming compounds

Pastor, V., Balmer, A., Gamir, J., Flors, V., & Mauch-Mani, B.

Frontiers in Plant Science. publication date Jun 2014

Immune-stimulated plants are able to respond more rapidly and adequately to various biotic stresses allowing them to efficiently combat an infection. During the priming phase, plant are stimulated in absence of a challenge, and can accumulate and store conjugates or precursors of molecules as well as other compounds that play a role in defense. These molecules can be released during the defensive phase following stress. These metabolites can also participate in the first stages of the stress perception. Here, we report the metabolic changes occuring in primed plants during the priming phase. **β**-aminobutyric acid (BABA) causes a boost of the primary metabolism through the tricarboxylic acids (TCA) such as citrate, fumarate, (S)-malate and 2-oxoglutarate, and the potentiation of phenylpropanoid biosynthesis and the octodecanoic pathway. On the contrary, *Pseudomonas syringae* pv tomato (*Pst* AvrRpt2) represses the same pathways. Both systems used to prime plants share some common signals like the changes in the synthesis of amino acids and the production of SA and its glycosides, as well as IAA. Interestingly, a product of the purine catabolism, xanthosine, was found to accumulate following both BABA- and PstAvrRpt2-treatement. The compounds that are strongly affected in this stage are called priming compounds, since their effect on the metabolism of the plant is to induce the production of primed compounds that will help to combat the stress. At the same time, additional identified metabolites suggest the possible defense pathways that plants are using to get ready for the battle.