Environmental influences and genetic regulation on systemic acquired resistance

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Summary

Systemic acquired resistance (SAR) is induced by a localized leaf inoculation with pathogenic microbes and constitutes a state of elevated, broad spectrum disease resistance to microbial pathogen within the entire plant foliage. Activation of SAR enables the plant to respond more quickly and vigorously to subsequent microbial attack (SAR priming). For SAR to happen, a signal must be generated and translocated to the leaves distal to the site of pathogen infection. In the last decade, a number of potential SAR signals such as non-protein amino acid pipecolic acid (Pip), methyl salicylate (MeSA), glycerol-3-phosphate (G3P), dihydroabetinal (DA), DIR1 and azelaic acid (AzA), have been proposed. Pip is considered as an endogenous regulator of SAR and plants defective in production of Pip (*ald1*) are compromised in SAR. SAR establishment and the associated priming of defense responses are regulated by Pip. Exogenous Pip application strongly increases pathogen resistance in wild-type and in *ald1* plants and promotes the plants into a primed SAR-like state (Pip-priming). Moreover, Pip positively regulates biosynthesis of another immune regulator salicylic acid (SA) and primes the plants for accumulation of phytoalexin camalexin and expression of defense genes such as PR1. SA, itself is not a mobile signal, however its accumulation in systemic leaves is required for SAR establishment. Pip orchestrates SAR via salicylic acid (SA)-dependent and -independent pathways. Our research group has recently discovered that Pip is further N-hydroxylated to a SAR-activating metabolite N-hydroxypipecolic acid (NHP) by flavin-dependent monooxygenase1 (FMO1). NHP is the main regulatory metabolite which mediates SAR against bacterial and oomycete pathogens and NHP induces expression of defense genes and it primes the plants for effective defense activation towards pathogens.

In this thesis, we investigated whether and to which extend the putative SAR signals (G3P, MeSA, AzA, and DIR1) interact with Pip in resistance induction and contribute to SAR establishment in an Arabidopsis-*Pseudomonas syringae* interaction. To address these questions, we first examined the SAR establishment in a series of Arabidopsis mutants impaired in the production of G3P (*gli1-1* and *gly1-1*), MeSA (*bsmt1-1*), DIR1 (*dir1-1*), and also plants defective in azelaic acid signaling pathway (*azi1-2*). We observed that irrespective to the time of pathogen attack, SAR is established in the putative SAR signaling mutants, indicating that in contrast to previous reports from other publications, G3P, AzA, MeSA, and DIR1 are not central SAR regulators under our laboratory conditions. In addition, irrespective to the time of infiltration, virulent and avirulent *Pseudomonas syringae* strains induce SAR in all SAR signaling mutant lines. *gli1-1* mutant lines showed a weaker SAR than wild-type, suggesting that GLI1 is required for full SAR establishment upon morning and evening infiltration of *Psm*.

Furthermore, irrespective to the time of *Psm* infiltration (morning or evening), all SAR signaling mutants accumulate Pip and SA (as two main SAR regulators) in their local (infiltrated) and systemic (non-infiltrated) leaves. *gly1-1* mutant lines accumulate local and systemic Pip to significantly lower level than wild-type. Therefore, a connection between G3P and Pip biosynthesis is likely to exist. To further understand if there is a connection between putative signals and Pip signaling, we tested how SAR signaling mutants (*gli1-1*, *gly1-1*, *azi1-2*, *dir1-1*, and *bsmt1-1*) respond to exogenous Pip (Pip-induced resistance (Pip-IR)). We observed that these was no contribution of MeSA, AzA, DIR1, and G3P to Pip-IR and Pip-IR developed similarly in all SAR signaling mutants and wild-type plants. Next, we examined whether there is an interplay between SA and each individual SAR signals. All SAR signaling mutants also positively respond to exogenous SA, indicating that SA acts downstream of MeSA, AzA, DIR1, and G3P for induction of resistance towards *Psm*.

In second and third chapters of this study, we investigated the role of environmental factors (light and nitrogen supply) on Pip biosynthesis, defense priming, and SAR. We confirmed that SAR is a light-dependent process. Pip biosynthesis and its regulatory role in defense priming are positively influenced by light. In addition, SA biosynthesis and SA-induced PR1 expression depend on a light-related factor. Although the length of Light applied to Arabidopsis plants after *Psm* infiltration negatively correlates with the basal bacterial growth, the production of defense metabolites (Pip and SA) are positively and directly regulated by light. Similar to light effect, adequate nitrogen (N) supply in plants, stimulates the pathogen-induced Pip accumulation and is a prerequisite for effective SAR induction. It is more likely that nitrogen supply positively affects the systemic accumulation of *Psm*-induced SA. In contrast, a higher concentration of nitrate causes a reduced level of SA at the site of pathogen attack. Our study provides the framework for future studies on how plants adapt SAR and defense priming responses to changing environmental conditions.

Contents

1 Introduction

1.1 Plant-pathogen interactions

Plants are main life-energy producers, able to convert light energy into chemical energy and oxygen for consumption of all living organisms on earth. Living in a dynamic atmosphere, plants are exposed to variable biotic and abiotic stresses. Abiotic stresses include changes in light and temperature, nutrient deprivation, drought, and salinity. Biotic stresses caused by living organisms includes interacting with other neighboring plants, pathogenic viral and microbial factors (bacteria, viruses, fungi, oomycetes, etc.), pests and herbivores (insects, mammals, etc.) (Mansfield et al., 2012; Dean et al., 2012; Kamoun et al., 2015; Scholthof et al., 2011). Depending on the mode of feeding, plant pathogens are classified as biotrophs, necrotrophs, and hemibiotrophs. While biotrophic pathogens feed on living plant cells without causing major injury to the cells, necrotrophs kill the living cells and consume nutrients released from injured cells. Hemibiotrophs have an initial biotrophic phase which is followed by a necrotrophic phase. Based on different lifestyles and mode of reproduction, each pathogen employs a unique tactic to enter the plant for manipulation. Outcome of a plant and microbe interaction can be a *compatible* response (which is an interaction that causes disease on a host plant), an *incompatible* response (which is usually associated with hypersensitive responses in a host), and a *non-host* interaction (in which a normally virulent pathogen on a specific host is unable to infect the other different host) (Thordal-Christensen, 2003; Glazebrook, 2005; Göhre and Robatzek, 2008). Unlike animals, plants are sessile and lack specialized mobile immune cells, nevertheless plants rely on their "innate" immune machinery and evolve highly specific constitutive and inducible defense mechanisms to detect and respond to invading organisms at local side of attack (basal defense responses) and systemically throughout the whole plant foliage (systemic acquired resistance) (Jones and Dangl, 2006; Spoel and Dong, 2012).

Basal defense responses (innate immunity) Plant basal resistance (innate immunity) is the first line of inducible defense responses that protect plants against entire groups of pathogens. This is due to the presence of plants preformed physical and chemical barriers which restrict the pathogen attachment, invasion and, colonization. The first-line defensive physical and structural barriers in plants include cell wall and cuticle. All plant cells consist of a primary cell wall and a secondary cell wall which is developed inside the primary cell wall after cell stops growing. Plant cell walls are composed of high molecular-weight polysaccharides and highly glycosylated proteins and lignin which all make a rigid barrier to pathogen invasion (Somerville et al., 2004; Nawrath, 2002b). In addition, as a part of induced defense mechanisms, plant cells also respond to microbial attack through deposition of cell wall appositions (callose deposition), so-called papillae which are formed between plasma membrane and inside cell wall to impede cellular penetration at the site of infection (Voigt, 2014).

The cuticle is a waxy layer covering outer surface of aerial epidermis parts of plants such as leaves, flowers, and fruits. The structural component of the cuticle is cutin which is an insoluble polymer composed of hydroxy and hydroxyepoxy fatty acids and embedded in a mixture of non-polar lipids called waxes. Beside its role in plant transpiration and protection prior to irradiation and mechanical damages, the cuticle protects the plant against herbivore and pathogen attacks (Kolattukudy, 1985; Yeats and Rose, 2013; Nawrath, 2002b). For a phytopathogen, entering into the host tissue is a vital step in causing infection and establishing virulence. Therefore, it is important to bypass the mechanical barriers either by direct penetration into the plant or by entry through physical injuries or natural openings such as stomata and/or hydathodes (Muthamilarasan and Prasad, 2013). To penetrate into host tissue, certain phytopathogenic fungi produce cutinases which are cutin-degrading enzymes (Kolattukudy, 1985; Ryan and Jagendorf, 1995), while other fungi might require a factor from the surface wax of their host to stimulate fungal penetration processes (Tsuba et al., 2002; Hansjakob et al., 2011; Mario et al., 2014). Penetrating pathogens, subsequently encounter the chemical barriers of plant immune responses.

In general, plant chemicals consist of *primary metabolites* such as sugars, proteins, and amino acids which are directly involved in plant growth, developmental, and reproduction stages and *secondary metabolites* which contribute to plant defense mechanisms (Piasecka et al., 2015). Based on their mode of biosynthesis and action, phytochemicals are categorized as *phytoanticipins* (which are produced and stored constitutively in vacuoles and translocate to the site of pathogen detection) and *phytoalexins* (which are synthesized to respond to the infection) (Piasecka et al., 2015; Dixon, 2001). Phytoanticipines include saponins (such as terpenoids), amino acid-derived glucosinolates (aliphatic-, indolic-, and benzyl-glucosinolates), cyanogenic, and benzoxazinone glucosides. Hydrolysis of glucosinolates by myrosinases lead to release of toxic antimicrobial products such isothiocyanates and nitriles (Halkier and Gershenzon, 2006; Piasecka et al., 2015). Phytoalexins are defined as low molecular antimicrobial compounds including camalexin (in Brassicaceae), terpenoids, and phenylalanine-derived phytoalexins (Ahuja et al., 2012; Piasecka et al., 2015; Dixon, 2001).

Camalexin: the major phytoalexin in Arabidopsis Camalexin (3-thiazol-2'-yl-indol) is a major tryptophan-derived phytoalexin in Arabidopsis which accumulates in response to infection with various *Pseudomonas syringae* strains as well as fungi and oomycetes (Großkinsky et al., 2012). In addition, several abiotic stresses such as heavy metal ions treatment and UV-B irradiation triggers camalexin accumulation (Großkinsky et al., 2012). Biosynthesis of camalexin involves several cytochrome P450 enzymes and it is connected to the biosynthesis pathway of glucosinolates and the phytohormone IAA (auxin) via the intermediate metabolite indole-3-acetaldoxime (IAO_x) (Glawischnig et al., 2004). IAO_x is further converted to indole-3-acetonitrile (IAN) in reaction catalyzed by P450 (CYP71A13) (Glawischnig et al., 2004). The final step of camalexin biosynthesis is mediated by CYP71B15 (PAD3), which catalyzes the decarboxylation of dihydrocamalexic acid (DHCA) to form camalexin (Nafisi et al., 2007; Schuhegger et al., 2006) (Figure 1.1).

The synthesis of camalexin is regulated by a mitogen-activated protein kinases tha involves MPK3/MPK6 cascade. Activation of MPK3/MPK6 by upstream MAPK kinase (MAPKK) or MAPKK kinase (MAPKKK) can sufficiently induce camalexin synthesis in the absence of pathogens (Ren et al., 2008). The mode of action of camalexin has been investigated by membrane integrity assays and it was shown that camalexin disrupts the integrity of bacterial membranes and its toxicity is a consequence of pathogen

membrane disruption. For instance, it inhibits prolin uptake by *Pseudomonas syringae* and causes ion leakage (Rogers et al., 1996). Arabidopsis CAMALEXIN (PHYTOALEXIN) DEFICIENT (PAD) mutants did not affect the plants to restrict the growth of avirulent *Pseudomonas syringae* strains. However, they enhanced susceptibility towards virulent strains (Glazebrook and Ausubel, 1994). Mutations in PAD1, PAD2, and PAD4 cause increased susceptibility to *Psm* E4326, whereas mutations in PAD3 and PAD5 have no effect on resistance to *Psm* E4326 (Glazebrook et al., 1997; Rogers et al., 1996).

Induced defense responses Besides plants basal constitutive defense layers and pre-invasive antimicrobial metabolites, a pathogen faces the second obstacles which are referred to plant inducible defense mechanisms. Inducible defense mechanisms are activated upon recognition of non-self general and specific microbial components known as elicitors and effectors which lead to activation of PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI), respectively.

1.1.1 PAMP-triggered immunity (PTI)

PTI is activated upon recognition of pathogen- or microbial-associated molecular patterns (PAMPs/MAMPs) or host-derived damage-associated molecular patterns (DAMPs) by plant pattern-recognition receptors (PRRs) located at the cell surface (Zipfel, 2008; Bittel and Robatzek, 2007; Altenbach and Robatzek, 2007; Lotze et al., 2007). PAMPs/MAMPs are indispensable, highly conserved among classes of pathogens and thus, they are not easily modified by mutagenesis. The well-recognized MAMPs are flagellin and lipopolysaccharides (LPS) from bacteria, chitin and ergosterol from fungi, and cell wall components such as heptaglucoside from oomycetes (Boller, 1995; Dow et al., 2000; Zipfel and Felix, 2005; Granado et al., 1995; Felix et al., 1993). Pattern-recognition receptors (PRRs) are classified into two groups: *Receptor kinases (RKs)* and *receptor-like proteins (RLPs)* (Shiu and Bleecker, 2001). A plant RK contains a ligand-binding ectodomain (ECD), a single-pass transmembrane domain, and a cytoplasmic kinase domain, whereas an RLP is essentially an RLK lacking a cytoplasmic kinase domain. PRRs are more divided into subfamilies which are based on special domains and ECD motifs such as Leucine-Rich Repeat (LRR) domain (binds preferentially to proteins and peptides), Lysine Motifs (LysM) (binds to carbohydrate-based ligands), Lectin domain (bind extracellular ATP or bacterial lipopolysaccharides (LPS)), or Epidermal Growth Factor (EGF)-like domain (recognizes plant cell-wall derived oligogalacturonides). Types of these domains determine the ligand-binding specificity (Tang et al., 2017; Couto and Zipfel, 2016). Recognition of MAMPs/PAMPs by PRRs is associated with several PTI responses including rapid ion fluxes across the plasma membrane (increased intracellular Ca^{+2}), MAP kinase (MAPK) activation, production of reactive-oxygen species (ROS) and oxidative burst, rapid changes in defense gene expression, callose deposition and cell wall reinforcement, stomatal closure, production of salicylic acid (SA), and accumulation of phytoalexins (Tena et al., 2011; Felix et al., 1999; Zipfel, 2008; Asai et al., 2002; Navarro et al., 2004; Kaku et al., 2006; Mishina and Zeier, 2007b; Grant et al., 2000).

The best two well-known MAMPs are flagellin and elongation factor TU (EF-TU). Flagellin is the main building block of eubacterial flagella. The highly conserved N-terminal domain of flagellin, a 22 amino acid long stretch peptide (flg22), is recognized by FLAGELLIN SENSING 2 (FLS2) which is a plasma

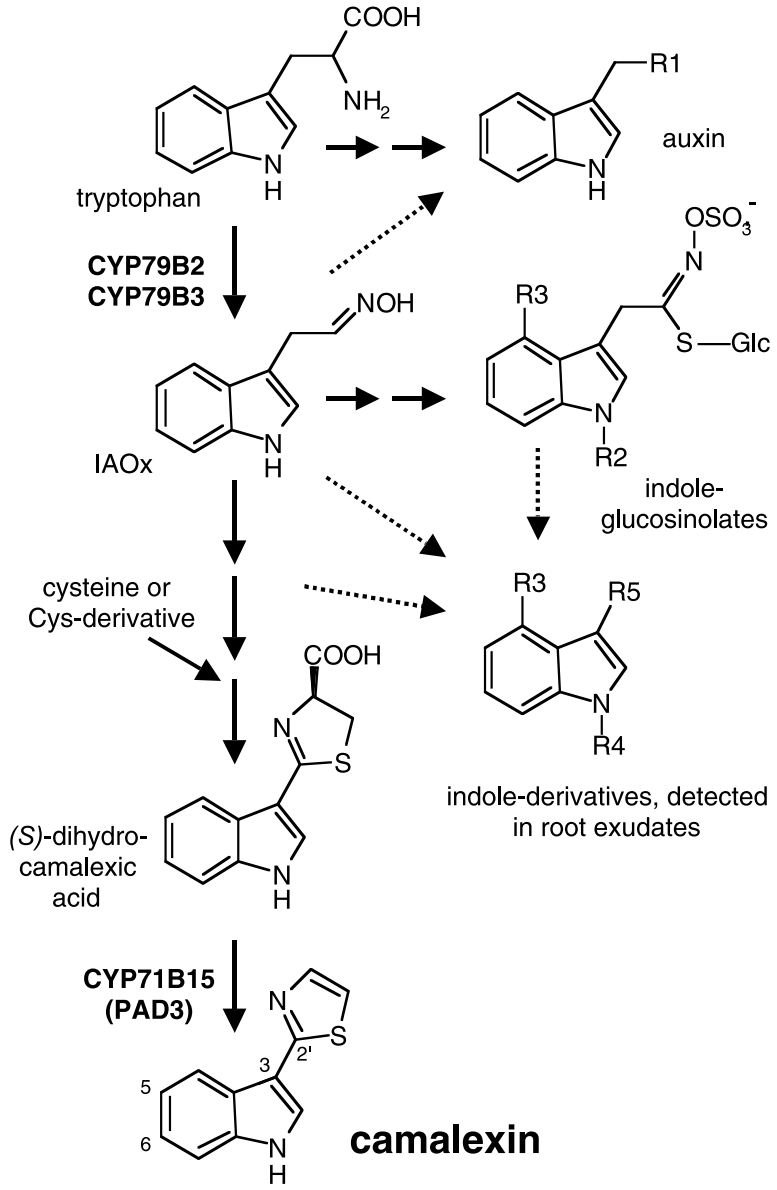

Figure 1.1: Camalexin biosynthesis pathway: The biosynthesis of camalexin and related indolic compounds in Arabidopsis thaliana. CYP79B2 and CYP79B3 catalyze the conversion of tryptophan to indole-3-acetaldoxime (IAOx), an intermediate of indole glucosinolate and camalexin biosynthesis. The last step in camalexin synthesis is the decarboxylation of dihydrocamalexic acid by CYP71B15 (PAD3). IAOx probably is also a precursor of indole-3-carbaldehyde (R5 = CHO), indole-3-carboxylic acid (R5 = COOH), and indole-3-aceto-nitrile (R5 = CH_2CN) derivatives, as well as auxin (IAA, the predominant auxin, R1 = COOH; indole propionic acid, R1 = CH_2COOH; indole butyric acid, R1 = $(CH_2)_2COOH$). R2 = H or OCH_3; R3 = H, OCH_3; R4 = H, CH_3, or OCH_3. (Glawischnig, 2007)

membrane localized, Leucine-Rich Repeat-receptor kinase (LRR-RK) in Arabidopsis (Gómez-Gómez and Boller, 2000; Felix et al., 1999; Zipfel et al., 2004). Mutant *fls2* plants showed susceptibility toward *Pseudomonas syringae pv. tomato* DC3000 (*Pst*) upon bacterial inoculation onto leaf surface (but not into leaf tissue) (Bittel and Robatzek, 2007). Upon ligand binding, FLS2 recruits BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) as co-receptor, to form a hetero-dimer resulting in rapid phosphorylation of both FLS2 and BAK1 and activation of downstream defense responses via MAPK signaling cascades (Chinchilla et al., 2007; Asai et al., 2002). BAK1 is an LRR-RK which acts as co-receptor with BRASSINOSTEROIDS INSENSITIVE 1 (BRI1) for Brassinosteroids perception (plants growth-promoting steroid hormones) and signal transduction leading to plant growth (Kim and Wang, 2010). Arabidopsis *bak1* mutants do not interfere flg22 binding, however they show abnormal flg22- triggering responses (Chinchilla et al., 2007).

Another well-characterized MAMP is bacterial elongation factor thermo unstable (EF-Tu) which is recognized by Arabidopsis LRR-RK type receptor termed as EF-Tu receptor (EFR). Arabidopsis plants specifically recognize the N terminal of this protein, an N-acetylated peptide comprising 18 amino acids, termed elf18 which is an active elicitor of defense responses. Like FLS2, EF-Tu relies on BAK1 for activation of defense signaling after perception of elf18 such as oxidative burst and ethylene biosynthesis (Kunze, 2004). Recognition of EF-Tu is restricted to *Brassicaceae*. Arabidopsis *efr* mutants show enhanced susceptibility to the bacterium *Agrobacterium tumefaciens* and therefore, EF-Tu-induced responses reduce transformation by *Agrobacterium* (Zipfel et al., 2006).

1.1.2 Effector-triggered immunity (ETI)

Pathogens must circumvent pattern-triggered immunity (PTI) to successfully manipulate host cells. Therefore, phytopathogens secret specific type of elicitors, called effectors, into cytoplasms of host cells where they play dual roles, either targeting plant immunity signaling pathways (inhibit PTI) or inducing host genes to enhance plant susceptibility (activate effector-triggered susceptibility (ETS)) (Jones and Dangl, 2006). Gram-negative bacterial such as *Pseudomonas syringae* pathogens acquire a type III secretion system (TTSS) to deliver effectors directly into plant host cells (Buttner and He, 2009). *P. syringae* incapable of delivering effectors into host cells are non-pathogenic, thus TTSS effectors are required for pathogenicity.

TTSS effectors might target PTI through direct interaction with receptor-like kinases and/or PAMP receptors to interfere with downstream defense signaling, for instance effector AvrPto (AvrPto1) from *Pst* which directly interact with receptor-like kinases FLS2 and EFR to block PAMP/MAMP-induced defense responses (Zhou and Chai, 2008; Grant et al., 2006). To enhance host susceptibility, effectors might interfere with plant hormones, for example *P. syringae* AvrPtoB effector modulates abscisic acid (ABA) signaling and AvrRpt2 enhances auxin accumulation in Arabidopsis (Torres-Zabala et al., 2007; Chen et al., 2007). Also, *Psm* effector HopI1 suppresses the production of salicylic acid by modifying host chloroplast (Jelenska et al., 2007). The action of effectors renders the plant to be susceptible to infection and is called effector-triggered susceptibility (ETS) (Jones and Dangl, 2006).

In response to pathogen effector proteins, plants have evolved nucleotide binding leucine-rich repeat (NB-LRR) resistance proteins (R proteins), to directly or indirectly monitor the presence of effectors. Plant R proteins are conserved and they are similar to mammalian NOD-Like Receptors (NLRs), which are generally characterized by the presence of nucleotide-binding and leucine-rich repeat (NB-LRR) domains. Based on N-terminus motifs of these proteins, there are two types of NLRs in plants: i) coiled-coil (CC) and ii) toll-like interleukin (TIR). In Arabidopsis and *Pseudomonas syringae* pathosystem, an example for TIR-R protein/Avr protein interaction is RPS4/AvrRps4 and examples of CC-R protein/Avr protein interactions are RPM1/AvrRpm1 and AvrB, RPS5/AvrPphB, and RPS2/AvrRpt2. Arabidopsis gene ENHANCED DISEASE SUSCEPTIBILTY 1 (EDS1) and its interaction partners PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101) have been shown to be required for TIR-NB-LRRs such as RPP2, RPP4, RPP5, RPP21, and RPS4 (Aarts et al., 1998). EDS1 regulates these genes to confer resistance to the biotrophic oomycete *Peronospora parasitica* and to *Pseudomonas* bacteria expressing the avirulence gene AvrRps4 (Aarts et al., 1998). In contrast, plasma-membrane localized protein NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) is required for the function of CC-NB-LRR receptors such as RPS2, RPS5, and RPM1 to mediate resistance against *Pseudomonas syringae pv. tomato* DC3000 containing cognate effector genes AvrRpt2, AvrPphB, and AvrRpm1 respectively (Aarts et al., 1998; Coppinger et al., 2004).

Most NB-LRR either detect the effectors *directly* through ligand-receptor binding or *indirectly* through the interaction of effector by another host proteins (guard or decoy model). As an example for indirect effector detection, NB-LRR proteins RPM1 and RPS2, which are R proteins for recognition of *Psm* effectors AvrRpm1/AvrB and AvrRpt2 respectively, are associated with a membrane-localized protein RPM1-INTERACTING 4 (RIN4) (Grant et al., 2006). RPS2 and RPM1 are inactive when linked to RIN4. Cleavage of RIN4 by AvrRpt2 effector releases RPS2 for defense activation and AvrRpm1 and AvrB trigger resistance of RPM1 by inducing phosphorylation of RIN4 (Zhou and Chai, 2008; Schreiber et al., 2016). As a consequence of host R protein and effector interaction, a robust immune response will be established termed as effector-triggered immunity (ETI). Activation of ETI is associated with several immune responses such as accumulation of SA and pathogenesis-related (PR) proteins, increased levels of reactive oxygen species (ROS), and localized programmed cell death which is also called hypersensitive responses (HR). In this case, multiplication of the pathogen is limited. Thus, some effector genes have been defined as avirulence (Avr) genes. Therefore, gene-for-gene resistance occurs when a pathogen carrying an avirulence (Avr) gene, is recognized by host plant that carries the matching resistance gene (R-gene). In incompatible interaction, as a consequence of host-triggered hypersensitive responses (HR), bacterial growth is suppressed after a short proliferation phase while in compatible interaction, the virulent pathogen is able to proliferate in host cells.

1.1.3 Plant-pathogen co-evolution

Plant and pathogen continually evolve mechanisms to escape the limitations imposed on one another. The interplay between plant and pathogen follows a zig-zag model defined by Jones and Dangl, 2006 (Figure 1.2). According to this model and as explained previously, the primary immune response is referred to as PAMP-triggered immunity (PTI) and has evolved to recognize PAMP/MAMP from pathogen. In a co-evolution of host-microbe interactions, pathogens have evolved the ability to produce virulence factors (effectors) to suppress PTI and to trigger susceptibility in plants termed as effector-triggered

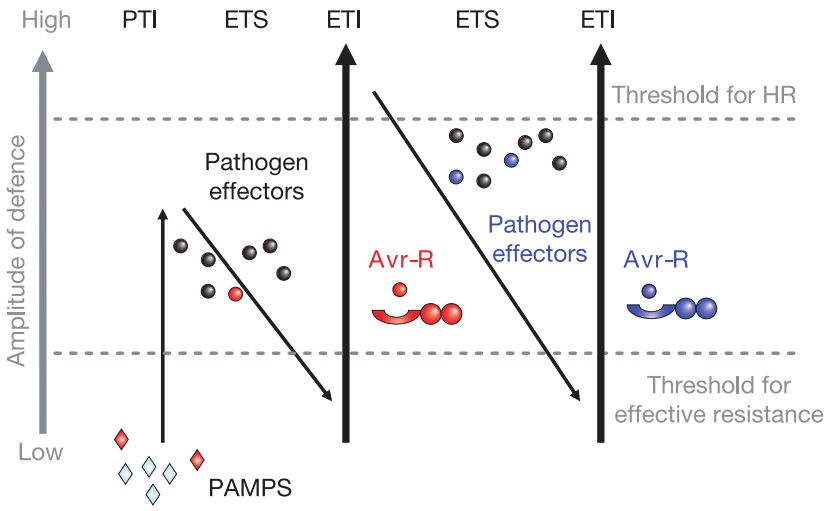

Figure 1.2: A zigzag model illustrates the quantitative output of the plant immune system. In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI–ETS–ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/ PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favors new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI. (Jones and Dangl, 2006)

susceptibility (ETS). Due to respond to pathogen effectors, plants acquired surveillance proteins (R proteins) to monitor effectors directly and indirectly and to prevent the action of effectors by triggering hypersensitive responses (HR) and establishing effector-triggered immunity (ETI) (Jones and Dangl, 2006; Chisholm et al., 2006). According to the Zig-Zag model, disease susceptibility is a consequence of the suppression of host immunity during the evolutionary arms race between plants and pathogens (Keller et al., 2016). This model fits to host interactions with biotrophic pathogens but it is less applicable for pathogens with necrotrophic lifestyle. Logically, the next step in evolutionary arms race is for pathogen to inhibit ETI due to establishing another susceptible phase. For instance, *P. syringae* HopD1 effector has an ETI-suppressing activity on Arabidopsis (Block et al., 2014). Therefore, an additional ramification of ETI in zig-zag model defined as effector-triggered immune pathology (ETIP) which better fits evolutionary arms race between plants and necrotrophic pathogens (Keller et al., 2016).

1.2 Systemic acquired resistance (SAR)

Plant immune responses are not only restricted to the sites of pathogen attack. Localized infection of plants induces long-lasting defense mechanisms against a broad spectrum of pathogens in whole plant foliage. This phenomenon is known as systemic acquired resistance (SAR) (Durrant and Dong, 2004; Fu and Dong, 2013; Mishina and Zeier, 2007b). The term (systemic acquired resistance) was first explained by Ross, 1961 who observed an enhanced immunity in uninfected leaves of tobacco following initial infection with tobacco mosaic virus (TMV). SAR responses include four distinct phases i) induction of SAR ii) signal generation and translocation iii) signal perception and defense priming iv) manifestation of SAR (Fu and Dong, 2013; Shah and Zeier, 2013).

Induction of SAR in infected leaves Several studies have described SAR in a number of plants such as tobacco, tomato, cucumber, and model plant Arabidopsis (Ross, 1961; Guedes et al., 1980; Metraux et al., 1990; Fu and Dong, 2013; Durrant and Dong, 2004). In Arabidopsis, SAR is induced in whole plant foliage after localized infection with a virulent or avirulent pathogen, or after recognition of PAMPs, as well as localizing insect eggs (Cameron et al., 1994; Hilfiker et al., 2014; Mishina and Zeier, 2007b). Induction of MTI and cell-death associated responses such as production of ROS are shown to be essential for signal generation and SAR induction (Wang et al., 2014; Mishina and Zeier, 2007b; Alvarez et al., 1998). Induction of SAR can occur in a variety of plants after infecting with bacterial, viral and fungal pathogens (Sticher et al., 1997).

Signal generation and translocation At the site of pathogen attack, a signal/signals must be generated to move from local infected leaves to systemic leaves. The identity of the exact signal/signals remains elusive. The timing of signal movement to induce systemic SAR is shown to be different depending on host plants, the nature of the pathogen, and the density of inoculum (Mishina and Zeier, 2007a). In a *Pseudomonas syringae* and Arabidopsis interaction, the signal might be transferred from the inoculated leaves between 24 and 48 hours (Mishina and Zeier, 2007a). Based on grafting experiment described in Jenns and Kuc, 1979, the signal is not likely to be cultivar-, genus-, or species-specific. Several studies suggest that SAR signal movement to distal leaves is through vasculature (Jenns and Kuc, 1979; Guedes et al., 1980) and also signal movement through plasmodesmata is shown to be essential for phloem loading and long distance transporting of signal (Carella et al., 2015; Lim et al., 2017; Kiefer and Slusarenko, 2003).

Several candidate SAR signals have been identified so far (Dempsey and Klessig, 2012; Shah and Zeier, 2013; Shah et al., 2014). Earlier studies hypothesized defense hormone SA to act as a SAR signal because it was accumulated in phloem exudate from infected leaves (Malamy et al., 1990; Metraux et al., 1990). However, grafting experiment in tobacco showed that SA-deficient rootstock is still able to generate and transmit the SAR signal to wild-type scion (Vernooij et al., 1994). Moreover, it was demonstrated in cucumber that exit of the SAR signal from inoculated leave was prior to SA accumulation in phloem (Rasmussen et al., 1991). Other putative candidate SAR signals include methyl salicylate (MeSA), an SFD1/GLY1-derived glycerol-3-phosphate (G3P) or G3P-dependent signal, lipid-transfer protein DIR1, dicarboxylic acid azelaic acid (AzA), abietane diterpenoid dehydroabietinal (DA), non-protein amino acid pipecolic acid (Pip), and N-Hydroxypipecolic Acid (NHP) (Dempsey and Klessig, 2012; Park et al., 2007b; Chanda et al., 2011; Maldonado et al., 2002; Jung et al., 2009; Chaturvedi et al., 2012; Návarová et al., 2012; Hartmann et al., 2018). [Pip and NHP are explained in section 1.3.2 on page 20 and other putative SAR signals are described in section 1.3.3 on page 25].

Signal perception and defense priming As it was previously mentioned, the role of SA as a mobile SAR signal is ruled out, however systemic accumulation of SA is essential for SAR manifestation. Park et al., 2007b explained that methyl salicylate (MeSA) esterase activity of SA-BINDING PROTEIN 2 (SABP2), which converts MeSA into SA, is required for SAR signal perception in systemic tissue. A transcriptional co-activator, termed NONEXPRESSOR OF PR GENES 1 (NPR1) is identified to interact specifically with SA and regulates SA-dependent responses such as PR1 expression (Cao et al., 1994; Dong, 2004; Wu et al., 2012). In a non-induced state, NPR1 is present in cytoplasm as oligomer formed

through intermolecular disulfide bonds. when SAR is induced and level of SA is high, NPR1 will switch to its monomeric form and will be then translocated in nucleus to activate defense gene expression (Mou et al., 2003; Attaran and He, 2012).

In addition to NPR1-mediated SA-signaling, putative SAR signal Pipecolic acid (Pip) is shown to regulate SAR in a SA-dependent and -independent manner (Bernsdorff et al., 2016). Results from Pip-induced SAR has revealed an existence of Pip and SA amplification loop in systemic tissue. Perception of SAR signal in systemic tissue triggers ALD1-dependent accumulation of Pip. Accumulated Pip will then activate FLAVIN DEPENDENT MONOOXYGENASE 1 (FMO1)-based SA-dependent and -independent signaling responses (Bernsdorff et al., 2016). This leads to a major transcriptional reprogramming in systemic tissue which is associated with activating of defense-related genes and suppressing of plant growth and development genes. SAR-activated plants are primed for more rapid and effective defense responses towards subsequent pathogen attack (Gruner et al., 2013; Návarová et al., 2012; Bernsdorff et al., 2016). This phenomenon is designated as defense priming which is an integral part of systemic responses and is dependent on functional ALD1 and FMO1 (Conrath et al., 2015; Návarová et al., 2012). FMO1 catalyzed the conversion of L-Pip to form N-hydroxypipecolic acid (NHP) which is recently found to be a critical regulator of SAR (Hartmann et al., 2018). NHP is accumulated in *Psm*-inoculated and systemic leaves of Arabidopsis and its biosynthesis completely depends on ALD1 and FMO1 genes and it is positively stimulated by immune regulators EDS1 and PAD4 genes (Hartmann et al., 2018). Although functional SA is not required for NHP generation, SA modulates the accumulation of NHP. Pip-induced defense mechanisms, priming of defense responses, and SAR-associated transcriptional reprogramming are dependent on functional FMO1, indicating the NHP biosynthetic pathway constitutes a core and indispensable element of SAR (Hartmann et al., 2018). [More details about ALD1 and FMO1 are explained in section 1.3.2, page 20 and section 1.3.2, page 22. Priming is explained in section 1.4, page 37].

Manifestation of SAR As previously explained, primed-status of plant triggers faster and stronger defense responses towards subsequent pathogen attacks (Conrath et al., 2015). Established SAR in plants, provide enhanced resistance towards other bacterial, viral and, fungal pathogen attacks. For instance, in Arabidopsis, initial pathogen-induced SAR causes resistance to virulent strains of *P. syringae* and the oomycete *H. arabidopsidis* (Cameron et al., 1994). The SAR-induced resistance can be used as a tool to reduce the severity of disease in some crops such as wheat (*Triticum aestivum*) in which an initial treatment with SAR-inducing bacteria *Bacillus mycoides* causes enhanced resistance to cereal fungal pathogen *Fusarium culmorum* (Moya-Elizondo and Jacobsen, 2016). In future, this strategy can be used in agriculture to protect main crops against pathogens.

1.3 SAR regulatory metabolites

1.3.1 Salicylic acid (SA)

SA biosynthesis In plants salicylic acid (2-hydroxy benzoic acid) is synthesized via two pathways: i) phenylalanine ammonia lyase (PAL) and ii) isochorismate (IC) pathway (Mauch-Mani and Slusarenko, 1996; Wildermuth et al., 2001). Both pathways utilize chorismate , the end product of shikimate pathway, as precursor. The PAL pathway operates in cytosol while IC pathway occurs in chloroplast. In PAL pathway, cinnamate is produced from phenylalanine through PAL activity and will be converted to SA via formation of benzoate. Silencing of PAL genes in tobacco or chemical inhibition of PAL activity in Arabidopsis, cucumber and potato reduces pathogen-induced SA accumulation (Chen et al., 2009). In Arabidopsis, IC pathway is the major SA-synthesis pathway which is responsible for 90% of pathogen-induced SA production (Garcion et al., 2008; Wildermuth et al., 2001). The first step of this pathway, is conversion of chorismate to isochorismate via ISOCHORISMATE SYNTHASE (ICS) activity. In bacteria such as *Pseudomonas aeruginosa*, the final step of SA synthesis is catalyzed by ISOCHORISMATE PYRUVATE LYASE (IPL) which is a key enzyme for conversion of isochorismate to salicylate (Wildermuth et al., 2001; Gaille et al., 2002; Gaille et al., 2003) (Figure 1.3). However, no gene similar to bacterial IPL has been reported in plants. Two Arabidopsis genes, PBS3 (AVR $_{PphB}$ SUSCEPTIBLE 3) and EPS1 (ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1), might contribute to the last step of SA biosynthesis. PBS3 encodes a member of the acyl-adenylate/thioester forming enzyme family, and EPS1 encodes a member of the BAHD acyltransferase superfamily (Van Verk et al., 2011; Chen et al., 2009).

In Arabidopsis, two isochorismate genes ICS1 (also known as SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2)) and ICS2 have been identified (Chen et al., 2009). ICS2 participates in synthesis of limited amount of SA which is only detectable in the absence of ICS1 (Garcion et al., 2008). Two allelic SA-deficient mutants, *sid2-2/eds16-1* and *sid2-1*, were mapped closed to ICS locus in chromosome 1 of Arabidopsis and it is proved that SID2 encodes a chloroplast-localized ICS1. Thus, *sid2-2* mutant, a fast-neutron-generated mutant, fail to express ICS1 upon infection with *Golovinomyces orontii* or a virulent strain of *Pseudomonas syringae* (Wildermuth et al., 2001). The mutant *sid2-1* is generated by ethyl methane sulfonate (EMS) treatment and it also contains a mutation in ICS1. Both *sid2-1* and *sid2-2* mutants, disrupt chorismate binding domain of ICS1 and are defective in basal and systemic resistance as well as ICS1 expression, SA accumulation and PR1 expression (Wildermuth et al., 2001; Nawrath and Métraux, 1999). Biosynthesis of SA is also regulated by several transcription factors such as NTM1-LIKE 9 (NTL9) and CCA1 HIKING EXPEDITION (CHE) (Zheng et al., 2015). NTL9 regulates stomata closure during pathogen attack, by inducing SA-biosynthesis genes as well as SA synthesis-related genes such as PAD4 and EDS1. CHE, as a central circadian clock oscillator, is required for oscillation in SA levels and for pathogen-induced SA accumulation in systemic tissues during SAR. It may also regulate ICS1 through other transcription activators CALMODULIN BINDING PROTEIN 60g (CBP60g) and SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) (Zheng et al., 2015).

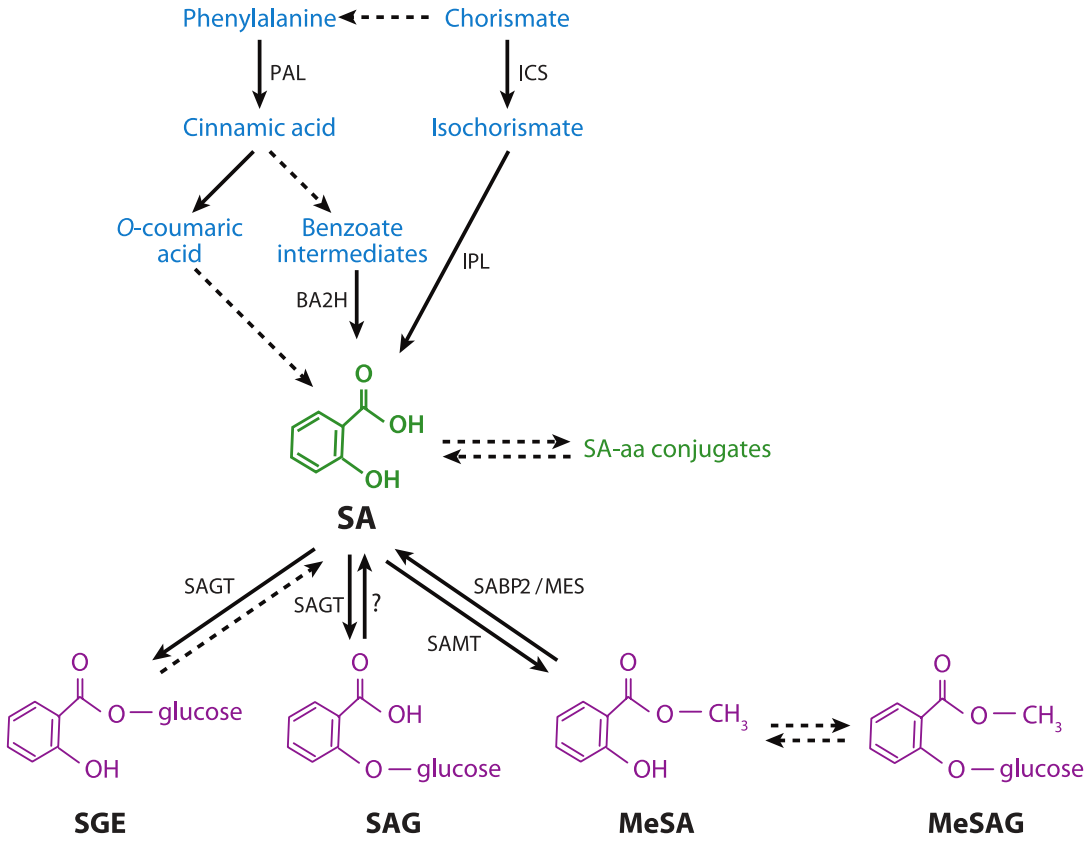

Figure 1.3: Simplified schematic of pathways for SA biosynthesis and metabolism as adapted from (Hedden et al., 2006). Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SA, salicylic acid; SAGT, SA glucosyltransferase; aa, amino acid; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase; SGE, salicyloyl glucose ester; SAG, SA O-β-glucoside; MeSA, methyl salicylate; MeSAG, methyl salicylate O-β-glucoside. (Vlot et al., 2009)

SA modification Once synthesized, free SA can undergo variety of chemical modifications. Most of Free SA is converted to an inactive form which will be stored in vacuole, like SA 2-O-β-glucoside (SAG) and less abundant like salicyloyl glucose ester (SGE). Arabidopsis encodes two pathogen-inducible SA glucosyltransferases (SAGTs): UGT74F1 which forms only SAG and UGT74F2 which forms both SAG and SGE (Dean and Delaney, 2008). Both SA-glucosyltransferases are able to transfer a glucose moiety to phenolic hydroxyl group of SA to produce SA 2-O-β-glucoside (SAG) while, UGT74F2 transfers a glucose to the carboxyl group of SA, to produce SA-glucose easter (SGE) (Figure 1.3) (Petersen et al., 2000; Dempsey et al., 2011). SAG synthesized in cytosol, is transported to vacuole where it is stored as an inactive form until it converts back to SA (Hennig et al., 1993; Dean et al., 2003; Dean et al., 2005). Free SA may be also converted to other forms such as Methylsalicylate (MeSA) and/or its glucosylated derivative MeSAG (Figure 1.3) (Dean et al., 2003; Dean et al., 2005; Seskar et al., 1998). More details about MeSA and its role on plant defense as a putative SAR signal, are explained in section 1.3.3.1 on page 25. SA can also be conjugated to amino acids via acyl-adenylate/thioester-forming enzyme (GH3.5). GH3.5 is involved in conjugation of amino acids to SA and the auxin indole acetic acid (Park et al., 2007a). Arabidopsis overexpressing GH3.5 lines, show higher SA accumulation, higher PR1 expression and consequently more resistance towards *Pseudomonas syringae pv. tomato* DC3000 (Park et al., 2007a). However, loss of function of GH3.5, is partially compromised in SAR and therefore, GH3.5 was proposed to be a positive regulator of SA signaling (Zhang et al., 2007c).

SA and plant defense responses The discovery of a functional role of SA in plant disease resistance dated back in 1979 when White and coworkers demonstrated that infiltration of aspirin or SA in the leaves of resistant tobacco cultivar Xanthi-nc prior to infection with tobacco mosaic virus (TMV), resulted in 90% reduction in lesion formation and induction of PR genes (White, 1979). Later on, SA treatment was found to induce PR gene expression and resistance to bacterial, viral, and fungal pathogens in different plant species. In TMV-resistant tobacco, SA was accumulated 20 fold in the inoculated and 5 fold in the systemic leaves (Malamy et al., 1990). Additional evidence supporting the signaling role of SA in plant defense arose from increase of 10-100 fold in level of SA in phloem exudate of cucumber inoculated with tobacco necrosis virus, *Colletotrichum lagenarium* or *Pseudomonas syringae* which these elevated levels preceded SAR development (section 1.2) (Metraux et al., 1990).

Arabidopsis mutants in constitutive expressor of PR genes 1/5/6 (*cpr1*, *cpr5*, and *cpr6*), exhibit enhanced SAR which is correlated with elevated level of SA and constitutively expression of ICS1 and PR1 (Wildermuth et al., 2001). In contrast, transgenic Arabidopsis or tobacco expressing bacteria NahG gene (a salicylate hydroxylase converting SA to catechol) fail to accumulate high level of SA and PR1 expression and SAR development upon virulent or avirulent pathogens (Delaney et al., 1994; Vernooij et al., 1994; Gaffney et al., 2000). However, disease resistance and PR expression in these plants were restored by treatment with SA synthetic analog, 2,6-dichloro-isonicotinic acid (INA) (Vernooij et al., 1995). Susceptibility to pathogens and defective in SAR development, are also observed in Arabidopsis plants defective in SA-synthesis genes such as SID2/EDS16 (encodes ICS1) or SID1/EDS5 (a member of multi-drug and toxin extrusion (MATE) family involving in transport of precursors of SA) and in tobacco plants with suppressed PAL expression (Nawrath and Métraux, 1999; Pallas et al., 1996; Nawrath, 2002a). Similar to NahG plants, the resistance in SA-defective plants, is also restored by treatment with INA or exogenous SA.

SA signaling have been shown to be required for manifestation of SAR and as explained previously, Arabidopsis *ics1* mutants (which are unable to produce SA) and transgeic NahG plants (which express bacterial SA-degrading enzyme salicylate hydroxylase) are defective in expressing SAR (Wildermuth et al., 2001; Vernooij et al., 1994). Due to its accumulation in phloem and its requirement for SAR, SA itself was initially considered as a mobile signal for SAR induction (Yalpani et al., 1991). However, grafting experiment using tobacco plants expressing the bacterial NahG gene, which encodes the SA-degrading enzyme SA hydroxylase, rejected this hypothesis. Tobacco mosaic virus (TMV)-infected NahG rootstocks were still capable of generating the signal for induction of SAR in non-transgenic scions, despite their inability to accumulate SA (Vernooij et al., 1994). Therefore, SA is not the SAR signal but it is required in signal transduction (Vernooij et al., 1994).

SA-mediated signaling and its regulation As explained, SA signaling plays an important role in plant defense responses. Several regulatory proteins such as ENHANCED DISEASE SUSCEPTIBILITY 1/4/5 (EDS1, EDS4, EDS5) and PHYTOALEXIN DEFICIENT 4 (PAD4) involve in upstream pathways of SA signaling to activate SA production in Arabidopsis. *eds1*, *eds4*, and *pad4* mutants show reduced level of SA in infected leaves with *Pseudomonas syringae* (Zhou et al., 1998; Gupta et al., 2000; Feys et al., 2001). EDS1 forms a distinct complex with its interacting component PAD4, which may play a role in transducing redox signaling to produce SA (Rustérucci et al., 2001; Wiermer et al., 2005). EDS5 (SID1), which acts downstream of PAD4, encodes a protein which transports precursors for SA biosyn-

thesis (Nawrath, 2002a). In the context of SAR, Pip/FMO1 signaling module, regulates SAR in both SA-dependent and SA-independent pathways (Bernsdorff et al., 2016). Pip-derived N-hydroxypipecolic acid (NHP), which is recently-described as the major SAR-activating metabolite, is shown to act synergistically with SA (Hartmann et al., 2018). The biosynthesis of NHP is positively regulated by EDS1 and PAD4 and it requires SA to provide strong protection against *P. syringae* or *Hyaloperonospora arabidopsidis (Hpa)* attack (Hartmann et al., 2018). Exogenous application of Pip or NHP resulted in lower induction of resistance in SA-deficient *sid2* plants when compared to wild-type (Hartmann et al., 2018). This suggests that NHP positively amplifies SA-inducible defense responses (Hartmann et al., 2018). Pip- and NHP-inducible resistance are still observable in *sid2*, suggesting that there is a SA-independent branch of Pip and NHP signaling pathways (Hartmann et al., 2018; Bernsdorff et al., 2016).

Moreover, other factors and signaling cascades acting upstream and/or downstream of SA and affect SA accumulation include nitric oxide (NO), reactive oxygen species (ROS), and Mitogen-activated protein kinase (MAPK) signaling cascade. Nitric oxide (NO) activate SA biosynthesis pathway by inducing PAL and conversely, SA activates NO synthesis and NO-regulated enzymes (Zottini et al., 2007; Neill et al., 2002; Durner and Klessig, 1995; Clark et al., 2000). Reactive oxygen species (ROS) like H_2O_2 causes an intracellular accumulation of benzoic acid (BA) to be converted to SA and on the other hand, SA regulates ROS level through binding to SA-BINDING PROTEIN 3 (SABP3) and inhibiting its antioxidant activity (Leon et al., 1995; Slaymaker et al., 2002).

Mitogen-activated protein kinase (MAPK) cascade function downstream of pathogen-recognition receptor (PRR) upon PAMP perception and downstream of SA. Some members of MAPKs signaling cascade negatively regulate SA signaling like MAP kinase 4 (MPK4). Transposon inactivation of Arabidopsis MPK4 exhibit constitutive systemic acquired resistance (SAR) and elevated SA (Petersen et al., 2000). In addition, some MAP kinases positively regulate SA signaling pathway in plant immunity such as MAP kinase kinase 7 (MAPKK7) which positively triggers accumulation of SA and regulates basal and systemic resistance (Zhang et al., 2007b).

NPR1 (NON-EXPRESSOR OF PR1) (also known as NIM1 (NONIMMUNITY 1)) is a master regulator of SA-mediated induction of defense genes. NPR1 acts downstream of SA because Arabidopsis *npr1* mutants are unable to induce PR1 gene even after SA treatment (Chern et al., 2008). Nuclear localization of NPR1 is essential for its function. In absence of pathogen, NPR1 protein forms an oligomer localized in cytoplasm. Pathogen/PAMP exposure, induces SA accumulation and NPR1 can directly bind to SA. SA controls the nuclear translocation of NPR1 through cellular redox changes which alter NPR1 into its monomeric form to be localized in nucleus (Mou et al., 2003; Dong, 2004). In the nucleus, NPR1 interacts with members of the DNA-binding proteins TGA-family of transcription factors (TGA2, TGA3, TGA5, TGA6, and TGA7) and activate PR1 gene expression (Despres et al., 2000).

SA and systemic acquired resistance (SAR) Salicylic acid (SA) is required for activation of defense responses at the site of pathogen attack, as well as distant pathogen-free organs to induce systemic acquired resistance (SAR) (Refer to section 1.2). SA signaling have been shown to be required for manifestation of SAR and as explained previously, Arabidopsis *ics1* mutants (which are unable to produce SA) and transgenic NahG plants (which express bacterial SA-degrading enzyme salicylate hydroxylase) are defective in expressing SAR (Wildermuth et al., 2001; Vernooij et al., 1994). Due to its accumulation

in phloem and its requirement for SAR, SA itself was initially considered as a mobile signal for SAR induction (Yalpani et al., 1991). However, grafting experiment using tobacco plants expressing the bacterial NahG gene, which encodes the SA-degrading enzyme SA hydroxylase, rejected this hypothesis. Tobacco mosaic virus (TMV)-infected NahG rootstocks were still capable of generating the signal for induction of SAR in non-transgenic scions, despite their inability to accumulate SA (Vernooij et al., 1994). Therefore, SA is not the SAR signal but it is required in signal transduction (Vernooij et al., 1994).

1.3.2 Pipecolic acid (Pip) and N-hydroxypipecolic acid (NHP)

The Lysin-derived non protein amino acid Pipecolic acid (Pip) is a critical SAR signal and an important regulator of several inducible defense responses such as PTI, ETI, BABA-induced resistance, and particularly SAR (Návarová et al., 2012; Zeier, 2013; Vogel-adghough et al., 2016; Bernsdorff et al., 2016). Pip was first detected in white clover (*Trifolium repens*) (Morrison, 1953) and bean (*Phaseolus vulgaris*) (Zacharius et al., 1954), and later on it was isolated from several plant species such as strawberry, tobacco (*Nicotiana tabacum*), rice (*Oryza sativa*), potato (*Solanum tuberosum*), Soybean, fungi, and animals (Yatsu and Boynton, 1959; Pálfi and Dézsi, 1968; Wickwires et al., 1990; Vogel-adghough et al., 2016; Suharti et al., 2016; Chang, 1976; Abeysekara et al., 2016; Charles, 1986; Murthy and Janardanasarma, 1999). Pip is shown to play a postsynaptic role or as weak inhibitory neurotransmitter GABA-agonist in brains of rat and mouse (Chang, 1976; Charles, 1986; Murthy and Janardanasarma, 1999). In human, Pip was described as a diagnostic marker of pyridoxine-dependent epilepsy in plasma and cerebrospinal fluid (Plecko et al., 2005).

In plants, Pip was previously described as flower-inducing substance in the aquatic plant *Lemna gibba* and as an indicator of abnormal protein metabolism in diseased plants (Fujioka et al., 1987; Pálfi and Dézsi, 1968). In addition, Pip is estimated to contribute to root nodulation in legumes (Chen et al., 2014). Later on, in 2012 Zeier and colleagues highlighted the role of Pip as a critical regulator of inducible plant immunity and defense priming (Návarová et al., 2012). Upon inoculation of Arabidopsis leaves with the SAR-inducing pathogen *Pseudomonas syringae pv. maculicola*, a massive change in the level of free amino acids was observed in inoculated, non-inoculated, and petiole exudate of inoculated leaves (Návarová, 2012). In inoculated leaves, an strong increased level was observed for Lys, the aliphatic amino acids Val, Leu, Ile, and β-Ala, and the aromatic amino acids Phe, Tyr, Trp, and His. Moreover, GABA, Cys, Asn, Ala, Gly, Ser, and Orn showed a moderate but still significant increase, whereas Asp showed a decreased level upon *Psm* inoculation (Návarová, 2012). *Psm* inoculations triggered an increased level of Pip (~ 70 fold higher) and α-amino adipic acid (Aad) at the site of infection, however Pip was the only amino acid which was enriched in petiole exudate upon *Psm* infection, suggested that Pip might be the putative SAR signal which perhaps moves from inoculated to distal leaves in the context of long-distance signaling (Návarová, 2012). Recently, a Pip-derived metabolite N-hydroxypipecolic acid (NHP) is identified which its infiltration in Arabidopsis wild-type or *fmo1* leaves, induces the systemic responses in these plants, suggesting that NHP is potential to move to the upper leaves (Hartmann and Zeier, 2018). However, further experimental investigations are needed to clarify the mobility of SAR signal and its role in long distance signaling. [NHP and its role in SAR is explained in section 1.3.2 on page 22.]

Biosynthesis of Pipecolic acid There is a vast amount of literature on biosynthesis pathway of Pip and various approaches have proposed L-lysin as a precursor for Pip biosynthesis (Gupta and Spenser, 1969; Chang, 1976; Wickwires et al., 1990; Murthy and Janardanasarma, 1999; Zabriskie and Jackson, 2000; Návarová et al., 2012; Hartmann et al., 2017). In response to *Psm* inoculation, Pip accumulates in Arabidopsis plants and the first step of Pip biosynthesis includes a transamination step, for which an aminotransferase is required (Návarová et al., 2012). In Arabidopsis ABERRANT GROWTH AND DEATH2 (AGD2; AT4G33680) encodes an aminotransferase which catalyzes the conversion of L-tetrahydrodipicolinate to LL-diaminopimelate as final transamination step in L-lysine biosynthesis (Hudson, 2005). Arabidopsis *agd2-1* mutants, showed elevated level of SA and a mild dwarfism phenotype and it is likely that Arabidopsis AGD2 is indispensable for plant development and it may repress the defense responses (Song et al., 2004a). In response to bacterial pathogen attack in Arabidopsis AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1; At2g13810), which is homolog to AGD2 with 62% protein sequence identity, is shown to mediate the first step of Pip biosynthesis by direct transamination of α-amino group of Lys to oxoacids such as pyruvate or α-ketoglutarate (Figure 1.4) (Návarová et al., 2012; Hartmann et al., 2017; Song et al., 2004a; Song et al., 2004b; Hartmann et al., 2017). This reaction might lead to formation of intermediate ε-amino-α-ketocaproic acid (KAC) which spontaneously undergoes cyclization and isomerization steps to form the final product of ALD1 which is Δ^2-piperidine-2-carboxylic acid (P2C), alias 2,3- dehydropipecolic acid (2,3-DP) (Figure 1.4) (Hartmann et al., 2017).

Both AGD2 and its close homolog ALD1 encode transaminases acting on similar sets of amino acids, but in opposite directions. In contrast to *agd2*, *ald1* mutant has reduced level of SA and is more susceptible to *P. syringae* (Song et al., 2004a). In response to pathogen, expression of ALD1 is partially regulated by PAD4, and they both act additively to control the accumulation of defense metabolites such as SA and camalexin and expression of PR1 (Song et al., 2004b). In vitro, the purified and recombinant ALD1 produces DP, when L-lysine as the amino acid substrate, an oxoacid as acceptor, and pyridoxal phosphate as a cofactor are present (Hartmann et al., 2017). Beside L-lysin as a preferable substrate for ALD1 (Song et al., 2004a), ALD1 catalyzes the transamination of several other amino acids such as L-Met, L-Leu, and L-Arg in vitro, however the final products of this reaction are either not detectable in planta or not associated with the function of ALD1 in plants. Thus, the biosynthesis of 2,3-DP from L-lysin is the major function of ALD1 in planta (Hartmann et al., 2017).

In Arabidopsis, *ald1* mutants have found to be susceptible to *Pseudomonas syringae* infection and are compromised in SAR and basal resistance to *Psm* (Návarová et al., 2012; Song et al., 2004b; Song et al., 2004a). Following pathogen attack, Pip is significantly accumulated in inoculated leaves, in leaves distal to the site of pathogen attack, and especially in petiole exudate from inoculated leaves (Návarová et al., 2012). *ald1* mutants fail to accumulate pathogen-induced Pip and therefore SAR is compromised in these lines. Exogenous application of Pip restores SAR defect of *ald1* plants and it induces SAR-related defense priming which is abolished in *ald1* (Návarová et al., 2012). Exogenously applied 2,3-DP to *ald1* mutant, will still be converted to Pip, showing that Pip deficiency of *ald1* is due to inability of this mutant to form the 2,3-DP intermediate (Hartmann et al., 2017).

In the second step of Pip biosynthesis, ALD1-derived DP is then reduced to Pip by Arabidopsis gene annotated as ORNITHINE CYCLODEAMINASE/ μ-CRYSTALLIN (ORNCD1) which is also designated as SAR-DEFICIENT 4 (SARD4; At5g52810) (Figure 1.4). SARD4 gene shows high sequence similarity to the mammalian ketimine reductase CRYM (Zeier, 2013; Ding et al., 2016; Hartmann et al., 2017).

Loss of function of SARD4 leads to delayed and reduced level of local Pip and markedly reduced level of Pip and SA in systemic leaves (Hartmann et al., 2017). *sard4* mutant lines showed significant lower basal resistance to *Psm* compared to wild-type Col-0, however, this mutant line was not compromised in SAR (Hartmann et al., 2017). SAR competency of *sard4* is attributed to its low but significant amount of local Pip which is able to trigger functions contributing to SAR in systemic leaves and then reduced systemic level of Pip in this line would contribute to the absolute strength of SAR (Hartmann et al., 2017; Ding et al., 2016). ALD1 and SADR4 are regulated by SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g) genes which encode two master transcriptional factors of plant immunity and regulate the accumulation of Pip and SA (Sun et al., 2015; Sun et al., 2017). Recently, TGACG-BINDING FACTOR 1 (TGA1) and TGA4 are shown to regulate Pip and SA by modulating the expression of SARD1 and CBP60g (Sun et al., 2017). L-lysin metabolism to Pip and NHP is illustrated in Figure 1.4.

Flavin-dependent monooxygenase 1 (FMO1) and generation of N-hydroxypipecolic acid (NHP) The pathogen-induced lysin catabolism pathway leads to ALD1-dependent Pip production. Pip is further N-hydroxylated to N-hydroxypipecolic acid (NHP) by activity of a flavin-dependent monooxygenase (Figure 1.4) (Hartmann et al., 2018). In plants, animals, and fungi, FLAVIN-DEPENDENT MONOOXYGENASES (FMOs) oxidizes either Nitrogen- or Sulfur-containing functional groups of small metabolites. In Arabidopsis, FMOs consist of 29 members and are categorized into three subgroups: i) YUCCA clade are capable of converting tryptamine to N-hydroxyl-tryptamine in vitro and are implicated in auxin biosynthesis (Zhao et al., 2001) ii) S-oxygenation subgroup ($FMO_{GS\text{-}OX}$) which oxidize the sulfide group of Met-derived methylthioalkyl glucosinolates to sulfoxide moieties, thereby generating methylsulfinylalkyl glucosinolates (Li et al., 2008) iii) Pathogen defense clade (FMO1 and a Pseudogene) (Olszak et al., 2006; Schlaich, 2007). Establishment of biological SAR and the associated transcriptional reprogramming of systemic leaf tissue are proved to be regulated by Pip and its downstream acting component FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1; At1g19250), therefore FMO1 is considered as an essential regulator of SAR (Bernsdorff et al., 2016; Mishina and Zeier, 2006).

FMO1 functions as Pipecolic Acid N-Hydroxylase in planta and in vitro and it catalyzed N-hydroxylation of L-Pip to generate N-hydroxypipecolic acid (NHP) (Figure 1.4) (Hartmann et al., 2018). Upon *Psm*-inoculation in 1° leave, NHP is produced at 10 hours post inoculation onwards and it reaches its highest amount at 24 hpi which coincides with the time of Pip accumulation (Hartmann et al., 2018). This level will be reduced at 48 hpi in inoculated leaves. Pathogen induces accumulation of NHP in systemic leaves starting at 24 hpi and reached its highest level at 48 hpi which is the time point that SA and Pip accumulate in systemic leaves to develop SAR (Hartmann et al., 2018). NHP generation is totally dependent on ALD1 and FMO1 and it is also regulated by EDS1/PAD4 regulatory genes (Hartmann et al., 2018). EDS1 and PAD4 positively stimulate the upregulation of ALD1 and FMO1 (Návarová et al., 2012; Mishina and Zeier, 2006; Song et al., 2004b).

Exogenous Pip and NHP induce a significant lower resistance in SA-deficient *sid2* plants comparing to wild-type, indicating a positive interplay between NHP and SA pathways (Bernsdorff et al., 2016; Hartmann et al., 2018). Moreover *sid2* plants shows a stronger expression of pathogen-induced FMO1 in comparison to wild-type (Bernsdorff et al., 2016). NHP requires inducible SA biosynthesis to induce full defense responses against *Psm* and *Hyaloperonospora arabidopsidis (Hpa)* (Hartmann et al., 2018). The additive effect of SA and Pip/NHP also exists in basal immunity since *sid2ald1* (defective in pro-

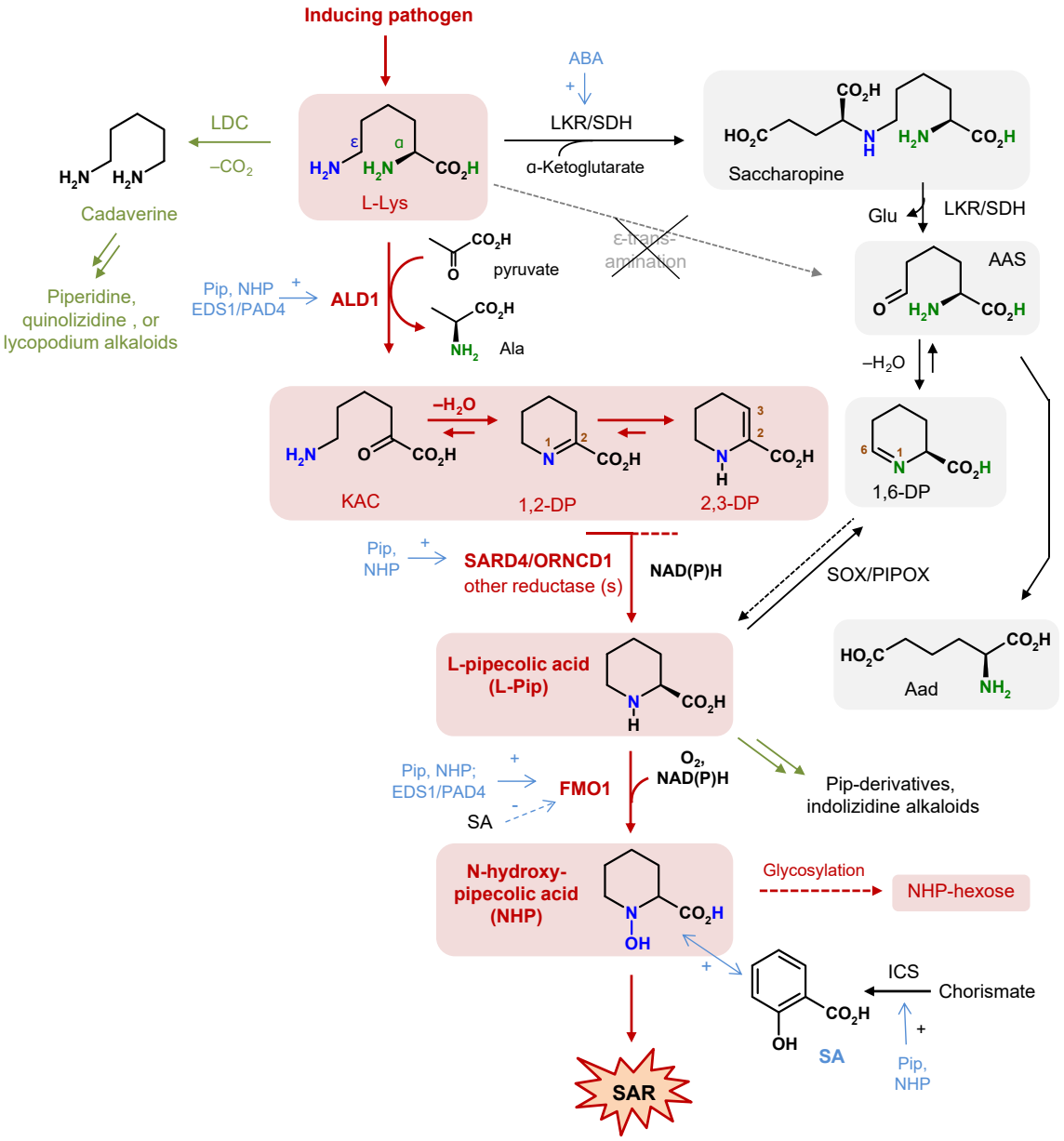

Figure 1.4: L-lysine catabolic pathways in plants. The formation of N-hydroxypipecolic acid (NHP), a critical regulator of systemic acquired resistance (SAR), occurs by three consecutive enzymatic steps from L-lysine (L-Lys): an α-transamination reaction catalyzed by the aminotransferase ALD1, reduction of dehydropipecolic acid (DP) intermediates, which are formed by dehydrative cyclization of the transamination product ε-amino-α-ketocaproic acid (KAC), to pipecolic acid (Pip) by SAR-deficient 4 (SARD4) and other reductase activities, and N-hydroxylation of L-Pip to NHP by FMO1. The NHP pathway is activated by an initial inducing leaf inoculation and systemically protects plants from subsequent infection. Biochemical steps associated with the NHP pathway are highlighted in red. Regulatory aspects are depicted in light blue (+, positive regulation; -, negative regulation). A second L-Lys catabolic pathway generally found in plants constitutes the saccharopine pathway via α-aminoadipic acid semialdehyde (AAS) to α-aminoadipic acid (Aad) (highlighted in grey). Only a restricted number of plant species realize the depicted cadaverine branch and specific metabolic conversion reactions from Pip (displayed in green). Other abbreviations: SA, salicylic acid; ABA, abscisic acid; LKR/SDH, lysine-ketoglutarate reductase/saccharopine dehydrogenase; SOX/PIPOX, sarcosine oxidase/pipecolate oxidase; LDC, lysine decarboxylase; ICS, isochorismate synthase; EDS1, enhanced disease susceptibility1; PAD4, phytoalexin-deficient4; ORNCD1, ornithine cyclodeaminase/μ-crystallin. (Hartmann and Zeier, 2018)

duction of both SA and Pip/NHP) shows a more compromised basal resistance to bacterial infection than each of the respective single mutants (Bernsdorff et al., 2016). Recent development on signaling pathways in basal resistance and SAR shows that Pip and SA act synergistically and independently

from each other to induce PR1 expression and to mediate basal resistance to *Psm* (Bernsdorff et al., 2016). In the context of SAR, Pip orchestrates SAR and priming responses via SA-dependent and -independent activation pathways (Bernsdorff et al., 2016). This is concluded after observing a moderate SAR in *sid2* plants exhibiting that SAR response can be triggered independently of SA but dependently on Pip (Bernsdorff et al., 2016). Since the role of Pip in SAR is highly dependent on FMO1 downstream of Pip, a Pip/FMO1 switch was estimated to be indispensable for SAR activation and priming events and SA amplifies the Pip-triggered SAR responses (Bernsdorff et al., 2016). Exogenous application of NHP and Pip are still able to induce resistance in SA-deficient *sid2* mutants, showing a SA-independent branch of NHP and Pip signaling pathways (Bernsdorff et al., 2016; Hartmann et al., 2018).

In Arabidopsis, basal resistance against virulent *Psm* and specific resistance against *Psm AvrRpm1* are not compromised in *fmo1* mutants, however FMO1 is required for a proper basal immunity. FMO1 contributes to the EDS1/PAD4 pathway in local defense signaling and its local induction is independent of the SA-signaling pathway and NDR1-mediated signaling (Bartsch et al., 2006; Mishina and Zeier, 2006; Koch et al., 2006). *fmo1* mutant lines are fully compromised in SAR and these lines fail to accumulate systemic Pip, NHP, SA, and to express defense-related genes (Návarová et al., 2012; Mishina and Zeier, 2006; Hartmann et al., 2018). Since *fmo1* fails to establish Pip-induced resistance (Pip-IR) but not SA-IR to *Psm*, it is concluded that FMO1 functions downstream of Pip and upstream of SA in SAR activation (Návarová et al., 2012; Mishina and Zeier, 2006). Therefore, a functional FMO1 is indispensable for induction of SAR and NHP plays an essential role in SAR establishment against bacteria (*P. syringae*) and oomycete (*Hyaloperonospora arabidopsidis (Hpa)*) (Hartmann et al., 2018). Exogenous application of NHP, but not Pip, overrides the defect of NHP-deficient *fmo1*.

Pip is considered as an endogenous mediator of defense priming. Exogenous Pip renders the plants into a primed SAR-like state which means the plants are able to respond more quickly and vigorously to subsequent pathogen attack. This phenomenon is designated as defense priming (Conrath et al., 2015). Pip, when applied exogenously, positively regulates accumulation of SA and camalexin and increases transcript levels of defense-related genes such as ALD1, FMO1, and PR1 in wild-type (Návarová et al., 2012; Bernsdorff et al., 2016). The priming effect of Pip on defense responses is dependent on ALD1 and FMO1, showing a crucial role of NHP in defense priming as an integral part of SAR (Návarová et al., 2012; Hartmann et al., 2018). In addition, Exogenous Pip, enhances the transcription of almost 700 genes in systemic leaves of Arabidopsis, and these genes are categorized in group of SAR $^{+}$ genes, which are systemically up-regulated upon biologically SAR induction (Bernsdorff et al., 2016; Hartmann et al., 2018). The transcriptional reprogramming of Pip is also dependent on a functional FMO1, indicating the important role of NHP in activation of defense responses in transcription level (Hartmann et al., 2018). [More details about defense priming is explained in section 1.4 on page 37].

Besides being a critical mobile SAR signal and a regulator of plant defense by priming function, Pip is also a significant element of a positive feedback loop which mediates signal amplification in systemic leaves (Zeier, 2013; Bernsdorff et al., 2016). Pip boosts its own biosynthesis by up-regulating ALD1 in systemic leaves and it potentiates FMO1 transcript (Zeier, 2013). This leads to ICS1 expression and accumulation of systemic SA which in turn regulate Pip accumulation in the feedback loop. PAD4 and NPR1 are considered as positive downstream regulators of this feedback loop (Zeier, 2013). Pip induces expression of ALD1, SARD4 and FMO1 in an FMO1-dependent manner, showing that NHP has a positive feedback on this biosynthesis pathway to amplify the signaling mechanisms in SAR establishment (Hartmann et al., 2018).

1.3.3 Elusive SAR signals

The L-lysin derived non-protein amino acid Pipecolic acid (Pip) is considered as a putative SAR signal (Návarová et al., 2012). Since Pip plays an important role in SAR and defense priming, thus Pip and Pip-derived NHP were described in previous section 1.3.2, on page 20.

1.3.3.1 Methylsalicylate (MeSA)

MeSA is a component of floral scent and functions as pollinator attractants (Wildermuth, 2006; Effmert et al., 2005). MeSA is normally absent in tissues but is highly induced upon pathogen attack in Arabidopsis and tobacco and a significant amount of MeSA is also emitted to the environment (Park et al., 2007b; Shulaev et al., 1997; Koo et al., 2007; Attaran et al., 2009). Methylation of free carboxyl group of SA yields MeSA which is catalyzed by a gene encoding for an enzyme with benzoic acid and/or SA carboxyl methyltransferase activity (AtBSMT1, At3g11480) in Arabidopsis (Wildermuth, 2006). In normal condition, this gene is highly expressed in flowers and leaves trichomes and hydathodes and its expression are upregulated by treatment with antibiotic alamethicin and methyl jasmonate and by herbivory. This gene also involves in defense mechanisms against certain biotic and abiotic conditions (Chen et al., 2003). In addition to movement through the phloem, MeSA can serve as an airborne signal that is emitted from infected plants and is able to activate defense mechanisms in uninfected leaves of the same plant and/or in neighboring plants. Thus, MeSA appears to be a major communication signal within and between the plants (Shulaev et al., 1997; Koo et al., 2007; Vlot et al., 2008b).

Although there are so many debates on the role of MeSA in defense, the fact that MeSA is not able to induce tobacco mosaic virus (TMV)-resistance and PR1 expression in NahG plants, depicts that MeSA itself is not biologically active and therefore it has to be converted to SA for activation of defense mechanisms (Seskar et al., 1998). On the other hand, transgenic Arabidopsis plants overexpressing OsBSMT1 (BSMT1 from *Oryza sativa*) showed susceptibility to *Psm* and it was due to high levels of MeSA and inability to accumulate SA or its glucoside form (SAG) (Koo et al., 2007). Together this data show that MeSA alone can not induce a defense response and it needs to be converted to active SA. SA-BINDING PROTEIN 2 gene (SABP2, At1g26360) which converts MeSA into active SA with its SA-inhibitory methyl esterase activity was identified in tobacco (Forouhar et al., 2005). Grafting experiments showed that SABP2-silenced scions grafted onto wild-type or SABP2-silenced rootstocks of tobacco, failed to establish SAR against TMV (Park et al., 2007b). This is in line with data from Vlot et al., 2008a which showed silencing of several methylesterases in Arabidopsis (AtMES), which are SABP2 orthologs, compromised SAR. Therefore, it was hypothesized that for SAR to happen, the methyl esterase activity of SABP2 is required in systemic leaves and SA-mediated inhibition of this esterase activity is required in primary infected leaves (Park et al., 2007b; Park et al., 2009).

SAR was also blocked when SA methyltransferase (SAMT) was silenced in primary leaves of tobacco which were due to low level of MeSA in these leaves. In addition, tobacco plants infected with TMV were able to transmit a SAR signal despite presence of a bacterial SA hydroxylase (SH) encoded by NahG gene (Park et al., 2007b). Moreover, MeSA treatment of lower leaves was able to induce SAR in upper untreated leaves. It was also shown that Arabidopsis *bsmt1* mutants which failed to accumulate

MeSA upon pathogen infection, also failed to accumulate systemic SA and its glucosidic form and did not develop SAR and surprisingly AtBSMT1 overexpression lines exhibited a compromised SAR as well (Liu et al., 2010). Furthermore, Klessig and colleagues considered MeSA as a mobile SAR signal and they proposed a model in which SA accumulating after TMV infection in tobacco, is converted to MeSA by methyltransferase activity of SAMT in local leaves and feedback inhibition of SABP2 in primary leaves ensures the sufficient amount of MeSA to be produced in infected leaves and to be transmitted to systemic leaves where it will be subsequently reconverted to active SA by methyl esterase activity of SABP2 to further activate downstream defense mechanisms and SAR (Figure 1.5) (Park et al., 2007b; Park et al., 2009; Vlot et al., 2008b). With this data, it was reported that MeSA is a mobile SAR signal in Arabidopsis and tobacco (Park et al., 2007b; Park et al., 2009; Vlot et al., 2008b) and also involves in arachidonic acid-induced SAR in potato (Manosalva et al., 2010).

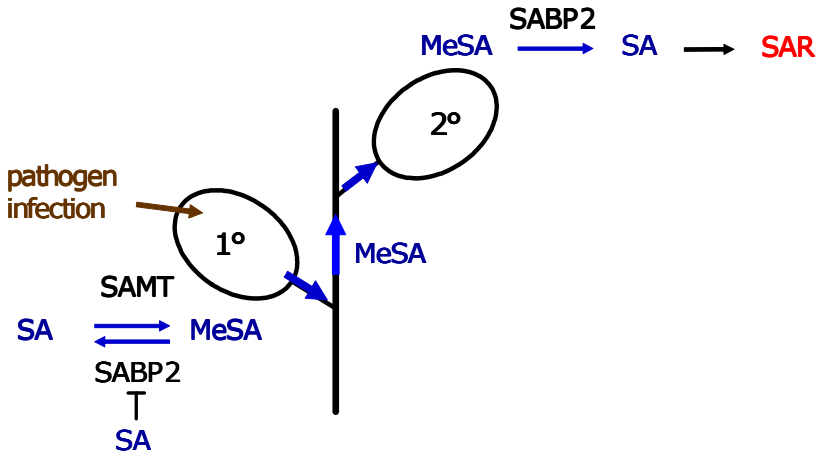

Figure 1.5: Proposed role of MeSA in establishment of SAR (according to Park et al., 2007b, figure provided by Jürgen Zeier)

However, contradictory results in Attaran et al., 2009 argued the role of MeSA in SAR signaling and showed that Arabidopsis *bsmt1* mutants (*bsmt1-1* and *bsmt1-2*) which failed to produce MeSA, did not affect the accumulation of systemic SA, systemic increase of PR1 expression, and a wild-type-like systemic resistance upon *Psm* treatment. Moreover, the local resistance against both compatible and incompatible strains of *Psm* in these mutant lines was the same as in wild-type (Col-0). Attaran et al., 2009 showed that pathogen-induced MeSA emission was significantly attenuated in JA-signaling mutant lines which highlighted the role of JA in MeSA regulation, though a functional role of JA in SAR had been previously ruled out (Attaran et al., 2009). Furthermore, Arabidopsis plants infected with *Pseudomonas syringae pv. tomato* cor$^-$ (*Pst* cor$^-$) triggered SAR to the same extent as infection with *Pst*, though *Pst* cor$^-$ suppressed the level of leave MeSA (Attaran et al., 2009). Coronatine is a phytotoxin and a bacterial virulence factor mimics jasmonates such as jasmonoyl-isoleucine (JA-Ile).

Based on genetic evidence in Arabidopsis, Zeier and colleagues concluded that MeSA produced in infected leaves is not the expected SAR signal and is not required for either biological SAR development or SA-analog 2,6-dichloroisonicotinic acid (INA)-induced SAR. In addition, the role of MeSA as a possible airborne signal for SAR was also excluded since *bsmt1* mutant plants failed to produced and emit MeSA, albeit their wild-type-like SAR phenotypes (Attaran et al., 2009). Based on this data, it was concluded that MeSA and JA signaling are dispensable for SAR induction in Arabidopsis (Attaran et al., 2009).

Following these contradictory observations, questions raised about the role of MeSA in long distance signaling. According to Griebel and Zeier, 2008, several inducible defense responses in Arabidopsis, such as accumulation of SA and expression of PR1, are regulated by light and dependent on the time of day when pathogen attack takes place. Therefore, in this context, subsequent investigations by Liu et al., 2011a examined a possible conditional role for MeSA in long distance signaling. Liu et al., 2011a linked the previously mentioned conflicting results to different experimental design such as developmental age of the plants, light intensity, and/or the strain of bacterial pathogen used in Attaran et al., 2009 and Liu et al., 2010. Klessig and colleagues suggested that the length of light exposure following the primary pathogen infection defines the extend to which MeSA is required for SAR. Based on their results, MeSA and its metabolizing enzymes were essential for SAR when primary infection of Arabidopsis with *Psm* or *Psm AvrRpt*^{2}*cor*$^{-}$ took place in the late afternoon followed by little or no light prior to the night. However, in morning-infection of plants followed by extended light exposure, MeSA was not necessarily required for SAR, thus it could potentiate SAR in morning (AM)-inoculated plants (Liu et al., 2011a). The same result was observed in tobacco and AM-inoculation restored partial SAR in tobacco lacking methyl esterase or methyltransferase activities. It is important to mention that Liu et al., 2011a also claimed that except *fmo1*, other SAR defective lines such as *dir1-1*, *gly1-1* which were previously reported by this group to be SAR-compromised in evening (PM)-inoculation, were shown to be partial SAR competent upon AM-inoculation (Liu et al., 2011a).

1.3.3.2 Diterpenoid Dehydroabietinal (DA)

Abietane diterpenoids are among natural components of plants with high demand in pharmaceutical and medical fields. They are components of resins extracted from angiosperms and conifers (Hanson, 2009; González, 2014). Diterpenoid Dehydroabietinal (DA) was identified in petiole exudate from treated leaves of Arabidopsis with an avirulent pathogen (Avr Pex) and considered as a putative SAR inducer in Arabidopsis, tobacco, and tomato plants (Chaturvedi et al., 2012). External DA, is able to induce SAR against virulent strains of *Pseudomonas syringae* and fungal pathogen *Fusarium graminearum* in Arabidopsis in a dose-dependent manner (more than 1 picomolar). Recovery of locally-applied deuterium-DA in systemic leaves of Arabidopsis within 15 min after application, demonstrates its systemic rapid movement (Chaturvedi et al., 2012). Pathogen inoculation does not really induce DA accumulation in leaves or petiole exudate, however, DA deriven from petiole exudate of leaves infiltrated with avirulent pathogen (Avr Pex), is enriched in a biological active high molecular weight (HMW) fraction (>100 kD) which is likely to be the SAR inducer. In contrast, petiole exudate from mock plants show low molecular weight (LMW) DA enrichment (<30 kD) which are unable to induce SAR (Chaturvedi et al., 2012).

Trypsin treatment of Avr Pex, not only reduce the SAR-inducing capacity, it also reduces the level of DA, indicating an association of DA with a trypsin-sensitive protein(s). DA-induced resistance depends on SA and SA-biosynthesis and -signaling pathways since transgenic NahG plants and double mutant *ics1ics2* exhibited attenuated SAR and it is also dependent on NPR1 (Chaturvedi et al., 2012). It is also considerable that DA induces local accumulation of MeSA and systemic induction of METHYL SALICYLATE ESTERASE 9 (MES9) expression which converts MeSA to SA (Chaturvedi et al., 2012).

Beside components of SA-signaling pathway, DA interacts with other potential SAR components as well. SFD1 (encoding plastidal G3P and suppressor of *ssi2*-conferred SAR) and Azelaic acid (AzA) and its induced gene AZI1, enhance DA effectiveness in SAR. This is proved by observing SAR activation when DA and AzA were co-applied at concentrations at which they were individually failed to promote SAR (Chaturvedi et al., 2012; Jung et al., 2009). Moreover, DA-induced resistance is dependent on two main SAR regulators DIR1 and systemic FMO1, since DA-induced SAR is compromised in both *dir1* and *fmo1*, thus DA-induced increase and decrease of systemic SA is observed respectively in these two lines (Chaturvedi et al., 2012; Dempsey and Klessig, 2012). So far, there is no gene identified in biosynthesis of DA and therefore no DA knockout lines are available to further corroborate the functional role of DA in SAR establishment.

1.3.3.3 Azelaic acid (AzA) and Azelaic acid induced 1 (AZI1)

Scanning for small metabolites collected in petiole exudate (Pex) of Arabidopsis plants infiltrated with avirulent strain of *Psm*, identified a nine-carbon dicarboxylic acid azelaic acid (AzA) with potential characteristics as a putative SAR signal (Jung et al., 2009). Although the exact AzA biosynthesis pathway is not known yet, it seems a chemical rather than an enzymatic pathway forms AzA in Arabidopsis. AzA is synthesized from 9-oxononanoic acid (ONA) in plastids where esterified ONA in galactolipids is generated from free radical-catalyzed oxidative fragmentation of poly unsaturated C18 fatty acids such as oleic (18:1), linoleic (18:2), and linolenic acids (18:3) (Zoeller et al., 2012). Further oxidation of ONA leads to formation of esterified AzA in galactolipids and hydrolytic release of fragmented fatty acids by lipase may lead to forming free AzA (Zoeller et al., 2012). Due to non-enzymatically biosynthesis pathway of AzA, no gene and therefore no knockout lines for AzA are identified (Zoeller et al., 2012).

Locally injected deuterium-labeled AzA was detected in Pex and systemic leaves of Arabidopsis. Although only 7% of ^{14}C-AzA was transported to distal leaves, it is sufficient to prove its systemic movement independently of the pathogen (Yu et al., 2013). Interestingly, transported AzA in systemic leaves is presented as derivatized products which suggest that AzA derivatization might occur before transport or rapidly after it reaches the distal leaves (Gao et al., 2014a; Yu et al., 2013).

Exogenous AzA is able to induce resistance to *Psm* in wild-type Arabidopsis in a concentration- and time-dependent manner, that means more than 10 µM of AzA is sufficient to induce resistance when applied at least 12 hours prior to pathogen infiltration (Jung et al., 2009). Exogenous AzA confers locally and systemically disease resistance to *Pseudomonas syringae pv. maculicola*. AzA-induced resistance requires SA and components of SA-signaling pathway (Jung et al., 2009). However, recently it has been discovered that unlike Arabidopsis plants, AzA treatment of tobacco (Nicotiana tabacum cv. Xanthi nc) does not alter local and systemic multiplication of compatible (*Pseudomonas syringae pv. tabaci*), incompatible (*Pseudomonas syringae pv. tomato*) bacteria and *tobacco mosaic virus* infections and this response was irrespective of light (Nagy et al., 2017). This is in line with previous studies by Zoeller et al., 2012 which showed that external local application of AzA to Arabidopsis does not prevent growth of *Pst* DC3000 in Arabidopsis.

Azelaic acid and long-distance signaling Although the exact mode of action of AzA in long-distance signaling is still unclear, Zoller and colleagues discussed the role of AzA as a marker for free radical-induced lipid fragmentation associated with oxidative membrane damage and cell death upon pathogen attack (Zoeller et al., 2012). In the context of plant resistance, activation of lipid peroxidation pathways is important since Wittek et al., 2014 showed that ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), as SA upstream regulator, contributes to SAR via directly and indirectly promoting ONA, AzA accumulation by activating lipid peroxidation pathways result in production of these compounds.

Later studies suggested a priming role for AzA in the context of SAR and it was shown that AzA is proposed to induce SAR by priming the plants to accumulate higher levels of SA and SA-mediated defense responses such as PR1 gene expression (Jung et al., 2009), and also by promoting G3P accumulation and up-regulating the transcription of G3P encoding genes GLY1 and GLI1 (Gao et al., 2014a). Furthermore, microarray data analysis identified AZELAIC ACID INDUCED 1 gene (AZI1; At4g12470), a putative lipid transfer protein (LTP), which is required for AzA- and biologically-induced SAR and priming of SA accumulation and signaling (Jung et al., 2009). In parallel to SA-derived signaling pathway, Wendehenne et al., 2014 and Wang et al., 2014 indicate presence of a feedback loop between nitric oxide (NO) and reactive oxygen species (ROS), upstream of AzA, which involves in SAR. ROS is associated with hydrolysis of C18 fatty acids leading to AzA formation (Wendehenne et al., 2014) and in turn AzA induces accumulation of G3P and functions upstream of G3P and DIR1/AZI1 feedback regulatory loop in SAR pathway (Yu et al., 2013) (Figure 1.6). [More details about mentioned feedback regulatory loop between G3P and DIR1/AZI1 are explained in section 1.3.3.5, page 35].

Transport of AzA is not associated with onset of SAR, because SAR defective mutants *dir1*, *azi1*, *gli1*, and *gly1* did not prevent AzA or AzA derivatives uptake and transport to distal leaves (Yu et al., 2013; Shah and Zeier, 2013). Notably, the exact role of AZI1 in AzA uptake and transport is still an issue of controversy since Cecchini et al., 2015 claimed that *azi1* showed significant decrease in ^{14}C-AzA uptake in distal tissues and therefore, AZI1 is partially contributed to AzA or AzA derivatives uptake. This contradictory results from Cecchini et al., 2015 and Yu et al., 2013 might be due to difference in AzA application methods. In addition, Zeier and colleagues did not observe AzA accumulation in petiole exudate of plants infected with *Psm* (Návarová et al., 2012), Thus, it is claimed that AzA translocation is not essential for SAR establishment but it rather enforces systemic immunity during SAR (Shah and Zeier, 2013).

AzA itself is not considered as a long-distance signal but rather it functions as an inducer for SAR signal(s) emission from primary infiltrated Arabidopsis leaves via AZI1 accumulation (Cecchini et al., 2015; Gao et al., 2014a; Jung et al., 2009). AZI1 is considered as a component of SAR which is induced by AzA treatment and encodes a lipid-protein transfer with no homology to DIR1 (section 1.3.3.4). AZI1 mutant lines were shown to be resistant to local bacterial infections but defective in long distance signaling and priming of SA and PR1 expression (Jung et al., 2009). Exogenous AzA and Col-0 Pex (Petiole exudate collected from Pathogen infiltrated Col-0 plants) were not able to restore impaired SAR in *azi1* lines (Jung et al., 2009). Since pathogen-induced Pex collected from *azi1* was inactive in wild-type, and the fact that AZI1 and its close paralog EARLY ARABIDOPSIS ALUMINIUM INDUCED 1 (EARLI1) are locally required for SAR, thus a possible role of AZI1 in production and/or translocation of SAR signal(s) rather than signal perception was estimated (Jung et al., 2009; Cecchini et al., 2015).

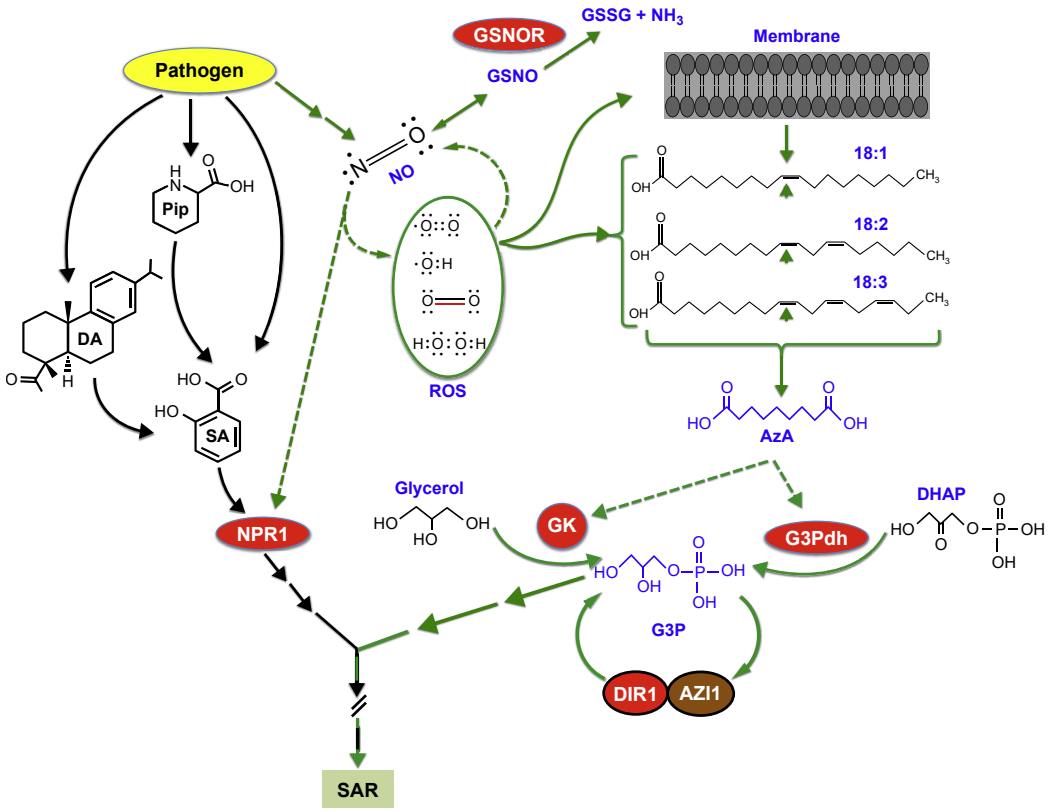

Figure 1.6: A simplified model illustrating chemical signaling during SAR. Infection by avirulent pathogen triggers independent signaling events that lead to the accumulation of salicylic acid (SA) and nitric oxide (NO). NO triggers synthesis of reactive oxygen species (ROS), which comprise of various species including superoxide radicals, singlet oxygen, hydroxyl radical, and hydrogen peroxide. These act in an additive manner to catalyze oxidation of free C_{18} unsaturated fatty acids (FAs) that are released from membrane lipids. NO, and ROS operate in a feedback loop, since mutants defective in ROS biosynthesis do not accumulate NO after pathogen inoculation. Hydrolysis of C_{18} FAs at the $\Delta 9$ double bond (indicate by an arrowhead) generates azelaic acid (AzA), which triggers the biosynthesis of glycerol-3-phosphate (G3P) by upregulating genes encoding the G3P biosynthetic enzymes, glycerol kinase (GK) and G3P dehydrogenase (G3Pdh). G3P and the lipid transfer-like proteins DIR1 (defective in induced resistance) and AZI1 (AzA insensitive) operate in a feedback loop and are interdependent on each other for stability. DIR1 interacts with AZI1 and overexpression of DIR1 and AZI1 can compensate for the lack of AZI1 and DIR1, respectively. The inability of the *azi1* and *dir1* mutants to accumulate pathogen-responsive G3P, together with the inability of *gly1* (defective in G3Pdh)/ *gli1* (defective in GK) mutants to accumulate DIR1/AZI1 proteins even when expressed as transgenes via the constitutive cauliflower mosaic virus 35S promoter, suggests that G3P, DIR1, and AZI1 might operate in a feedback regulatory loop. The *dir1* and *azi1* mutants accumulate normal levels of AzA, suggesting that the inability of *dir1* and *azi1* mutants to induce AzA-mediated SAR is related to their impaired G3P accumulation, not AzA biosynthesis. Reactivity of NO is partly regulated via its storage into GSNO (S-nitrosoglutathione), which can be reduced to glutathione disulfide (GSSG) and NH_3 by GSNOR (S-nitrosoglutathione reductase). The SA and NO/ROS pathways cross-talk at several levels and one of these steps include the S-nitrosylation of NPR1, a key positive regulator of SAR. (Wendehenne et al., 2014)

More advanced investigation on the role of AZI1 in priming and systemic immunity found that AZI1 and EARLI1 are localized in chloroplast outer membrane (OEM) and endoplasmic reticulum (ER) and both are required locally for SAR establishment and uptake/transport of AzA (Cecchini et al., 2015). AZI1 was shown to move in vesicle-like structures in association with ER transcytoplasmic strands connecting chloroplasts, ER and the plasma membrane (Cecchini et al., 2015). AZI1/EARLI1 make a complex together with DIR1 which is localized in perinuclear ER and plasmodesmata which this complex might facilitate contacting the organelles due to transmission of possible SAR signal(s) (Cecchini et al., 2015).

Beside its role in resistance against biotic stress, AZI1 contributes to abiotic stress as well, such as cold-tolerance in Arabidopsis (Xu et al., 2011). Furthermore, AZI1 has been identified as an interacting target for MITOGEN-ACTIVATED PROTEIN KINASE 3 (MAPK3) in Arabidopsis salt-stress responses and *azi1* mutants were shown to be hypersensitive to salt-stress (Pitzschke et al., 2014).

1.3.3.4 Defective in induced resistance 1 (DIR1)

Genetic screening of SAR-defective Arabidopsis Wassilewskija (Ws) mutant lines, identified a type of lipid transfer protein (LTP), DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1; At5g48485) as a key mobile component of SAR (Maldonado et al., 2002). DIR1 is expressed in phloem sieve elements and companion cells in seedling, flowers and untreated leaves of Arabidopsis and has a LTP-like structure, consists of 77 amino acids with eight cysteine residues (considered as typical LTP signature) and a large hydrophobic cavity that can bind with high affinity to two molecules of long-chain fatty acids (Lascombe et al., 2008; Champigny et al., 2011). Additionally, it also consists of a recognition motif (two prolin-rich SH3 domains) which can facilitate interaction between proteins (Lascombe et al., 2008; Champigny et al., 2011). DIR1 is localized in endoplasmic reticulum (ER) and cell periphery and contains an N-terminal signal sequence which directs it to the cell wall (Champigny et al., 2011; Cecchini et al., 2015).

dir1 mutants exhibited wild-type local resistance to virulent and avirulent strains of *Pseudomonas syringae*, but failed to express systemic PR1 gene and to develop SAR upon these pathogen inoculation, though *dir1* mutant was not defective in basal and systemic SA accumulation (Maldonado et al., 2002). For SAR induction, a cytosolic pool of DIR1 was shown to be important, since the defective-SAR response in *dir1* can be restored by DIR1, lacking ER signal sequence, to the same extent as entire protein (Champigny et al., 2011).

Avr Pex collected from *dir1-1* plants, fail to induce PR gene expression and resistance in wild-type Ws, however *dir1* were responsive to PR1 expression induced by Avr Pex collected from Wild-type (Maldonado et al., 2002). Based on this data, DIR1 was considered as a good candidate taking role in synthesis and/or translocation of the SAR signal. Chaturvedi and colleagues also showed that co-application of Avr Pex from *dir1* with that of *sfd1* or *fad7* is able to reconstitute SAR activity to *Psm* and PR1 transcription in wild-type which was previously failed when Avr Pex of *sfd1*, *fad7*, and *dir1* applied individually. Thus, DIR1 and a plastid glycerolipid-dependent factor might both be required for defense (Chaturvedi et al., 2008). Subsequent studies, focusing on G3P as a putative mobile SAR signal, reported that G3P-conferred SAR is dependent on DIR1 and these two components require each other for translocation to systemic leaves (Chanda et al., 2011). Later on, more data published in Yu et al., 2013, reported on existence a feedback regulatory loop between DIR1/AZI1 and G3P which is regulated by AzA upstream of this unit (More description of this regulatory loop is written in section 1.3.3.5, page 35). However movement and uptake of AzA were shown to be dependent on AZI1, but not DIR1 (Cecchini et al., 2015). Among other putative SAR signals, DA-induced resistance is also shown to be dependent on DIR1 and DIR1 is required for increasing of systemic DA-induced SA to confer SAR (Chaturvedi et al., 2012). On the other hand, DIR1 was also shown to limit systemic expression of AtBSMT1 upon *Psm* inoculation and thus limiting the conversion of SA to MeSA, and this way DIR1 is contributed to SAR by increasing systemic SA (Liu et al., 2011b).

Further investigation by Cameron and his colleagues showed that *dir1-1* mutant lines in Arabidopsis Ws-2 background, are not absolutely SAR-defective and these lines occasionally exhibit SAR-response to the pathogen (Champigny et al., 2013). They assumed the partially SAR-competence phenotype of *dir1-1*, is attributed to a DIR1 paralog, DIR1-like protein (At5g48490) which was detected in Pex of *dir1* plants and has already similar protein structure as *dir1*, but reduced capacity to move systemically during SAR (Champigny et al., 2013). Although DIR1 and DIR1-like expression are reduced in both inoculated and non-inoculated leaves during SAR induction, a low amount of these proteins were shown to be sufficient for SAR response in Arabidopsis Ws (Champigny et al., 2011; Champigny et al., 2013). However, a recent publication by Carella et al., 2017 has investigated the requirement of DIR1 and DIR1-like protein in Arabidopsis Col-0 accession (using *dir1-2* mutant lines generated in Col-0 background) and provided data has shown that unlike Ws, DIR1 but not DIR1-like protein is required for SAR establishment in Col-0. Based on this result, DIR1 and DIR1-like both display similar subcellular localization pattern in tobacco and both are able to form homo- and heterodimer with one another in yeast and in planta (Carella et al., 2017). Nevertheless, DIR1 is required for SAR establishment in Col-0 and Ws but DIR1-like is only partially contributed to SAR in Ws and does not significantly contribute to SAR in Col-0 (Champigny et al., 2013; Carella et al., 2017).

In 2011, Klessig and his colleagues assume a conditional role of DIR1 in the context of SAR. According to Liu et al., 2011a, the time of inoculation influenced SAR in *dir1-1* mutants, showing SAR was restored in *dir1-1* after morning inoculation with *Psm AvrRpt2 cor*$^{-}$ compared to *dir1-1* plants inoculated in the evening.

Phylogeny DIR1 analysis provided evidence that DIR1 is conserved and DIR1 orthologs exist in Brassica family (*Arabidopsis thaliana*, *Arabidopsis lyrata*) as well as agricultural relevant plants such as tobacco, tomato, cucumber, and soybean (Isaacs et al., 2016) and functional role of DIR1 orthologs in SAR has been also proved in tobacco (*Nicotiana tabacum*) and cucumber (*Cucumis sativus*) (Isaacs et al., 2016; Liu et al., 2011b).

1.3.3.5 Glycerol-3-phosphate (G3P)

Biosynthesis pathways of G3P Glycerol-3-phosphate (G3P) is a three-carbon sugar and the main component of membrane glycerolipid biosynthesis. In Arabidopsis thaliana, there are two metabolic pathways for G3P biosynthesis. In Arabidopsis, G3P is synthesized either through phosphorylation of glycerol which is catalyzed by GLYCEROL INSENSITIVE 1 (GLI1; At1g80460) (also known as NON-HOST RESISTANCE TO *P. s. phaseolicola 1* (NHO1)) or it is synthesized via NADH-dependent G3P dehydrogenase (GPDH) activity of GLY1 (At2g40690) which encodes dihydroxyacetone phosphate (DHAP) reductase. DHAP is derived from glycolysis through activity of triosephosphate isomerase on glyceraldehyde-3-phosphate or it can also be derived from conversion of glycerol to dihydroxyacetone (DHA) via glyceroldehydrogenase (GlyDH) which is followed by phosphorelation of DHA to DHAP regulated by DHA kinase (DHAk). GLY1 is also known as SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1 (SFD1 in Arabidopsis accession Nössen) and it carries a mutation in SFD1 gene in Arabidopsis accession Columbia. SFD1 contains a chloroplastic transit signaling peptide and has DHAP reductase activity (referred as G3P dehydrogenase) which this activity is required for plastid lipid metabolism contributing to plant defense mechanisms and SAR (Lorenc-Kukula et al., 2012; Nandi et al., 2004).

In addition to plastidal Glycerol-3-phosphate dehydrogenase (GPDH), GLY1 (At2g40690), Arabidopsis genome encodes four other GPDH isoforms. Two cytosolic (At2g41540, At3g07690), one mitochondrial (At3g10370), and one plastidal GPDH (At5g40610). There are so many reports on characterization and biological function of G3P dehydrogenases (GPDH), but their direct contribution to plant disease resistance remains to be elucidated. Although there is no direct contribution of cytosolic (At3g07690) and plastidal (At5g40610) GPDHs to the total plastidal lipid pool, these two GPDHs are shown to be SAR defective like GLY1 (Chanda et al., 2011). Mitochondrial G3P dehydrogenase is essential for G3P shuttle and glycerol metabolism in Arabidopsis as well as yeasts and animals. Transporting of metabolites and reducing equivalents between cytosol and mitochondria are important for mitochondrial metabolism. Metabolite exchange between these two compartments depends on the concentration of metabolites and redox status of cytosol which is regulated by mitochondrial GPDHs. G3P shuttle is regulated by two components: i) NAD-dependent cytosolic G3P dehydrogenase (GPDHc) which catalyzes DHAP to G3P ii) a membrane-bound FAD-dependent GPDH in outer surface of inner mitochondrial membrane which catalyzes conversion of G3P to DHAP and forms $FADH_2$ from FAD (Shen et al., 2003; Shen et al., 2006).

G3P-mediated Lipid biosynthesis G3P is catabolized by converting to glycerol via Glycerol-3-phosphatase (GPP) or it will be utilized for biosynthesis of glycerolipids which are the most abundant type of lipids in plant cells. Higher plants consist of two pathways for glycerolipid biosynthesis: prokaryotic pathway in chloroplast inner membrane and eukaryotic pathway in endoplasmic reticulum (ER) (Ohlrogge and Browse, 1995). Fatty acid synthesis in plastids leads to formation of palmitic acid (16:0)-, Stearic acid (18:0)- and, oleic acid (18:1)-acyl carrier protein (ACP) (Ohlrogge and Browse, 1995). These fatty acids are involved in synthesis of glycerolipid through prokaryotic pathway in plastid or they are exported as CoA thioesters to ER for eukaryotic glycerolipid biosynthesis. Conversion of stearic acid (18:0) to oleic acid (18:1) is mediated by stearoyl-acyl-carrier-protein-desaturase (SUPPRESSOR OF SA-INSENSITIVITY 2) SSI2/FAB2 (Shanklin and Cahoon, 1998). Therefore *ssi2* mutant plants have low level of oleic acid and these plants exhibit dwarf phenotypes, shown spontaneous lesion formation, accumulate high level of SA and express SA-mediated, NPR1-dependent and -independent defense responses (Nandi et al., 2003; Kachroo et al., 2001).

In parallel, constitutive activation of SA-signaling pathway in *ssi2* causes deactivation of JA-mediated signaling pathways and inability of JA to induce PDF1.2 and consequently susceptibility of Arabidopsis mutants *ssi2* to *Botrytis cinerea* (Nandi et al., 2003; Kachroo et al., 2001; Kachroo et al., 2004). *ssi2*-conferred phenotypes and defense responses are shown to be suppressed by *sfd* mutant alleles. *sfd1*, *sfd2* and, *sfd4* suppress *ssi2*-conferred phenotype and defense responses and consequently suppresses *ssi2* resistance to *Psm* (Nandi et al., 2003). In addition, a major step of glycerolipid biosynthesis in plastids are acylation of oleic acid 18:1 fatty acid (product of SSI1) by G3P which is regulated by ACYLTRANSFERASE1 (ACT1) (Kachroo et al., 2004). Therefore, *ssi2* plants carrying a loss-of-function mutation in plastidal G3P-acyltransferase (*act1*) and concurrently contain elevated level of 18:1 are restored in *ssi2*-triggered phenotype (Kachroo et al., 2003). Plastidal GLY1-encoded GPDH mutant plants, containing low level of G3P to quench 18:1 fatty acid, are shown to restore *ssi2*-triggered phenotype in an age-dependent manner. Exogenous application of glycerol to *ssi2* and *ssi2gly1-3* plants regenerates G3P pool and therefore it causes the reappearance of *ssi2*-like phenotype (Kachroo et al., 2004). As a consequence, balance between plastidal fatty acid and G3P pool is an important factor for mediating defense signaling pathways and in this context, the role of ACT1 and GLY1 is considerable. More details

on G3P and fatty acid metabolism in plants is shown in Figure 1.7.

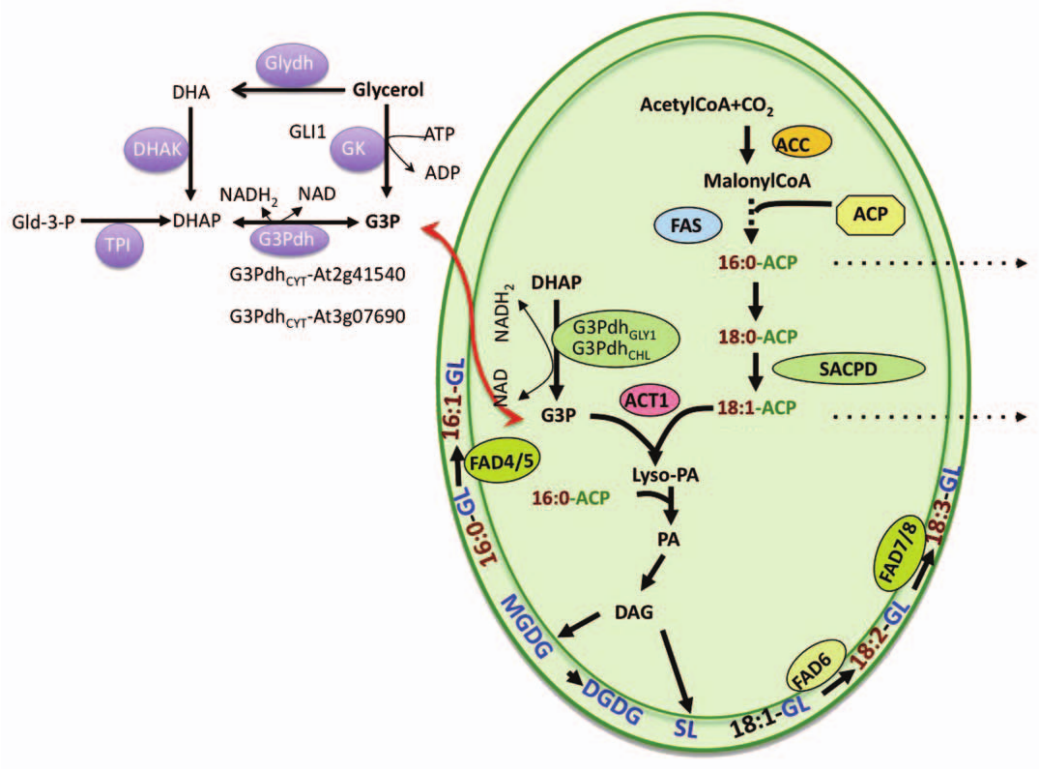

Figure 1.7: A condensed scheme of glycerol-3-phosphate (G3P) metabolism in plants. Glycerol is phosphorylated to G3P by glycerol kinase (GK; GLI1). G3P can also be generated by G3P dehydrogenase (G3Pdh) via the reduction of dihydroxyacetone phosphate (DHAP). DHAP is derived from glycolysis via triosephosphate isomerase (TPI) activity on glyceraldehyde-3-phosphate (Gld-3-P) or from the conversion of glycerol to dihydroxyacetone (DHA) by glycerol dehydrogenase (Glydh) followed by phosphorylation of DHA to DHAP by DHA kinase (DHAK). G3Pdh isoforms are present in both the cytosol and the plastids (represented by the oval). GLY1 is one of the two plastidial G3Pdh isoforms that plays an important role in plastidial glycerolipid biosynthesis. In the plastids, G3P is acylated with oleic acid (18:1) by the ACT1-encoded G3P acyltransferase. This ACT1-utilized 18:1 is derived from the stearoyl-acyl carrier protein (ACP)-desaturase (SACPD)-catalyzed desaturation of stearic acid (18:0). The 18:1-ACP generated by SACPD either enters the prokaryotic lipid biosynthetic pathway through acylation of G3P or is exported out (dotted line) of the plastids as a coenzyme A (CoA)-thioester to enter the eukaryotic lipid biosynthetic pathway. Membranous fatty acid desaturases (FAD) catalyze desaturation of FAs present on membranous glycerolipids. Other abbreviations used are: GL, glycerolipid; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; Lyso-PA, acyl-G3P; PA, phosphatidic acid; PG, phosphatidylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SL, sulfolipid; DAG, diacylglycerol. (Mandal et al., 2011)

G3P and plant defense mechanisms In recent years, there has been an increasing interest in plants resistance mechanisms towards different type of plant-pathogen interactions such as non-host, gene-for-gene resistance and especially specific form of resistance such as SAR. There are so many reports on contribution of G3P and its metabolism in defense against different type of pathogens. As previously mentioned, G3P in synthesized via phosphorelation of glycerol by glycerol kinase activity of GLI1 (NHO1) or from DHAP by the DHAP reductase activity of GLY1. NHO1 (GLI1) is shown to be required for non-host and gene-for-gene resistance against *Pseudomonas* bacteria and also fungi such as *Botrytis cinerea* in Arabidopsis. However, *nho* mutants have no effect on growth of virulent *Pseudomonas* strains *P.s. maculicola* and *P.s. tomato* DC3000 (Lu et al., 2001). *P.s. tomato* DC3000 inoculation suppresses the transcript level of NHO1. This suppression is likely to be regulated by JA-signaling pathway and COI1 is required for NHO1 suppression by DC3000 (Kang et al., 2003).

SUPPRESSOR OF FATTY ACID DESATURASE 1 (SFD1) was discovered to be involved in lipid metabolism and it was shown to be required for SAR establishment but not in basal resistance against pathogen in Arabidopsis. As previously mentioned, in Arabidopsis accession Nössen, this gene catalyzes synthesis of G3P by its DHAP reductase activity. *sfd1* mutant lines are compromised in SAR and impaired in accumulation of systemic SAR-associated SA and PR1 gene expression after inoculation of local leaves with avirulent pathogen (Nandi et al., 2004). In addition to SFD1, Arabidopsis FATTY ACID DESATURASE 7 (FAD7), SFD2 and MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE 1 (MGD1) which highly involved in plastidal glycerolipid synthesis are shown to be important for SAR activation (Chaturvedi et al., 2008). *fad7*, *sfd2* and, *mgd1* mutant lines are compromised in SAR and Avr Pex collected from these lines fail to promote resistance against *Psm*. However, co-application of Avr Pex from *dir1* with *sfd1* or with *fad7* was able to promote resistance in Wild-type plants. This suggests that a plastid glycerolipid-dependent factor along with DIR1-encoded lipid transfer protein in Pex are required for SAR establishment (Chaturvedi et al., 2008).

Previous studies had shown a major role of G3P for synthesis of plastidal lipids. In 2011, G3P was described as a critical SAR inducer and plants defective in G3P synthesis were shown to be SAR defective (Chanda et al., 2011; Mandal et al., 2011; Gao et al., 2014a). *gly1* and *gli1* were shown to be SAR defective but this effect was restored by applying exogenous G3P to these plants. Notably, application of exogenous G3P alone caused a slight increase in SAR in wild-type plants and the SAR-inducing capacity of G3P was more increased when it was mixed with petiole exudate of plants infiltrated with $MgCl_2$ or *AvrRpt2* pathogen (Chanda et al., 2011). Although applied G3P does not increase the level of SA, G3P-conferred SAR is dependent on basal SA (Gao et al., 2014a). Unlike *sfd1*, *gly1* and *gli1* were still able to accumulate SAR-associated systemic level of SA and PR1 expression (as well as azelaic acid and jasmonic acid) and impaired SAR in these lines were contributed to low G3P level, but not fatty acid or lipid flux (Chanda et al., 2011). Moreover, G3P-conferred SAR was shown to be DIR1-dependent because G3P mixed with petiole exudate collected from *dir1* infiltrated with $MgCl_2$ or *Avr* was not able to induce SAR in wild-type Ws or in *dir1* (Chanda et al., 2011). In this context, DIR1 is likely to play a role in translocation of G3P to distal leaves since the infiltrated C_{14}-labeled G3P was not able to move to distal tissues but its co-infiltration with DIR1 caused translocation of radiolabeled G3P to systemic tissues (Chanda et al., 2011). Therefore, existence of a feedback regulatory loop between G3P, DIR1, and AZI1 was predicted and demonstrated in Yu et al., 2013 which is explained below.

Playing role in long-distance signaling, G3P levels are also shown to be associated with basal resistance of Arabidopsis against hemibiotrophic fungus *Colletotrichum higginsianum*. Infection of Arabidopsis with this fungus increases the level of G3P and plants impaired in G3P production (*gly1* and *gli1*) are susceptible to the fungus infection (Chanda et al., 2008). Since *gly1* shows more susceptibility compared to *gli1*, it is more likely that GLY1 is the major contributor to G3P pool and defense responses (Chanda et al., 2008; Venugopal et al., 2009).

Feedback regulatory loop between G3P, DIR1, and AZI1 in SAR G3P and DIR1 are both required for SAR establishment. An obvious interdependency of G3P and DIR1 was proved in Chaturvedi et al., 2008 when Avr-Pex from *sfd1* failed to establish SAR in wild-type unless it was mixed with Avr-Pex from *dir1*. DIR1 might facilitate the translocation of G3P to systemic tissue (Chanda et al., 2011). Furthermore, Azelaic acid (AzA), as a priming component of SAR, induces G3P biosynthesis in wild-type in

absence of pathogen. This suggests that AzA functions upstream of G3P in long distance signaling pathway (Yu et al., 2013). Notably, Like AzA, 18:1 and 18:2 fatty acids (as AzA precursors) application results in accumulation of G3P in wild-type, but *gli1* and *gly1* are insensitive to both AzA and 18:1, 18:2 fatty acids (Yu et al., 2013). AzA- and G3P-induced immunity during SAR are dependent on Lipid-transfer proteins (LTPs) DIR1 and AZI1 which are likely to function downstream of AzA and G3P and additionally, G3P regulates the stability of DIR1 and AZI1 transcripts. *dir1* and *azi1* accumulate wild-type level of AzA but reduced level of G3P and subsequently GLY1 and GLI1 transcripts. Overexpression of DIR1 and AZI1 compensate *azi1* and *dir1* respectively which proves that these two LTPs are interacting with self and with each other (Yu et al., 2013). Interestingly, AZI1 and DIR1 both are localized in plasmodesmata and endoplasmic reticulum. Together this data outlines that there is an interconnection loop between LTPs (AZI1 and DIR1) and G3P and upstream of this loop is AzA to mediate SAR (Yu et al., 2013) (Figure 1.8).

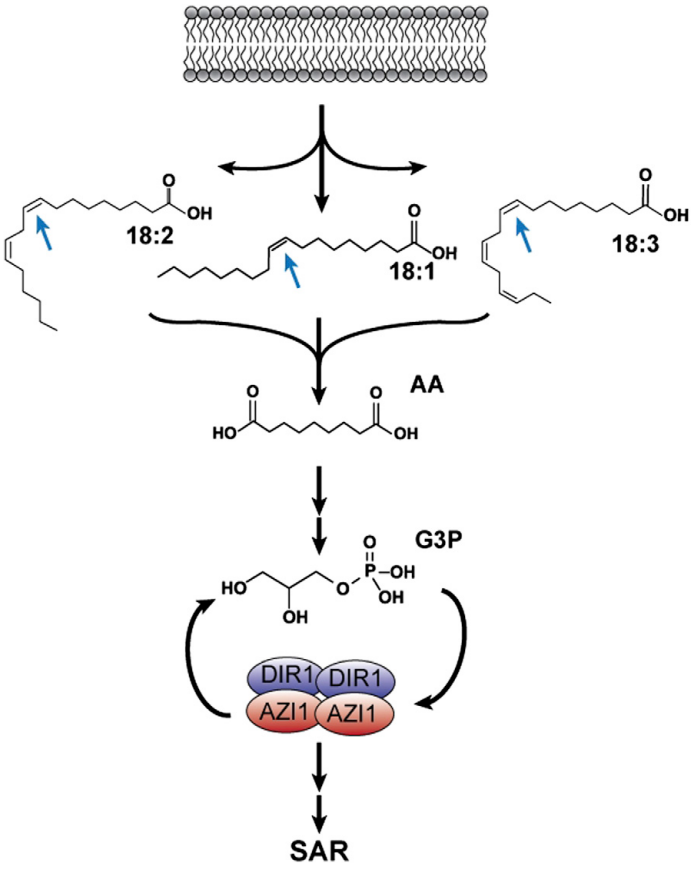

Figure 1.8: Model Illustrating AzA- and G3P- Mediated Systemic Signaling Inoculation of an avirulent pathogen triggers the release of free unsaturated C_{18} Fatty Acids (FAs), which undergo oxidative cleavage at carbon 9 to form AzA (shown by blue arrows). AzA induces SAR because it induces G3P biosynthesis by upregulating the transcription of GLY and GLI1 genes. G3P-mediated SAR is dependent on the cytosolic DIR1 and AZI1 proteins, which interact with each other and require G3P for the stability of their respective transcripts. Conversely, DIR1 and AZI1 are required for G3P biosynthesis, suggesting that G3P and DIR1/AZI1 regulate SAR via a feedback loop (Yu et al., 2013)

1.4 Priming

A primed status is a physiological condition in which the plant is able to respond stronger and faster to subsequent biotic (such as infection by a pathogen) and/or abiotic stresses and this is associated with basal and systemic immunity (Conrath, 2011). The primed status in a plant is induced upon recognition of pathogen-, microbe-, and damaged-associated molecular patterns (PAMPs, MAMPs, and DAMPs) and effectors, several natural and synthetic chemical compounds (such as β-aminobutyric acid (BABA), probenazole, benzothiadiazole (BTH), and salicylic acid (SA)), and mechanical and abiotic stimuli (such as wounding, cold, heat, and salt) (Kohler et al., 2002; Oostendorp et al., 2001; Mishina and Zeier, 2007b; Singh et al., 2014; Benikhlef et al., 2013; Chassot et al., 2008). It is noteworthy that BABA, which was not previously detected in plants, is demonstrated to be a natural product since it has been recently detected and quantified in plant tissues and endogenous moderate levels of BABA is shown to increase after infection with necrotrophic, biotrophic, and hemibiotrophic pathogens (Thevenet et al., 2017).

Defense priming establishes not only in the local tissue exposed by one of these priming factors (or stimuli) but also in systemic, untreated tissues. The defense priming processes comprise systemic acquired resistance (SAR), induced systemic resistance (ISR), β-amino butyric acid (BABA)-induced resistance (BABA-IR), and wound-induced resistance (Pieterse et al., 2014). Following perception of a stimulus, a plant undergoes several physiological, transcriptional, molecular, and epigenetic changes which prepare it for enhanced responses toward challenging stress. This biological process of acquiring priming, which takes place from the initial stimulation through the exposure to a challenging stress is defined as priming phase (Mauch-Mani et al., 2017). One of the important biological changes during priming phase is accumulation of dormant mitogen-activated protein kinases (MPKs). BTH-induced Arabidopsis exhibited accumulation of transcripts and proteins of MPK3 and MPK6 which react stronger towards mechanical stress (Beckers et al., 2009). Furthermore, BTH-treatment along with activated *P. syringae*-induced SAR is able to prime WRKY transcription factor genes WRKY29, WRKY6, and WRKY53. BTH-treatment alone had a minor effect on these gene expression levels (Jaskiewicz et al., 2011). In Arabidopsis, BABA-application or inoculation with *Pseudomonas fluorescens* WCS417r induced the expression of transcription factors associated with defense response mechanisms (Van der Ent et al., 2009).

In addition to transcriptional changes, there are several metabolic changes take place during priming phase. This involves the accumulation of defense-related hormones, hormone conjugates, phytoanticipins, and indolic glucosinolates (Gamir et al., 2014). For instance, two glycosylated form of SA (SAG and SGE) are accumulated during BABA-induced and natural priming by avirulent bacteria (Pastor et al., 2014). The priming role of SA was discovered when pretreatment of cultured parsley cells with SA, primed defense genes such as PAL for enhanced expression (Thulke and Conrath, 1998). Pretreatment of plants with SA or the SA-analogues, BTH and 2,6-dichloroisonicotinic acid (INA), induces local PR1 gene expression, a marker for SA-signaling in plants (Conrath et al., 2002; Kohler et al., 2002).

In the context of SAR, inoculation of first leaves of Arabidopsis with a SAR-inducing pathogen (e.g. *Psm*), leads to activation of several defense priming responses in systemic leaves (Návarová et al., 2012). This biologically-induced primed state, causes the plants to respond faster and stronger to the

subsequent pathogen attack. The priming responses in biologically-induced plants include increase in defense-related gene expression, SA biosynthesis and accumulation of the phytoalexin camalexin and the systemic activation of defense priming responses are totally depend on FMO1 (NHP biosynthesis gene) and ALD1 (Pip biosynthesis gene) (Návarová et al., 2012). Thus, Pip/NHP are considered as endogenous mediators of defense priming.

Application of 10 ml of 1 mM (= 10 µmol) Pip via the roots and prior to the pathogen inoculation, renders the plants into a primed SAR-like state which means the plants are able to respond more quickly and vigorously to subsequent pathogen attack (Návarová et al., 2012). This phenomenon is designated as defense priming (Conrath et al., 2015). Pip, when applied exogenously, positively regulates accumulation of SA, camalexin and increases transcript levels of defense-related genes such as ALD1, FMO1, and PR1 in wild-type plants (Návarová et al., 2012; Bernsdorff et al., 2016). Arabidopsis *ald1* plants, show attenuated basal resistance to *Psm* and they are totally compromised in SAR (Song et al., 2004b; Návarová et al., 2012). Feeding the plants with Pip, increases the resistance towards *Psm* and *Psm AvrRpm1* in wild-type Col-0 plants and it compensates the attenuated local resistance in *ald1* and its defect in SAR (Návarová et al., 2012). Exogenous Pip boosts the local and systemic SA biosynthesis in both wild-type and *ald1*, indicating the importance of Pip in systemic SA and in amplification of systemic defense responses (Návarová et al., 2012). Exogenous Pip increases ALD1 transcript levels, indicating a positive regulatory role of Pip on its own biosynthesis. Besides ALD1, Pip enhances *Psm*- triggered FMO1 and PR1 transcription (Návarová et al., 2012). These data highlights the role of endogenous Pip in defense priming as an integral part of SAR.

Moreover, exogenous Pip or NHP induces a moderate but still significant resistance in SA-deficient *sid2* plants, showing that Pip and NHP orchestrates SA-dependent and SA-independent priming of pathogen responses (Bernsdorff et al., 2016). Exogenous SA also induces the expression of pathogenesis-related gene 1 (PR1) and the SA-induced response is strongly potentiated when wild-type Arabidopsis is pretreated with Pip (Bernsdorff et al., 2016). This additive effect of Pip on SA-induced responses is absent in *fmo1*, indicating a functional role of NHP in amplification of SA-induced responses (Bernsdorff et al., 2016; Hartmann et al., 2018).

Among SAR putative signals, Pip and AzA are considered indispensable for systemic priming (Návarová, 2012; Jung et al., 2009). As Arabidopsis *ald1* mutants are compromised in SAR-associated priming response as well as BABA-induced resistance (BABA-IR), thus the role of Pip, as the main mediator of SAR, is crucial in SAR-induced priming and BABA-IR (Návarová, 2012). A priming role of AzA was proved by observing that AzA treatment of wild-type plants did not induce SA accumulation and PR1 expression, but rather primed their enhanced activation after a subsequent pathogen inoculation (Jung et al., 2009). Moreover, a mutation in AZA-responsive AZI1 gene led to the specific loss of bacterial- and AZA-induced priming of SA accumulation and exhibited attenuated SAR (Jung et al., 2009).

The priming function of Pip is not restricted to Arabidopsis and exogenous Pip is also able to prime tobacco for strong accumulation of SA and nicotine upon *Pseudomonas syringae pv tabaci (Pstb)* infection (Vogel-Adghough et al., 2013). Thus, exogenous Pip amplifies the immune responses of both tobacco and Arabidopsis to compatible (*Pstb*) and HR-inducing (*Psm*) bacterial pathogens (Vogel-Adghough et al., 2013).

Biologically activation of SAR by inoculation of 1° leaves of Arabidopsis with *Psm*, leads to transcriptional reprogramming in systemic leaves and several genes will be up- (SAR^+) or down-regulated (SAR^-) (Gruner et al., 2013). The group of SAR^+ genes, consist of genes associated with SA-associated defenses, signal transduction, transport, and the secretory machinery and the SAR^- group consists of genes activated by JA/ET-defense pathway, and genes associated with cell wall remodeling and biosynthesis of constitutively produced secondary metabolites (Gruner et al., 2013). The systemic transcriptional reprogramming is absent in *ald1* and *fmo1*, showing a functional role of Pip and NHP in this context (Gruner et al., 2013). Moreover, treatment of Arabidopsis plants with Pip, enhances the transcription of about 700 genes which are in the category of SAR^+ genes, thus exogenous Pip induces the transcription of immune regulatory genes (Hartmann et al., 2018). The transcriptional responses to exogenous Pip is fully depended on FMO1 (Gruner et al., 2013; Hartmann et al., 2018). Therefore, FMO1-generated NHP induces the defense-related genes upregulated by exogenous Pip (Hartmann et al., 2018).

The functional roles of Pip on activation of defense priming, induction of resistance and reprogramming the transcription of genes, are all dependent on FMO1 and its Pip-derived NHP product. Exogenous NHP causes changes in the expression of defense-related genes in systemic leaves to enhance resistance to bacterial pathogen (Chen et al., 2018). Arabidopsis wild-type and *fmo1* plants treated with NHP, accmulated the highest levels of ICS1, ALD1, SARD4, PR1, PR2, PR5, and SAG13 mRNA in treated lower and non-treated upper leaves compared with plants treated with water or Pip (Chen et al., 2018). This indicates the regulatory function of NHP on Pip or SA by induction of their related biosynthesis genes. In addition, NHP positively regulates transcription of FMO1 by a positive feedback loop and it induces SAR in wild-type and in *fmo1* plants whereas, exogenous Pip was not able to induce resistance in *fmo1* (Chen et al., 2018; Hartmann et al., 2018). Therefore, Pip-derived NHP is a major regulator of SAR, which increases the defense of plants by activating the immune-associating gene expression, priming the plants for effective defense responses, and amplifying the SA-induced resistance (Hartmann and Zeier, 2018).

Priming processes are also linked to epigenic changes which are likely to function as a long-term memory of previous stresses for plants. Alteration in chromatin structure by initial priming stimuli will cause the promoters of priming-related genes to be more accessible and to be easily activated. As Jaskiewicz et al., 2011 demonstrated that priming stimuli such as BTH-treatment or *Psm* inoculation caused an alteration in chromatin structure surrounding the promoter of WRKY29, WRKY6, and WRKY53. This modification is associated with trimethylation of histone (H3) at lysine 4 (H3K4me3) and it was blocked in the priming-deficient *npr1* mutant plants (Jaskiewicz et al., 2011).

Epigenic Changes such as DNA methylation can be inheritable and there are several reports showing higher resistance in the progeny of plants infected with a priming-induced pathogen (Holeski et al., 2012; Slaughter et al., 2012). For instance, it was shown by Roberts, 1983 that acquired resistance to tobacco mosaic virus (TMV) was transmitted to the progeny of hypersensitive tobacco. Chromatin modification might also function as a memory for SAR as Luna et al., 2012 showed an association between this transgenerational memory and acetylated histone H3 at lysine 9 (H3K9) at PR1, WRKY6, and WRKY53 promoters. *npr1* failed to establish transgenerational defense phenotype, suggesting a critical role of NPR1 in transgenerational SAR (Luna et al., 2012).

1.5 Environmental factors and plant defense responses

1.5.1 Effect of light on plant defense responses

Living in a dynamic environment, plants require a system to aware them of the situation in surrounding area. Light, as an environmental factor, acts as a source of information for plants which helps them adapt their growth, metabolism, and development to the changing environmental circumstances. By perceiving light quantity (light fluence rate) and quality (color, wavelength), plants obtain information about day length, the direction of light, the presence of competitors, and shading. Moreover, light is a major factor for photosynthesis to produce required energy and carbohydrates in plants. Light directly or indirectly affects the circadian clock, hormonal status, developmental status of the chloroplast, and regulation of gene expression in plants (Casal and Yanovsky, 2005; Devlin, 2000). It is noteworthy to mention that beside light, temperature is another determinant factor in plant immunity which is in close association with light and humidity. Similar changes in the temperature do not modulate the different plant and pathogen interactions in a same way (Hua, 2013).

Several pathways in plants were shown to be light-dependent such as the production of elicitor-induced phenylpropanoid-derived phytoalexin precursors in soybean cotyledons (Graham and Graham, 1996). Regulation of phenylpropanoid pathway by light was also observed in light-grown Arabidopsis roots, showing higher expression of phenylpropanoid genes and a higher level of phenylpropanoid-derived metabolites (Hemm et al., 2004). Moreover, according to Lam et al., 1995, the level of amino acids was shown to be different in light-grown and dark-adapted wild-type Arabidopsis as shown in Figure 1.9. In the light, when photosynthesis happens, under high C:N ratio, nitrogen (N) will be assimilated to glutamine which will be used as an nitrogen source for biosynthesis of other amino acids. In contrast, the level of asparagine, will be decreased in light and it will be accumulated in dark-adapted plants in which the ratio of C:N is low. Therefore, light and its role on photosynthesis affect the metabolisms of amino acids as well. [Note: This topic is also more explained in section of 1.5.2 on page 44]

In addition, light influences several aspects of plant and pathogen interplay. Several publications have appeared in recent years documenting the effect of light on pathogenicity and plant defense responses. For instance, fungal toxin fumonisin B1 induces light-dependent programmed-cell-death (PCD) in Arabidopsis protoplasts (Asai et al., 2000). Moreover, Light is required for PCD execution in Arabidopsis mutant *accelerated-cell-death 11 (acd11)*, because BTH induces cell death in SA-deficient *acd11* only in the light but not in the dark (Brodersen et al., 2002).

In 2004, Zeier and colleagues proved the light-dependency of several defense responses in an interaction between Arabidopsis and an avirulent strain of *Pseudomonas syringae* (*Psm AvrRpm1*). These responses include activation of phenylalanine ammonia-lyase, accumulation of salicylic acid (SA), expression of PR1, development of hypersensitive response (HR), and SAR establishment (Zeier et al., 2004). In contrast, pathogen-induced accumulation of jasmonic acid, production of phytoalexin camalexin, and transcriptional induction of a pathogen-inducible myrosinase were even more pronounced in the dark (Zeier et al., 2004). Plant defense responses were shown to be dependent on the time of day when pathogen attack takes place. According to Griebel and Zeier, 2008, morning and midday

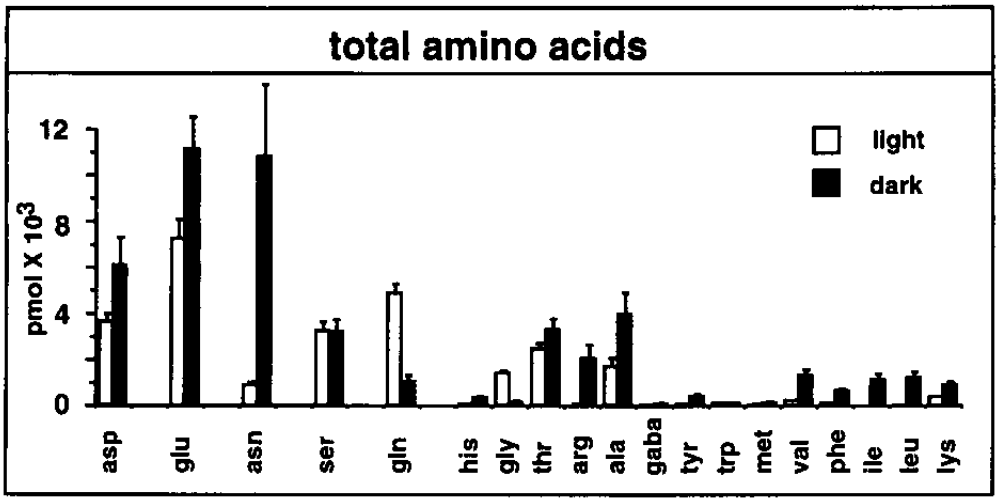

Figure 1.9: Amino acid levels in light-grown and dark-adapted wild-type Arabidopsis plants. Amino acids were extracted from Arabidopsis plants grown in light (empty boxes) or subsequently dark-adapted for 24 h (filled boxes), derivatized and separated by reverse phase HPLC. The standard three letter code is used for all amino acids. Each sample represents the average of three different plants (two leaves/plant) (Lam et al., 1995).

Arabidopsis-inoculation with *Psm* result in higher SA accumulation, faster PR1 expression, and a more pronounced hypersensitive response in comparison to evening or dark inoculations (Griebel and Zeier, 2008).

Light influences the establishment of SAR. Development of SAR in response to avirulent bacteria was completely lost when primary inoculation of Arabidopsis plants occurred in darkness (Zeier et al., 2004). However SAR in Arabidopsis plants was developed when primary infection with *Psm AvrRpm1* was occurred under medium (PFD 70 µmol photons m^{-2} s^{-1}) and high (PFD 500 µmol photons m^{-2} s^{-1}) light conditions (Zeier et al., 2004). Interestingly, SAR establishment under high light was not associated with systemic SA accumulation and PR1 expression, showing that even defense mechanisms under low and high light differs.

The length of light applied to the Arabidopsis or tobacco plants after pathogen attack, was previously considered as a determinant factor in development of SAR and requirement of MeSA (as a critical SAR signal) (Liu et al., 2011a). According to Liu et al., 2011a, MeSA was shown to be essential for SAR establishment when primary infection of pathogen occurred in the afternoon and plants exposed to little light after infiltration. In contrast, establishment of SAR in the morning-infiltrated plants with *Psm*, did not require MeSA.

Plant defense and Light signaling A link between defense responses and light signaling is likely to exist. Resistance to bacterial pathogens, *Pseudomonas syringae pv. maculicola* containing the avirulent gene AvrRpm1 (*Psm AvrRpm1*) and *Pseudomonas syringae pv. tomato* DC3000 containing the avirulent gene AvrRpt2 (*Pst avrRpt2*) is proved to be dependent on red and far-red light receptors phytochrome A and phytochrome B (PHYA and PHYB) (Genoud et al., 2002). Moreover, Arabidopsis blue light photoreceptor cryptochrome 1 (CRY1) is shown to positively regulate inducible resistance to *Pst avrRpt2* under constant light (Wu and Yang, 2010). Regulation of SAR involves FMO1-dependent red-light phytochrome

receptors (phytochromes A and B), while blue-light receptor mutants *cryptochrome1cryptochrome2 (cry1cry2)* and *phototropin1phototropin2 (phot1phot2)* are both capable of establishing a full SAR response (Griebel and Zeier, 2008). A cross-talk between phytochrome signaling and both SA perception and HR development was also previously demonstrated by Genoud et al., 2002. According to Genoud et al., 2002, Arabidopsis *phyAphyB* double mutants, showed a reduced HR development and increased susceptibility to *Pst avrRpt2* (Genoud et al., 2002). Moreover, development of HR and resistance to Turnip Crinkle Virus (TCV) and Tobacco Mosaic Virus (TMV) in Arabidopsis and tobacco respectively, depends on the light. Light is required for both biosynthesis and perception of SA since SA-treatment of Arabidopsis in dark or dim light resulted in an strongly reduced PR1 expression (Genoud et al., 2002).

In this context, the role of chloroplasts has to be considered since light-dependent redox status and ROS propagation from the chloroplasts also plays a key role in defense strategies against invading microorganisms (Delprato et al., 2015). There are several studies reporting on down-regulation of photosynthesis in response to various types of pathogens, forcing the plants to shift the metabolites from source to the sink which is due to high demand of energy in infected tissue (Bolton, 2009; Major et al., 2010).

Plant defense and circadian clock Several aspects of basal immunity are regulated by a circadian clock which is also linked to the light (Roden and Ingle, 2009; Sauerbrunn and Schlaich, 2004). It is shown that in Arabidopsis wild-type plants, increased resistance to *Pst DC3000* in the morning, results from clock-mediated modulation of PAMP-triggered immunity since leucine-rich repeat receptor kinase, encoding a flagellin receptor FLS2 (FLagellin-Sensitive 2), and downstream MAPK signaling components (MKK4/5, MAPK3/6, WRKY22 module) are circadian regulated with a peak expression in the morning (Bhardwaj et al., 2011). In Arabidopsis, CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), a circadian regulator, controls the rhythmic expression of R gene RPP4 which confers immunity against downy mildew disease by *H. arabidopsis* and peak expression of RPP4 and RPP4-dependent genes occurred at dawn, coinciding with the time of *H. arabidopsis* sporulation. Artificial infection by this pathogen at dusk caused susceptibility of plants compared with dawn-infection (Wang et al., 2011).

Since stomata opening (as a pathogen entrance way) is regulated by the circadian clock (Hotta et al., 2007), it is obvious that the entry of pathogens is lower during the night due to stomata being closed. As reported by Zhang et al., 2013, disruption of circadian clock in Arabidopsis, resulted in enhanced susceptibility to *Pseudomonas syringae* and the regulatory role of circadian clock on plant defense was described through its effect on stomata (as a barrier to restrict pathogen entry). However, Griebel and Zeier, 2008 suggested a negligible role of circadian rhythm in pathogen-induced SA accumulation, using pressure inoculation of *Psm AvrRpm1* into the leaves, thus bypassing stomatal defense responses. Moreover, Liu et al., 2011a also mentioned that exposure of MeSA mutant plants (*bsmt1-3*) to 3.5 h light before evening dark period was sufficient to induce SAR in these mutants, indicating that the duration of light, rather than the circadian rhythm impacts the interaction between Arabidopsis and *Psm AvrRpm1*.

It is noteworthy to mention that accumulation of defense-regulators jasmonates and salicylates is also rhythmic and circadian-regulated. Plant jasmonates show peak accumulation in the middle of the day corresponding to the peak time of herbivore attack, whereas SA (which often act antagonistically to jasmonates) has a peak accumulation at subjective night which is a few hours before the time of infection.

This observation can be linked to enhanced resistance of Arabidopsis to biotrophic bacteria when infection occurs in the early morning as opposed to the evening (Goodspeed et al., 2012). Figure 1.10 illustrates an overview of the interaction between plant immune system and light, clock, and temperature.

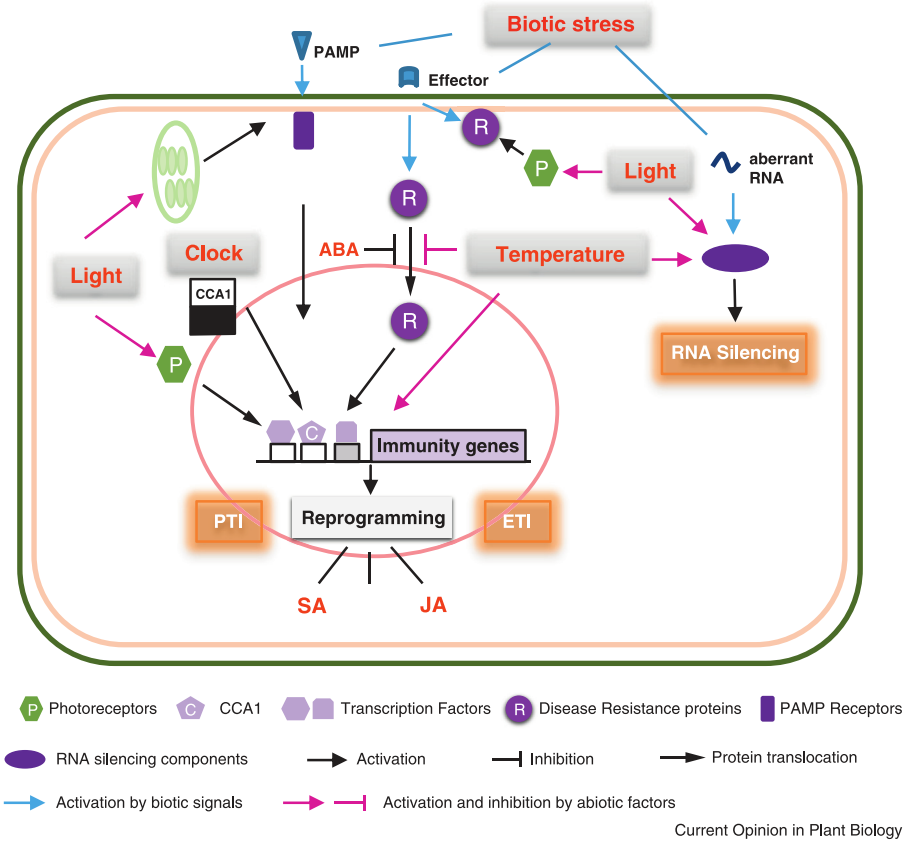

Figure 1.10: Modulating plant immune responses by light, clock, and temperature. Plant immunity has multiple defense mechanisms among those PTI, ETI, and RNA silencing are each triggered by PAMPs, effectors, and aberrant RNAs from viruses, respectively. Abiotic factors, such as light, clock, and temperature, have profound effects on plant immunity. Illustrated are major intersection points of these factors on defense mechanisms, especially those identified recently. Please note that not all players in abiotic responses and immune responses are depicted and interactions among the defense molecules (such as between SA and JA) or abiotic factors (such as between light and clock) are not illustrated. (1) Light affects PTI, ETI, and RNA silencing through photosynthesis and photoreceptor signaling. Various attributes of light including its quality and quantity are integrated into immune responses through distinct photoreceptors of red, far-red, blue, and UV-B lights. Light regulates expression of many defense response molecules including SA. Photoreceptors and their interacting proteins could directly modulate the protein stability of R proteins. (2) Circadian clock modulates expression of central genes in PTI and ETI and thus confers a clock regulation on plant immunity. Many defense genes and defense metabolites including JA are circadian regulated. (3) High temperatures often inhibit ETI and enhance RNA-silencing mediated resistance. Nuclear accumulation of some TIR-NB-LRR proteins is reduced by high temperatures as well as by ABA. High temperatures enhance expression and/or activities of components in RNA silencing machinery. Expression of defense genes including R genes can be regulated by temperature as well. (Hua, 2013)

Effect of light on pathogens virulence In addition to the effect on plant immune system, light can also act as a determinant of virulence in plant pathogens. Light can affect pathogen fitness, motility, adhesion, and virulence. Different light spectra affect the respiratory activity of non-phototrophic bacterium *Pseudomonas sp.* DR 5–09 (Gharaie et al., 2017). In *Agrobacterium tumefaciens* light induces reduction of flagella subunits FlaA and FlaB which consequently inhibits the motility of this bacteria (Oberpichler et al., 2008). Examples of the direct effect of light on virulence, are reduced root attachment of *A. tumefaciens* in tomato *(Solanum lycopersicum)* and smaller tumor formation in cucumber *(Cucumis sativus)*

in the presence of light compared with darkness (Oberpichler et al., 2008). In addition, light regulates motility, attachment, and virulence of *Pseudomonas syringae pv. tomato* DC3000 during an epiphytic phase of its life-cycle on the leave surface of Arabidopsis (Río-Álvarez et al., 2014). According to this study, the blue component of white light is responsible for the inhibition of swarming motility of *Pst* and provoke the attachment of this pathogen to the plant leave surface (Río-Álvarez et al., 2014).

1.5.2 Effect of nitrogen on plant defense responses

Nitrogen is an essential element involved in plant growth, development, and defense against biotic and abiotic stresses. In addition, nitrogen is a major constituent of proteins, nucleic acid and other organic compounds in plants which affects all levels of plant function, from metabolism to resource allocation (Krapp, 2015; Scheible, 2004). Nitrogen deprivation in Arabidopsis results in repression of a majority of genes assigned to photosynthesis, chlorophyll synthesis, plastid protein synthesis, induction of many genes for secondary metabolism, and reprogramming of mitochondrial electron transport (Scheible, 2004). Arabidopsis seedlings were grown on low (0.1 mM) nitrogen showed significant reduction of cotyledon size, fresh weight, chlorophyll, and anthocyanin content but a slight increase in endogenous sugars (Martin et al., 2002). It is worthy to note that distribution of metabolites under low and high nitrogen supply, is also related to leaf age. According to Hirel et al., 2005 in maize, leaf nitrate content was high in young vegetative N-fertilized plants compared with N-depleted plants, however, in mature plants, its relative amount was about five times lower with a preferential accumulation in the youngest leaves. Apparently, a total amount of free amino acids in young vegetative plants was approximately three times higher compared with that measured in mature plants (Hirel et al., 2005).

Plants absorb soil nitrogen (N) in inorganic forms of nitrate NO_3^- and/or ammonium NH_4^+ which will be then assimilated mostly in shoots to incorporate into amino acids. Nitrogen assimilation in plants involves a reductase series reducing nitrate NO_3^- to ammonium NH_4^+ via nitrite NO_2^- formation (Krapp, 2015; Ohyama, 2010). This process is further followed by transamination to form amino acids via glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Figure 1.11) (Masclaux-Daubresse, 2006).

Inorganic nitrogen is assimilated into the amino acids glutamine, glutamate, aspartate, and asparagine which are dominant compounds for production of other amino acids in plants (Lam et al., 1995). Amino acids are not only the structural components of proteins, they serve as precursors for production of large amount of metabolites required for plant growth and defense against biotic and abiotic stresses. Therefore, in plants nitrogen availability modifies the amino acid content and metabolite concentrations. In Arabidopsis Ws accession, grown under nitrogen-limiting conditions, showed less amino acid and more sugar content in rosette leaves and the levels of proline, asparagine, and glutamine were increased under high N (Lemaître et al., 2008). Based on the plant tissue and physiological conditions, the enzymes and genes involved in the amino acid metabolism pathway play different roles. For instance, in Arabidopsis, under light conditions (high C:N ratio) glutamine and glutamate are used to transport assimilated nitrogen from roots to shoots. In contrast, in dark-adapted Arabidopsis plants (low C:N ratio), asparagine is the predominant amino acid which is exported from leaves (Lam et al., 1995) (Figure 1.9 on page 41).

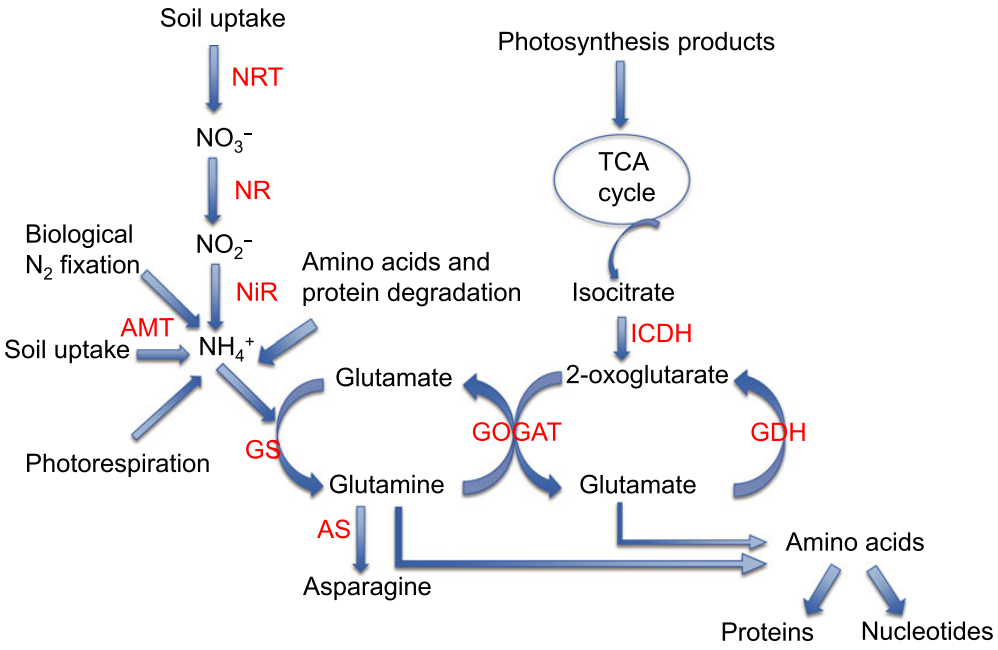

Figure 1.11: Nitrogen-assimilation pathway in higher plants. Inorganic nitrogen in the form of nitrate or ammonia becomes incorporated into amino acids and other organic molecules as depicted. The specific steps shown include: nitrate transporters (NRT), nitrate reductase (NR), nitrite reductase (NiR), ammonium transporters (AMT), glutamine synthetase (GS), glutamate synthase (GOGAT), asparagine synthetase (AS), glutamate dehydrogenase (GDH), and isocitrate dehydrogenase (ICDH). (Lu et al., 2016)

Nitrogen is a major constituent of amino acid formation and obviously the amino acid metabolism and amino acid-related defense responses are likely to be affected by external N supply. A most interesting approach to this issue has been proposed by Dietrich et al., 2004 confirming that constitutive and induced resistance to pathogens in Arabidopsis depends on nitrogen supply. According to this study, the activities of three anti-fungal enzymes (chitinases, chitosanases, and peroxidases) in Arabidopsis was strongly dependent on nitrogen supply and constitutive levels of the selected enzyme markers were much lower in plants cultivated under N-limiting condition (Dietrich et al., 2004). Previously, Stout and colleagues also could show a positive correlation between the level of total protein in leaflets of damaged-induced tomato (*Lycopersicon esculentum*) and nitrogen availability (Stout et al., 1998).

The amino acid metabolism impacts the host-pathogen interaction and there are so many reports showing that the level of amino acids in host is modified during the interaction. In crucifers, the whole amino acid pool increases upon *Xanthomonas campestris pv. campestris* infection (Kumar and Prasad, 1992). In Arabidopsis-*Pseudomonas syringae* interaction system, a massive change in the level of free amino acids is observed during SAR (Návarová et al., 2012). The level of Lys, the aliphatic amino acids (Val, Leu, Ile, β-Ala), and the aromatic amino acids (Phe, Tyr, Trp, and His) are strongly increased in *Psm*-infected leaves. There is a moderate but still significant increments for GABA, Cys, Asn, Ala, Gly, Ser, and Orn, whereas Asp shows a significant decrease in *Psm*-infiltrated leaves (Návarová et al., 2012). Most strikingly, the level of Pip and Aad which are faintly detectable in non-inoculated leaves, are strongly increased after *Psm* inoculation (Návarová et al., 2012). Pip is also strongly accumulated in petiole exudate of infected leaves and leaves distal to the site of the infection (Návarová et al., 2012). Further study could explain the increase level of Pip and its major role in SAR performance (refer to 1.3.2). However, the contribution of each specific amino acid to the outcome of host-pathogen interaction requires

more investigation. It is also possible that changing in the level of amino acids is as a result of protein degradation or autophagy which could happen in the cells as a consequence of pathogen attack (Hirota et al., 2018). Moreover, pathogen also impacts the host amino acid metabolism due to withdraw the nutrients and amino acids to its own advantage (e.g. increase in GABA in tomato leaves which is used as a nitrogen source for *Cladosporium fulvum* (Lemaître et al., 2008)). In addition, nitrogen metabolism and production of amino acids in the host can be also interfered with pathogen by producing toxins (e.g. tabtoxin and phaseolotoxin produced by *Pseudomonas syringae* which inhibits glutamine synthetase (GS)) (Bender et al., 1999).

Nitrogen also affects the root architecture. Under limited nitrogen, root system adapts itself by up-regulating the transporting system and stimulating the growth of lateral roots for more nitrate uptake (Remans et al., 2006). Other effects of nitrogen supply on root system include: i) a localized stimulatory effect of external nitrate on lateral root elongation, ii) a systemic inhibitory effect of high tissue nitrate concentrations on the activation of lateral root meristems, iii) a suppression of lateral root initiation by high C:N ratios, and iv) an inhibition of primary root growth and stimulation of root branching by external L-glutamate which all are discussed in Zhang et al., 2007a.

In addition, nitrogen drives the generation of nitric oxide (NO), as an important defense signal which has a central role in hypersensitive responses during pathogen attack. NO is generated during nitrogen assimilation and reducing NO_3^- to NO_2^- by nitrate reductase (NR). Thus, N availability plays a crucial role in NO-mediated defense responses (Mur et al., 2017).

Beyond the nitrogen concentration, the form of nitrogen applied to plants (either as nitrate NO_3^- or ammonium NH_4^+ fertilizers) affects the outcome of plant-pathogen interactions (Figure 1.12) (Gupta et al., 2013). NO_3^- feeding augments hypersensitive response (HR)-mediated resistance, enhances production of defense signals such as polyamines (spermine and spermidine), whereas NH_4^+ nutrition can compromise defense and it increases GABA level as a nutrient source for pathogen (Gupta et al., 2013; Mur et al., 2017). Production of nitric oxide (NO) by nitrate reductase (NR) and expression of SA-induced PR1 are reduced in NH_4^+-fed tobacco plants as well (Gupta et al., 2013).

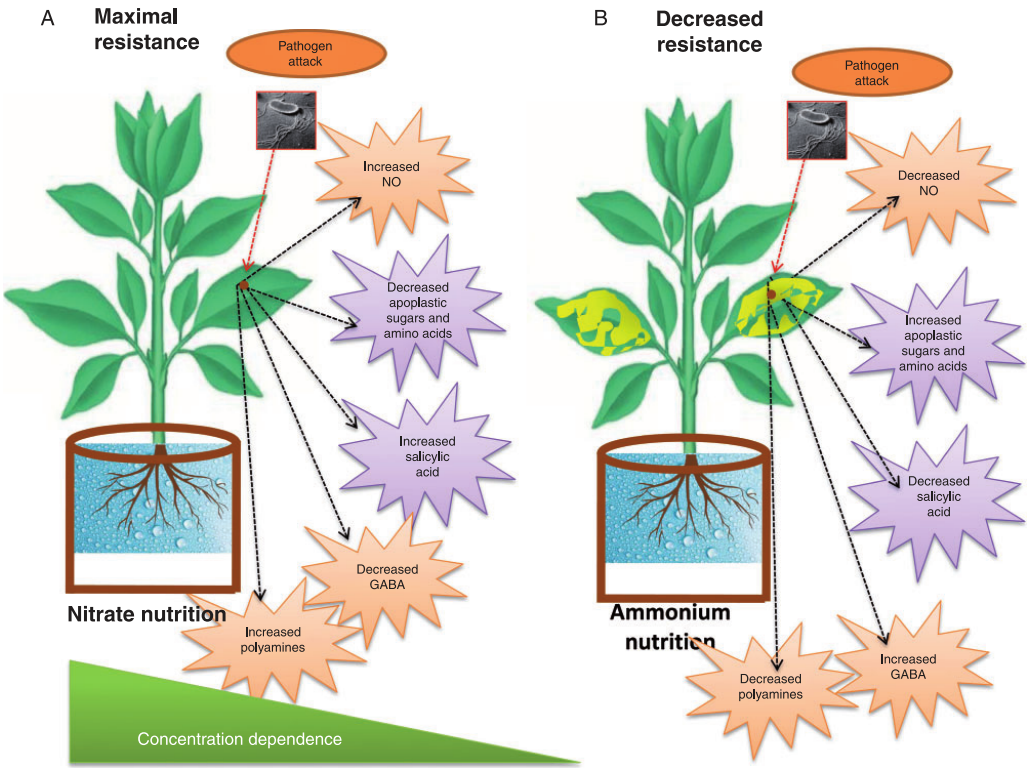

Figure 1.12: Effect of NO_3^- vs. NH_4^+ on plant resistance to pathogen infection. Growth on NO_3^- nutrition leads to increased levels of NO, SA, PR gene expression, induction of the polyamine pathway, a decrease in apoplastic sugars and amino acids, and an overall increase in plant resistance in a concentration-dependent manner. Growth on NH_4^+ nutrition leads to increased levels of apoplastic sugars and amino acids, reduced levels of SA and PR gene expression, induction of GABA biosynthesis and reduced plant defense response. (Mur et al., 2017)

1.6 Arabidopsis thaliana and *Pseudomonas syringae* pathosystem

Arabidopsis thaliana L. Heynh (thale cress) is an annual dicot from Brassicaceae family. Arabidopsis has a short life cycle (around 6 to 8 weeks) and is used as a model organism in plant research. Different ecotypes of Arabidopsis are columbia (Col-0), landsberg (Ler-0), wassilewskija (Ws) and C24. In this study, ecotypes Col-0 and Ws have been used. A big portion of Arabidopsis genome is sequenced (115.4 Mb of total 125 Mb) and this plant consists of approximately 30,000 genes that are located on 5 chromosomes. A large collection of Arabidopsis knockout mutants are available which can be used in different research areas working on Arabidopsis (The Arabidopsis Genome Initiative, 2000).

Pseudomonas syringae is a gram-negative rod-shaped bacterium consisting of polar flagella. This bacteria consists of a large number of pathovars that can be pathogenic to varieties of plant species such as *Arabidopsis thaliana*, *Nicotiana benthamiana*, and tomato (*Solanum lycopersicum*). This bacterium got its name after lilac tree (*Syringa vulgaris*), from which it was first isolated. Pathogenicity of *Pseudomonas* bacterium is dependent on effector proteins secreted into the plant cell by type III secretion system. Hemi- or biotrophic *Pseudomonas syringae* bacteria are able to enter plants through wounds or stomata to proliferate in the apoplast. *P. syringae* strains are host-specific and assigned to more than 50 pathovars. Different types of pathogen-host interactions such as non-host, compatible and incompatible

interactions exist between strains of *P. syringae* and Arabidopsis ecotypes (Katagiri et al., 2002). In this study, we used virulent *Pseudomonas syringae pv. maculicola* ES4326 (*Psm*) to study compatible interaction and (*P. syringae*) carrying plasmid containing avirulence genes (*Psm AvrRpm1*, *Psm AvrRpt2*, and *Pst avrRpt2*) were used for incompatible interactions.

2 Aim of thesis

Systemic acquired resistance (SAR) is induced by a localized leaf inoculation with pathogenic microbes and constitutes a state of elevated, broad-spectrum disease resistance within the entire plant foliage. SAR establishment and the associated priming of defense responses are regulated by the non-protein amino acid pipecolic acid (Pip) via salicylic acid (SA)-dependent and -independent pathways (Bernsdorff et al., 2016). Besides Pip and SA, a number of other potential SAR signals, such as methyl salicylate (MeSA), glycerol-3-phosphate (G3P), DIR1 and azelaic acid (AzA), have been proposed (Park et al., 2007b; Chanda et al., 2011; Maldonado et al., 2002; Jung et al., 2009). The major objective of this study was to investigate the long distance signaling with the focus on Pip, as a main SAR regulator, in the context of other elusive SAR signals. I wanted to find out whether and to which extent the individual SAR signals contribute to the establishment of SAR in Arabidopsis thaliana. To address this question, first I re-evaluated the significance of these putative signals in SAR and examined whether several factors such as the type of the pathogen and the time of pathogen infiltration (either morning or evening) may affect the contribution of each signal to the SAR. Furthermore, I wanted to know whether these putative signals are required for biosynthesis of Pip and SA in the context of light and dark. Therefore, I evaluated the accumulation of SA and Pip (as two main SAR regulators) in plants defective in production of an individual SAR signal to find out whether there is a connection between each of the potential SAR signals and biosynthesis of Pip and SA upon *P. syringae*-infiltration in the morning and evening.

In order to understand the interplay between Pip- and the SA signaling with each of the other reported SAR signals and to understand whether the putative signals acts up or downstream of Pip and SA, I analyzed the capacity of knockout mutants with defects in AzA, G3P, MeSA, and DIR1 signaling to activate Pip- or SA-induced resistance (Pip- and SA-IR).

In the second and third chapters of this study, I analyze the effect of environmental factors such as light and nitrogen supply on the defense responses associated with systemic acquired resistance. Considering Pip, as the main SAR regulator, I wanted to understand whether biosynthesis and priming function of Pip on the production of defense regulators (SA and camalexin), depend on external factors such as light or nitrogen supply. I further investigated whether the length of light period applied after pathogen attack may affect the induced resistance and the production of SA and Pip. Plants live in a dynamic nature, in which the intensity of light changes rapidly. To have a better perception of the activation of defense mechanisms in different light intensities, I further evaluated the production of defense metabolites (SA and Pip) under fluctuating light.

3 Results: Long Distance Signaling (LDS)

Systemic acquired resistance (SAR) is induced by a localized leaf inoculation with pathogenic microbes and constitutes a state of elevated, broad-spectrum disease resistance within the entire plant foliage. For SAR establishment, a putative SAR signal(s) must be produced and localized in leaves distal from the pathogen-infected site. Publications have appeared in recent years, reporting on several potential SAR signals such as methyl salicylate (MeSA), glycerol-3-phosphate (G3P), defective in induced resistance 1 (DIR1), azelaic acid (AzA), and pipecolic acid (Pip) (refer to section 1.3.3 on page 25). Among all, Pip has been considered to play a crucial role in SAR establishment and priming of defense responses (Návarová et al., 2012).

This chapter focuses on the characterization of putative SAR signals by using Arabidopsis long-distance signaling (LDS) mutant lines, defective in production of each individual SAR signal such as *gli1-1*, *gly1-1*, *bsmt1-1*, and *dir1-1* (in Ws background). AZI1 reported to be a component downstream of AzA-induced resistance, thus we used *azi1-2* mutant lines as well. We want to investigate the extent to which the putative SAR signals are required for SAR establishment and manifestation. Furthermore, the contribution of these signals to SAR when pathogen attacks in the different times of a day (morning and evening infiltration) and the ability of LDS mutant lines to produce main SAR regulators (SA and Pip) are investigated here. In addition, we analyze whether these signals are required for biologically-induced SAR in compatible or incompatible interactions. Moreover, the involvement of putative signals in Pip- and SA-induced resistance has been tested here.

3.1 Glycerol-3-phosphate (G3P) is dispensable for SAR establishment upon *Pseudomonas syringae* attack in the morning. G3P potentiates SAR establishment in the morning

Arabidopsis glycerol-3-phosphate, synthesized through G3P-dehydrogenase activity of GLY1 or glycerokinase catalyzed by GLI1, plays an important role in cellular mechanisms such as glycerophospholipids biosynthesis. To better investigate the role of G3P in SAR induction, we conducted comparative experiments in which induction of SAR in the morning (SAR assay/morning) was examined in G3P mutant lines (*gli1-1* and *gly1-1*) and the respective wild-type line (Col-0). Three local (1º) leaves of plants were infiltrated with either 10 mM $MgCl_2$ (as control buffer) or with *Psm* OD_{600}=0.005 at 9 AM. The second leaves were challenged-infected with *Psm lux* OD_{600}=0.001 two days later and bacterial growth was measured 2.5 days after 2º infiltration. Four individual experiments were performed for each line.

For each line, a total graph was produced which shows the average of the average values of individual experiments. Statistical ANOVA analysis was performed for each individual experiment and also across all the individual experiments in the total graph.

When primary leaves of Col-0 plants were pre-inoculated with *Psm*, a significant reduction of 12.5 fold bacterial growth in the subsequent challenge infection in systemic leaves was observed in comparison to $MgCl_2$-treatment, demonstrating the establishment of SAR (Figure 3.1 c, d). In contradiction with earlier findings (Chanda et al., 2011), our data indicated a significant SAR response in all of the four independent experiments for Col-0, *gli1-1*, and *gly1-1* (Figure 3.1). Our ANOVA analysis confirmed that in three out of four experiments, a lower fold-change reduction upon *Psm*-treatment and a weaker SAR response was observed in *gli1-1* when compared to Col-0 (Figure 3.1 a_1-a_4), suggesting that a functional GLI1 is required for full SAR establishment. Moreover, compared to $MgCl_2$-inoculated Col-0, a higher level of bacterial growth was observed in $MgCl_2$-inoculated *gli1-1* plants, showing a weaker basal resistance in *gli1-1* (in three out of four experiments) (Figure 3.1 a_1-a_4).

A SAR response with similar intensity as Col-0, was observed in two out of four experiments in *gly1-1* (Figure 3.1 b_1-b_4). Unlike *gli1-1*, there was no contribution of GLY1 to basal resistance and only in one out of four experiments, a higher local bacterial growth was observed in this line compared to Col-0.

Since the plasticity of SAR varies in each individual experiment, we provided a total graph for each line which shows the average of the average values of individual experiments. Statistical ANOVA analysis was performed across all the individual experiments in the total graph. Total SAR graphs showed a 12.5 fold-change reduction in bacterial growth upon *Psm* treatment in Col-0 (Figure 3.1 c, d), whereas a 3.1 and 5.2 fold-change reductions were observed in *gli1-1* and *gly1-1* respectively. This suggests that G3P produced by either GLI1 and GLY1 is not necessarily required for SAR establishment since defective plants in G3P (*gli1-1* and *gly1-1*) are not compromised in SAR. Our ANOVA analysis in the total graph indicates a similar SAR intensity in *gly1-1* as wild-type Col-0, suggesting that G3P produced by GLY1, is not essential for SAR establishment in our experimental system (Figure 3.1 d). However, SAR and basal resistance in *gli1-1* is weaker compared to Col-0, suggesting that a functional GLI1 is required not only for the establishment of a full SAR response but also for induction of basal resistance (Figure 3.1 c).

Consequently, Arabidopsis plants (*gli1-1* and *gly1-1*), impaired in the production of G3P, are capable of inducing SAR under our experimental setup. Based on our data, Glycerol-3-phosphate (G3P) is dispensable for SAR establishment upon *Pseudomonas syringae* attack in the morning. The cytosolic G3P produced by GLI1 is potentially required for full SAR establishment upon morning pathogen attack.

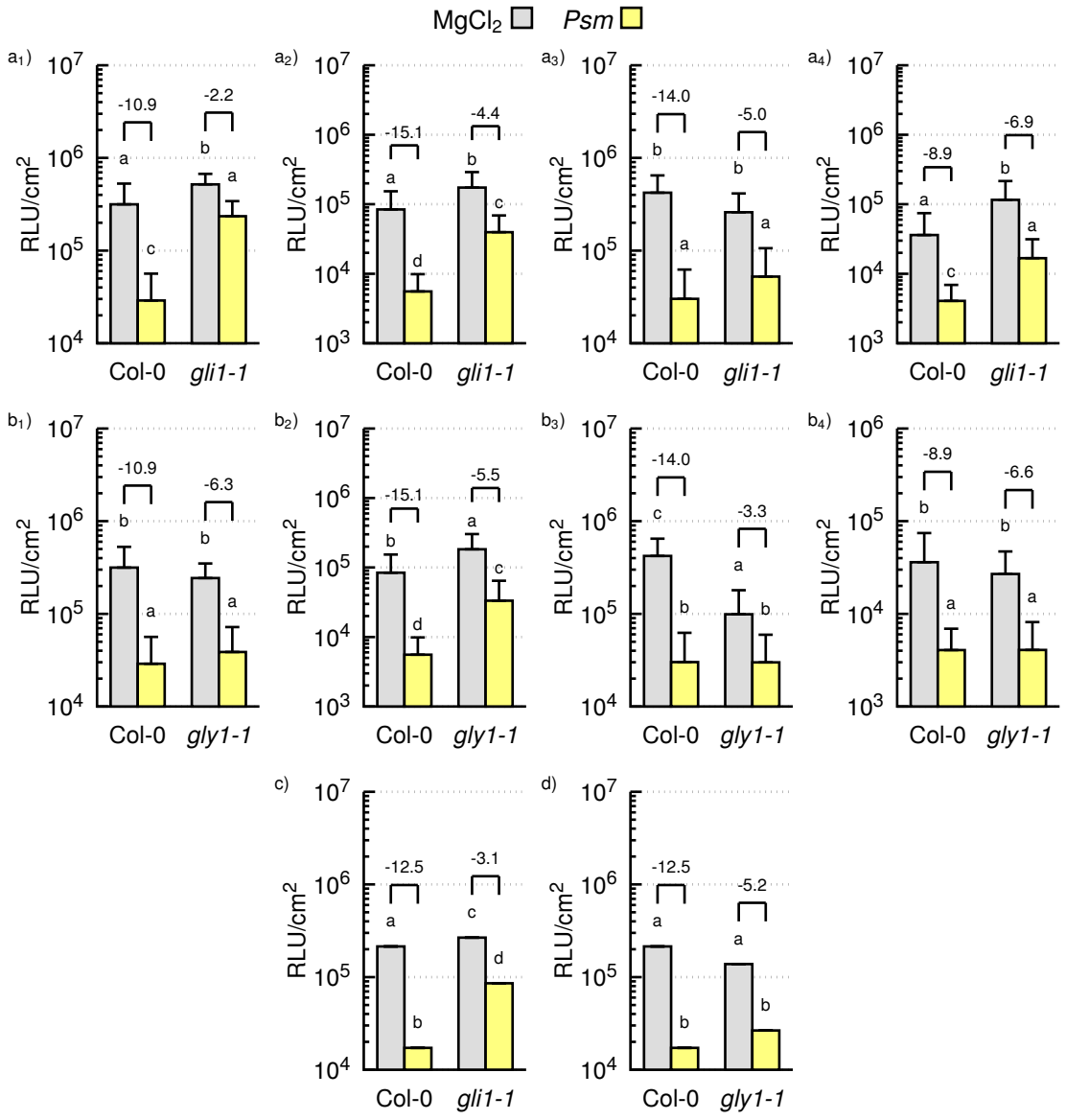

Figure 3.1: SAR establishment in the morning in Col-0, *gli1-1*, and *gly1-1* plants. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Four independent experiments for each mutant lines were performed and a total graph for each line was produced using the mean values of all independent experiments. a_1-a_4: *gli1-1* repetition experiments, b_1-b_4: *gly1-1* repetition experiments, c: *gli1-1* total graph, d: *gly1-1* total graph. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test).
a_1, a_2, b_1 and b_2 were done by Katrin Gruner.

3.2 Methylsalicylate (MeSA) is not required for *Psm*-induced SAR in the morning

There is a considerable amount of literature discussing the role of MeSA in SAR development as explained in the introduction section (section 1.3.3.1). MeSA was considered a mobile SAR signal in Park et al., 2007b, whereas MeSA was described as dispensable for SAR because of complete SAR establishment in *bsmt1* mutant lines defective in MeSA production Attaran et al., 2009. Further research by Liu et al., 2011a, proposed a conditional role of MeSA in SAR depending on the time of bacterial inoculation and discrepancies of previous studies were attributed to the differences in the experimental setup (such as age of the plants, light intensity, and/or the strain of bacterial pathogen) used by different research groups.

In this study, we first wanted to find out whether MeSA is required for SAR. Therefore, we conducted a comparative SAR assay/morning (as explained in section 3.1, on page 50) between mutant plants defective in production of MeSA (*bsmt1-1*) and the respective wild-type plants (Col-0). We repeated the experiment three times independently and a total graph was produced which shows the average of the average values of each individual experiments. Statistical ANOVA analysis was performed for each individual experiment and also across all the individual experiments in the total graph. As our data shows that despite the variation in the level of bacterial growth in individual experiments, SAR was developed in *bsmt1-1* in all three experiments (Figure 3.2). In one out of three experiments, (Figure 3.2 a_2), an almost similar fold-change reduction in bacterial growth upon *Psm*-treatment to Col-0 was observed and a wild-type SAR was developed in *bsmt1-1*.

Referring to our total graph, our ANOVA statistical analysis across all individual experiments, showed no difference in the induction of basal resistance between Col-0 and *bsmt1-1* suggesting that MeSA is not required for basal resistance (Figure 3.2 b). However, a slight weaker SAR in *bsmt1-1* compared to Col-0 was observed. Since MeSA alone can not induce a defense response and it needs to be converted to active SA, it is likely that lower amount of MeSA in *bsmt1-1* plants, affects the accumulation of systemic SA, therefore establishment of a wild-type-like SAR might require the adequate amount of MeSA either in local or in systemic leaves. In contrast to Liu et al., 2010, which reported a compromised SAR in *bsmt1-1* mutant lines, here we demonstrated that MeSA is not essential for SAR establishment and *bsmt1-1* plants are able to develop a significant SAR upon *Psm*-infiltration in the morning. We assume that the contribution of MeSA to SA-dependent signaling pathway is essential for full SAR establishment.

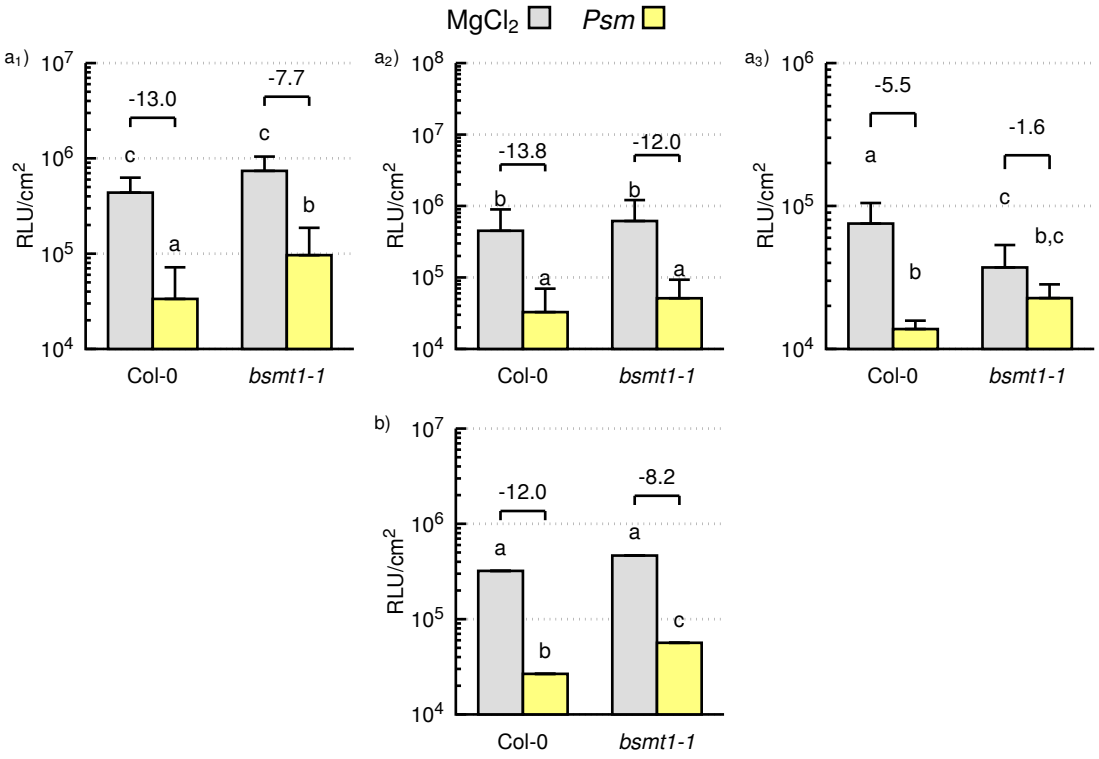

Figure 3.2: SAR establishment in the morning in Col-0 and *bsmt1-1* plants. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Three independent experiments for each mutant lines were performed and a total graph was produced using the mean values of all independent experiments. a_1-a_3: *bsmt1-1* repetition experiments, b: *bsmt1-1* total graph. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test).

3.3 Azelaic acid-induced resistance 1 (AZI1), and defective in induced resistance (DIR1) are dispensable for SAR establishment upon *Psm* attack in the morning

In 2009, Jung et al., 2009 reported that the nine-carbon dicarboxylic acid azelaic acid (AZA) confers local and systemic resistance in Arabidopsis and *Psm AvrRpt2* pathosystem. AzA has all expected properties of a mobile SAR signal because it shows increased accumulation in petiole exudate, confers resistance to local and systemic tissues and primes SA accumulation and SA-dependent gene expression. Furthermore, AZA is able to induce a lipid transfer protein (LTP)-like AZI1 (azelaic acid induced 1) which functions in AZA-conferred SAR signaling pathway. Two independent *azi1* mutant plants (SALK_017709 and SALK_085727) were found to be defective in SAR, although *azi1* shows normal susceptibility to local *Pseudomonas syringae* infection (Jung et al., 2009). Genetic screening of Arabidopsis T-DNA mutant lines which are impaired in long-distance signaling identified another lipid transfer protein named defective in induced resistance 1 (DIR1). Although there is no similarity between DIR1 and AZI1 proteins, both have been suggested to function in translocation of putative SAR signal(s) (Maldonado et al., 2002; Dempsey and Klessig, 2012). *dir1-1* failed to induce SAR in Arabidopsis plants infiltrated with *Pst avrRpt2* when compared to its respective wild-type Wassilewskija (Ws) as reported by (Maldonado et al., 2002).

In order to investigate the potential role of AZI1 and DIR1 in long-distance signaling, comparative SAR assay/morning (as explained in section 3.1, on page 50) was performed between Arabidopsis mutants *azi1-2* and *dir1-1* and their wild-type plants Col-0 and Ws respectively. We performed four and three independent experiments for *azi1-2* and *dir1-1* respectively.

As shown in Figure 3.3 a_1-a_4, in two-three experiments out of four, a similar SAR like in Col-0 was developed in *azi1-2*. Our total graph (Figure 3.3 c) produced based on the average of the average values of each individual experiments, showed almost a similar fold-change reduction in *azi1-2* (10.1) compared to Col-0 (12.5). *azi1-2* plants induced the same basal resistance to Col-0. Together, this data shows that *azi1-2* is able to induce SAR and basal resistance to the same degree as Col-0, thus in contrast to Jung et al., 2009, AZI1 is not essential for SAR development under our experimental setup.

A positive significant SAR was also established in *dir1-1* mutant lines in all three experiments we performed (Figure 3.3 b_1-b_3). Comparing to Ws, a higher fold-change reduction in the level of bacteria in *Psm*-treated *dir1-1*, was observed in two out of three experiments. Based on our ANOVA statistical analysis in *dir1-1* across all the individual experiments, we demonstrated that in comparison to wild-type Ws, *Psm* inoculation of *dir1-1* plants resulted in a higher fold-change reduction (15.2) in bacterial growth and consequently, the establishment of a stronger SAR in this line. A partial SAR-competent phenotype in *dir1-1* (in Ws background but not in Col-0 accession) was also previously reported by Carella et al., 2017, which was attributed to the occasional activation of a DIR1 paralog determined as DIR1-like protein which may sometimes contribute to SAR in the absence of a functional DIR1. In addition, a significantly higher reduction of bacterial growth in $MgCl_2$-treated *dir1-1* compared to that of Ws indicates a slightly higher basal resistance in plants defective in production of DIR1 compared to Ws.

Inconsistent with previous studies, our results prove that loss of AZI1 and DIR1 do not block the long-distance signaling network leading to SAR development. Here, we show that under our experimental condition, AZI1 and DIR1 are dispensable for SAR establishment during morning infiltration of *Psm*.

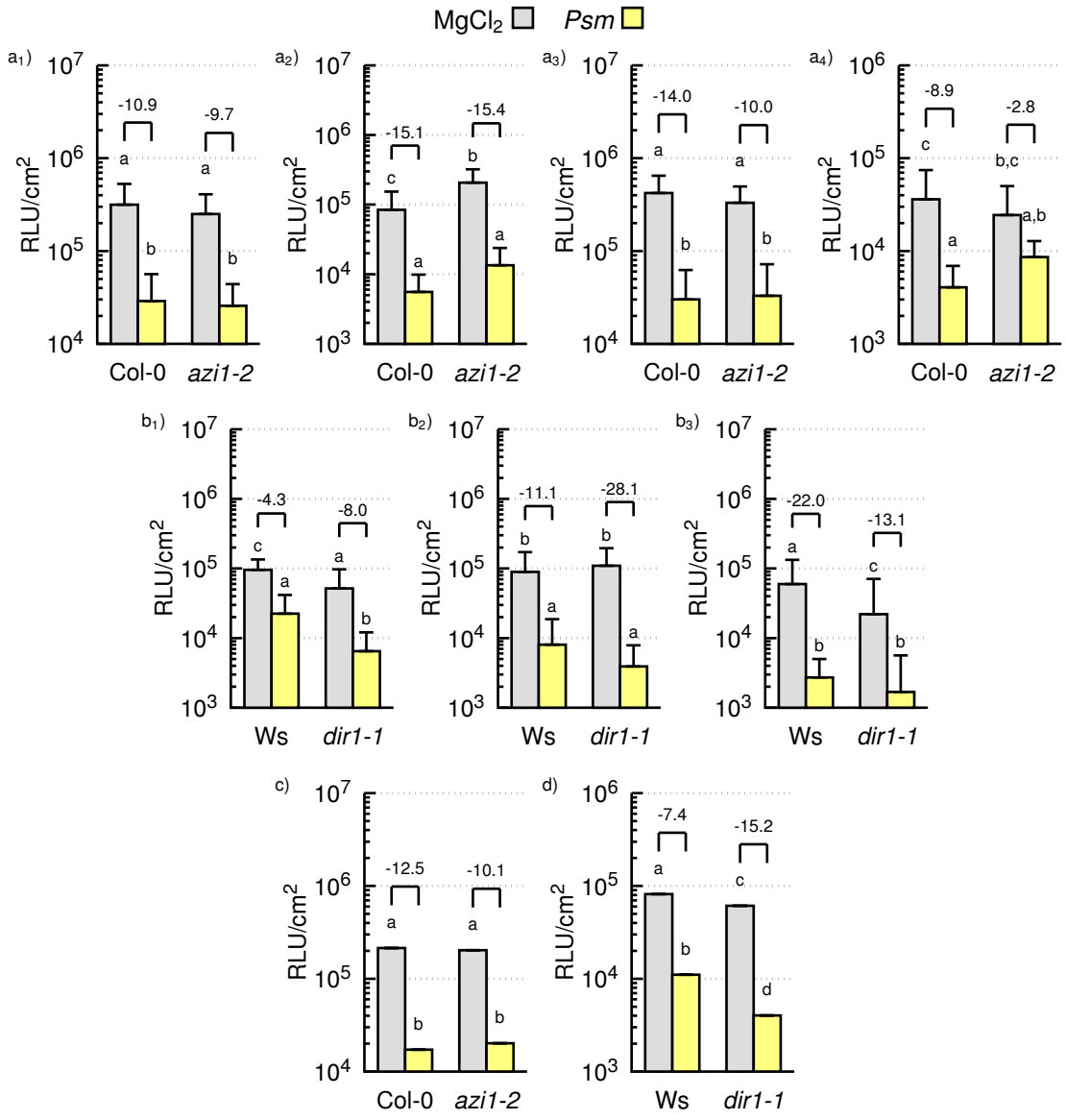

Figure 3.3: SAR establishment in the morning in Col-0, *azi1-2*, and *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Four and Three independent experiments were performed for *azi1-2* and *dir1-1* respectively, and a total graph for each line was produced using the mean values of all independent experiments. a_1-a_4: *azi1-2* repetition experiments, b_1-b_3: *dir1-1* repetition experiments, c: *azi1-2* total graph, d: *dir1-1* total graph. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test). a_1, a_2, b_1 and b_2 were done by Katrin Gruner.

3.4 SAR competency of putative SAR signal mutants, does not depend on the time of infiltration

Environmental factors such as light have been shown to affect plant defense mechanisms. In an Arabidopsis/*Pseudomonas syringae* interaction, several defense reactions have been shown to be light-dependent such as SA accumulation, pathogen-related (PR1) gene expression and, development of hypersensitive response. We already know that morning infiltration of a pathogen, results in higher accumulation of SA and faster expression of PR1 compared to evening infiltration (Zeier et al., 2004; Griebel and Zeier, 2008). Therefore, we hypothesized that activation of SAR signals might be also affected by the time when pathogen attack take place. As explained previously in section 3.2, in the context of light, a conditional role for SAR signals such as MeSA is possible.

To understand whether putative SAR signals play conditional roles in SAR depending on the time of pathogen attack, we examined induction of SAR in LDS mutant lines upon *Psm* infiltration in the evening. In this section, to test whether the time of pathogen infiltration and the presence of light after pathogen attack influence the contribution of putative signals to SAR, we performed a SAR assay as explained in section 3.1, on page 50, however we switched our previous time of pathogen infiltration from 9 AM in the morning to 7 PM in the evening (SAR assay/evening). Therefore, three local (1°) leaves of each plant were infiltrated either with 10 mM $MgCl_2$ (as control buffer) or with *Psm* (OD_{600}=0.005) at 7 PM. Since the light in our plant room is switched off at 7 PM, plants have been exposed to light at least for 10 hours before pathogen infiltration. To have a fresh potential bacterial culture, it was prepared from a bacterial plate on the morning of the same day of infiltration. Challenged-infiltration of systemic leaves with *Psm lux* OD_{600}=0.001 was performed 2 days later at 7 PM. The bacterial measurement was analyzed 2.5 days later after 2º infiltration. The evening-SAR assay was assessed in four independent experiments for each line and a total graph was produced from the average of the average values of each individual experiments. Statistical ANOVA analysis was performed for each individual experiment and also across all the individual experiments in the total graph.

Analysis of SAR in G3P-defective lines (*gli1-1* and *gly1-1*) indicated in all four individual experiments the inoculation of the pathogen in the evening resulted in a significant reduction of bacterial growth in *Psm* infiltrated leaves compared to $MgCl_2$-infiltrated leaves, showing induction of a positive SAR in these two lines (Figure 3.4).

Defense responses toward *Psm* in *gli1-1* differ in four experiments. In one experiment, we observed a similar wild-type-like SAR in *gli1-1* (Figure 3.4 a_4), while in another experiment a compromised SAR was observed in *gli1-1*, though a slight reduction of 2.3 fold-change in bacterial growth was observed in *Psm*-treated plants compared to mock-treated (Figure 3.4 a_1). Compared to Col-0 a weaker basal resistance in two out of four experiments was observed (Figure 3.4 a_2, a_3). The ANOVA analysis across all the experiments confirmed a weaker basal resistance in *gli1-1* compared to Col-0 and it indicated that SAR-induced in *gli1-1* is less effective compared to Col-0 (Figure 3.6 a).

Like *gli1-1*, *gly1-1* plants showed a Col-0-like SAR in one out of four experiments (Figure 3.4 b_4). Except in one experiment, (Figure 3.4 b_3), no significant difference in local resistance between *gly1-1* and Col-0 was observed, however, *Psm*-inoculation in the evening induced a weaker SAR in at least two out of four experiments. Total graph of *gly1-1* (Figure 3.6 b), showed a similar basal resistance in Col-0 and *gly1-1*, confirming that there is no contribution of GLY1 or plastidal G3P to basal resistance, however opposite to *gli1-1* mutants, a slightly higher SAR induction is observed in *gly1-1* compared to Col-0.

An induction of a wild-type-like SAR was also observed in *azi1-2* and *dir1-1* upon evening infiltration of *Psm*. Although SAR establishment in *azi1-2* varies in all four independent experiments (Figure 3.5 a_1-a_4), the *azi1-2* total graph demonstrates a similar induction of SAR in Col-0 and *azi1-2*, demonstrating that loss of function of AZI1 does not affect induction of SAR upon *Psm*-infiltration in the evening (Figure 3.6 c). Comparison analysis of evening-SAR in *dir1-1* and Ws in all four experiments (Figure 3.5 c_1-c_4) indicated that *dir1-1* plants behave similarly to Ws and in three out of four experiments, *Psm* induced the same degree of SAR in *dir1-1* and Ws as it is also proved in *dir1-1* total graph shown in Figure 3.6 e. One possible explanation would be that induction of SAR upon evening-inoculation of *Psm*, depends on an AZI1- and DIR1- independent signaling pathway or activation of only one of the LTPs-like (either AZI1 or DIR1) is sufficient for SAR induction in the evening.

It was found by Liu et al., 2011a, that MeSA played a conditional role in SAR, and when the primary infection occurred in the evening, MeSA and its metabolizing enzymes were essential for SAR development. In contrast, our results indicate that in three out of four experiments (Figure 3.5 b_1-b_4), a statistically-significant SAR was developed in *bsmt1-1* similar to Col-0. Our ANOVA analysis of *bsmt1-1* total graph (Figure 3.6 d), did not confirm the previous evidence by Liu et al., 2011a, and based on our results, in comparison to Col-0, even a higher induction of SAR was observed in *bsmt1-1* with a reduction of 10.7 fold -change in *Psm*-induced bacterial growth. A slightly reduced basal resistance in *bsmt1-1* compared to Col-0, suggest a functional role of MeSA in the induction of basal resistance when pathogen attacks in the absence of light.

Taking together, our data demonstrate that in contrast to earlier findings, MeSA, AzA, G3P, and DIR1 are dispensable for the induction of SAR and this effect is irrespective of the time of infiltration. A weaker SAR effect in *gli1-1* line in comparison with Col-0, indicate a small contribution of GLI1 to both morning and evening SAR establishment. As shown in Figure 3.7, in all plants pre-infiltrated with *Psm*, disease symptoms (chlorosis) are less pronounced compared to mock-treated plants which is an indicator of SAR establishment.

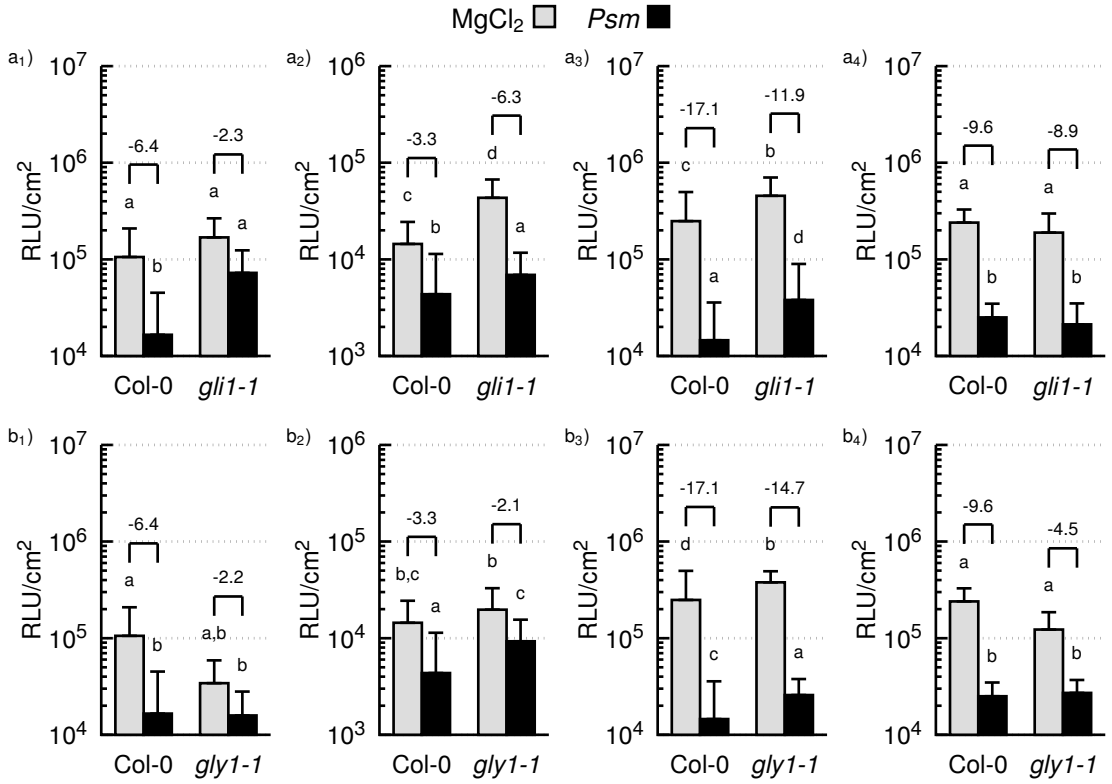

Figure 3.4: SAR establishment in the evening in Col-0, *gli1-1*, and *gly1-1*. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Four independent experiments were performed for each mutant line and a total graph (3.6) for each line was produced using the mean values of all independent experiments. a_1-a_4: *gli1-1* repetition experiments, b_1-b_4: *gly1-1* repetition experiments. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test). a_1 and b_1 were done by Karin Kiefer.

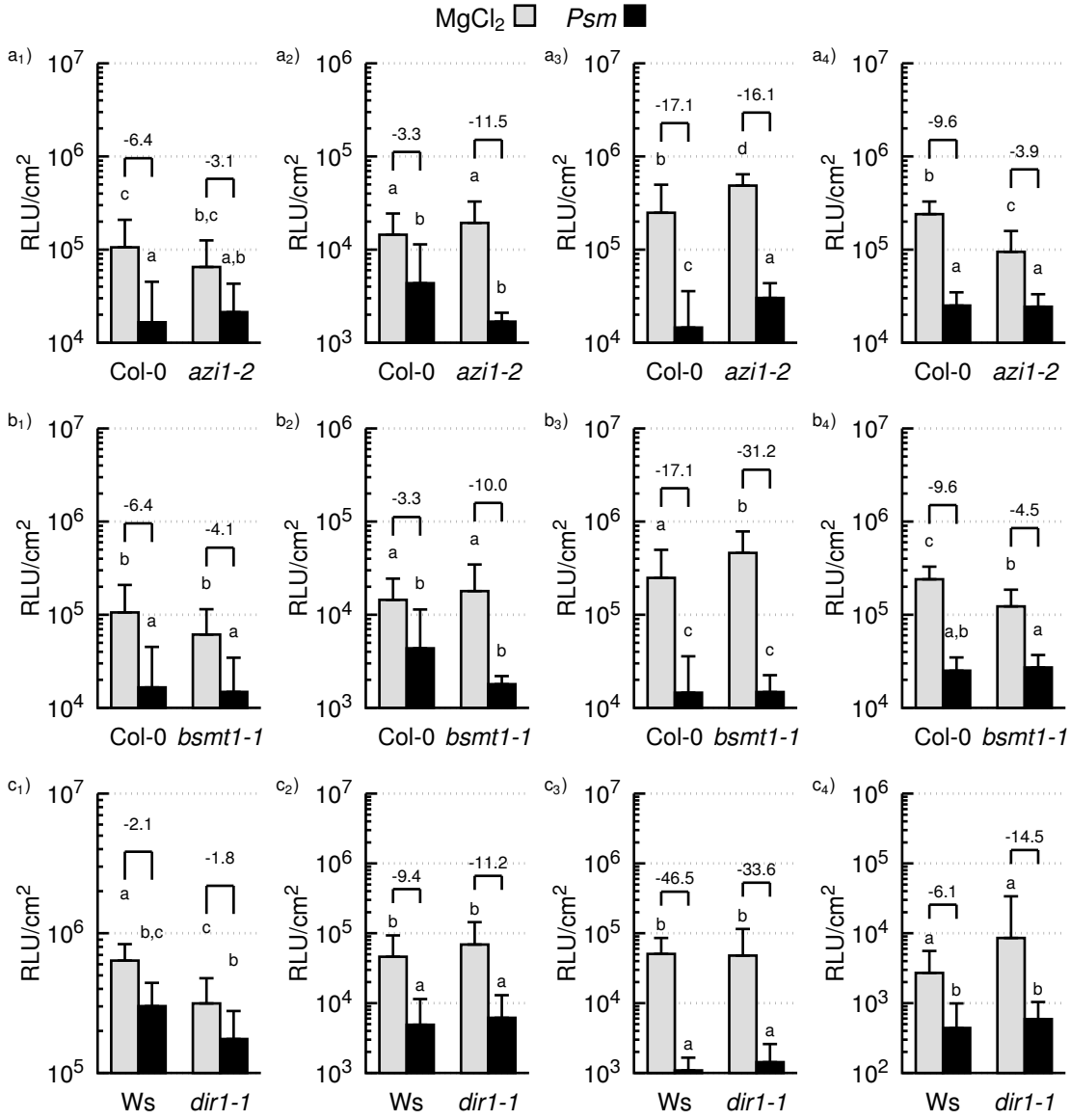

Figure 3.5: SAR establishment in the evening in Col-0, *azi1-2*, *bsmt1-1*, and *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Four independent experiments were performed for each mutant line and a total graph (3.6) for each line was produced using the mean values of all independent experiments. a_1-a_4: *azi1-2* repetition experiments, b_1-b_4: *bsmt1-1* repetition experiments, c_1-c_4: *dir1-1* repetition experiments. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test). a_1 and b_1 were done by Karin Kiefer.

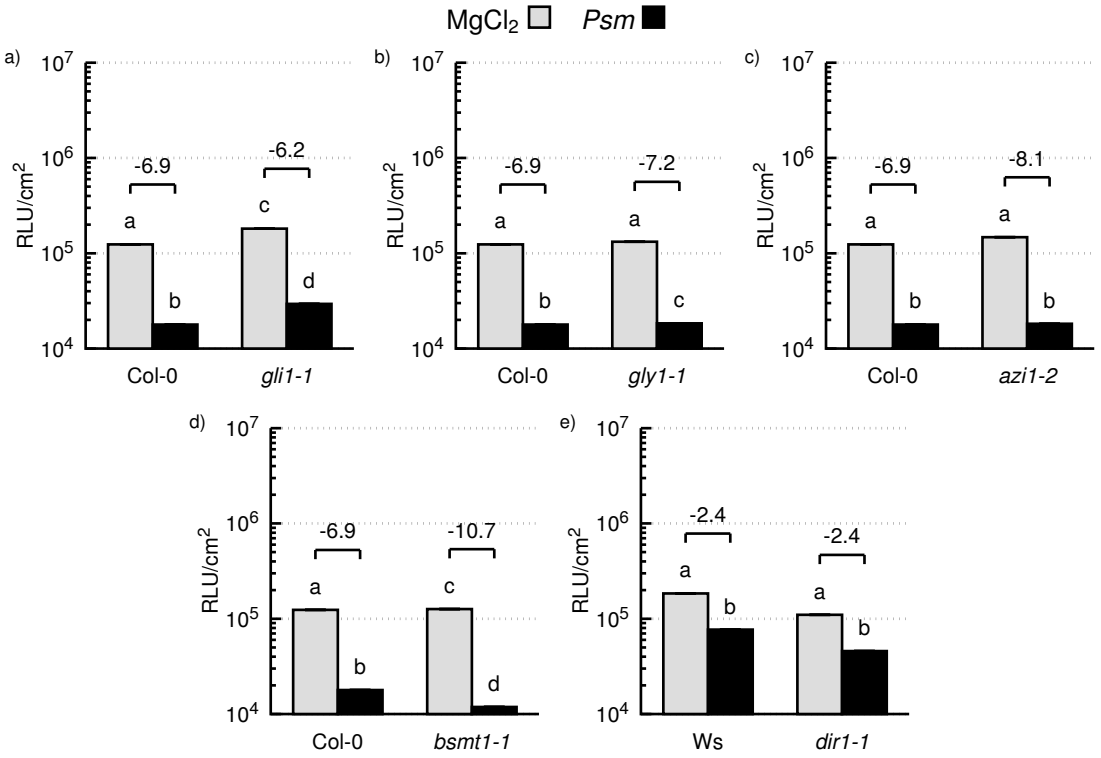

Figure 3.6: Total graphs for SAR establishment in the evening in LDS mutant lines. a:*gli1-1* total graph, b:*gly1-1* total graph, c:*azi1-2* total graph, d:*bsmt1-1* total graph, e:*dir1-1* total graph. SAR assay for each individual line was performed as explained in Figures 3.4 and 3.5 and total graph for each line was produced using the mean values of all independent experiments. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test).

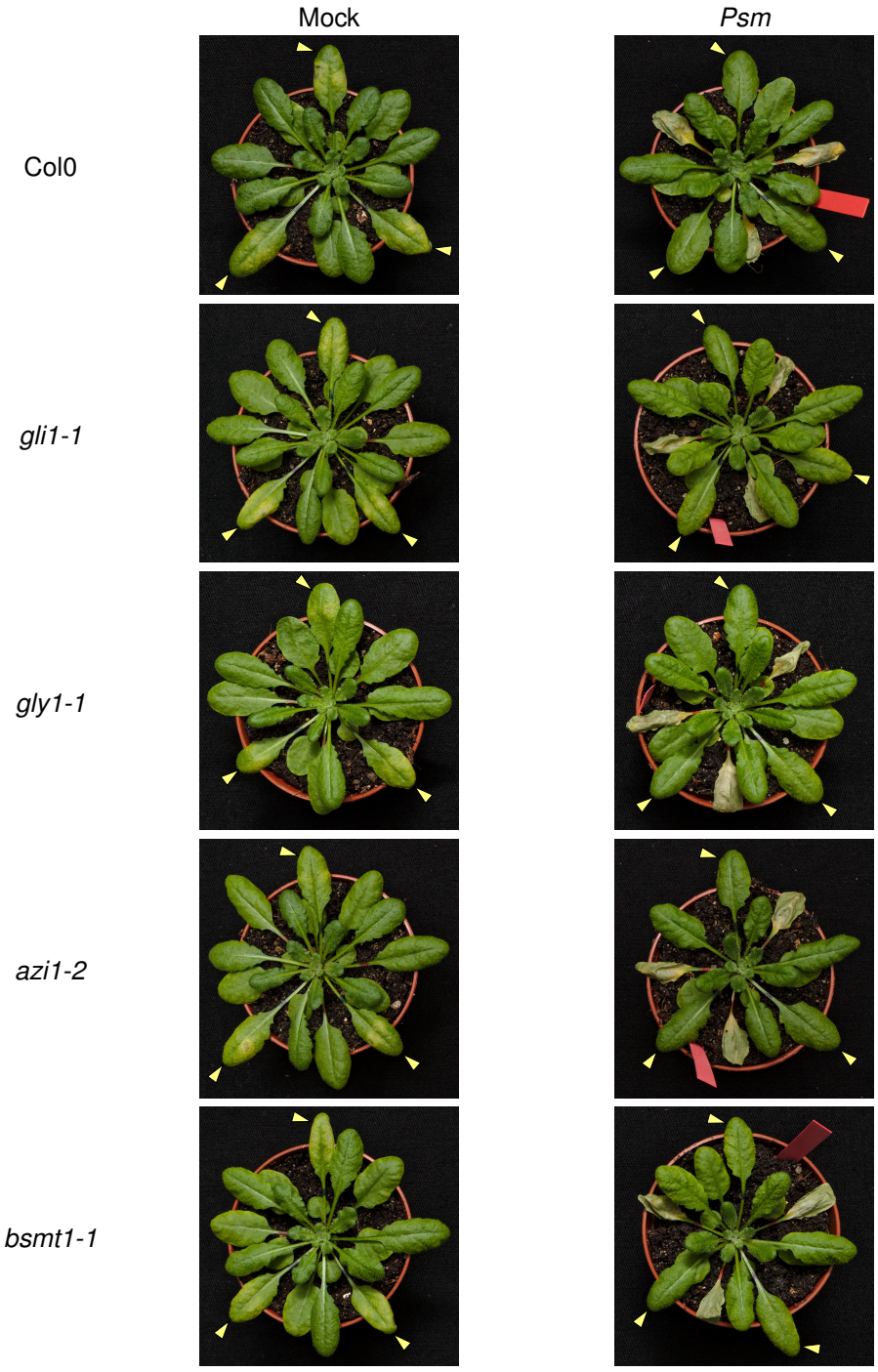

Figure 3.7: Plants picture shows SAR establishment upon *Psm* evening-infiltration in Arabidopsis Col-0 and LDS mutant lines. Arrows indicate systemic leaves 2.5 days after 2º infiltration with *Psm lux*.

3.5 Localized virulent and avirulent strains of *Pseudomonas syringae* induces SAR in all LDS mutant lines irrespective of the time of infiltration

Different virulent and avirulent strains of *Pseudomonas syringae* are able to induce SAR in Arabidopsis wild-type Col-0 and Ws (Mishina and Zeier, 2006). Establishment of SAR is highly dependent on the production of a signal(s) at the site of pathogen attack and transmission of the signal(s) to leaves above infection site. To investigate whether the presence of candidate signals are required for SAR responses towards different types of pathogen, we conducted an experiment in which LDS mutant lines were challenged with compatible and/or incompatible strains of *Pseudomonas syringae*. The following *Pseudomonas syringae* strains were used as challenging pathogens for SAR assay: *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*), HR-inducing bacterial strain *Psm AvrRpm1* harboring avrRpm1 avirulence gene and, *Psm AvrRpt2* carrying avrRpt2 avirulence gene.

Previously in section 3.6, we showed that putative signals are not required for SAR establishment upon morning or evening *Psm* infiltration. To investigate whether the putative signals play a conditional role based on the time of infiltration and the type of pathogen, we conducted combined SAR experiments (morning and evening SAR assays) in which infiltration with three pathogens was performed once in the morning (at 9 AM) and once in the evening (at 7 PM). Three local leaves were infiltrated either with $MgCl_2$ (as control buffer) and with *Psm* OD_{600}=0.005 or one of the two avirulent strains of *Psm AvrRpm1* (OD_{600}=0.005) or *Psm AvrRpt2* (OD_{600}=0.01). Plants were challenged with *Psm lux* OD_{600}=0.001 in three upper (2°) leaves 2 d later and the bacterial growth was scored 2.5 days after 2º infiltration.

As expected, irrespective of the time of the infiltration (morning or evening), local (1°) infiltration of col-0 plants with each of three strains of *P. syringae*, resulted in a significant reduction of *Psm lux* bacteria in 2° leaves, showing a positive SAR establishment (Figures 3.8 a_1 and 3.9 b_1). Morning infiltration of *Psm AvrRpt2* strain, induces a slightly but still significant weaker SAR in Col-0 compared to the other two strains ($P < 0.001$) (Figures 3.8 a_1). Evaluation of bacterial growth using *t*-test statistics, showed that morning-infiltration with each of the *Psm* strains, induced a wild-type-like SAR in *azi1-2* and *gly1-1* (Figure 3.8 a_1), indicating that AZI1 and GLY1 are not contributed to SAR establishment towards virulent and avirulent strains of *Psm* when pathogen attacks in the morning.

In contrast to *azi1-2* and *gly1-1*, it is likely that GLI1 is required for a full SAR development against different strains of *Psm* in the morning (but not evening), since a positive significant SAR ($P < 0.001$) was still observed in *gli1-1*, however a less reduction of bacterial growth in pathogen-inoculated leaves was observed when compared to Col-0 (Figures 3.8 a_1 and 3.9 b_1).

In comparison to Col-0, *bsmt1-1* mutant lines induced a weaker but still significant SAR ($P < 0.01$) toward virulent strain of *Psm* and HR-inducing *Psm AvrRpm1* in the morning (Figure 3.8 a_2). However, the weaker SAR effect in *bsmt1-1* is apparently as a consequence of its higher basal resistance than Col-0 in both morning and evening assays. Evening-infiltration of *azi1-2* and *bsmt1-1* with *Psm* and *Psm AvrRpm1* strains, resulted in reduced *Psm lux* growth and the establishment of a wild-type-like SAR (Figure 3.9 b_1). Surprisingly, *bsmt1-1* failed to induce SAR upon *Psm AvrRpt2* infiltration in the

evening, suggesting that MeSA plays a role in defense mechanisms against *Psm AvrRpt2* strain which are activating in the absence of light.

Irrespective to the time of bacterial infiltration (morning or evening), *dir1-1* mutant lines were SAR-competent to the same extent as in their wild-type Ws (Figure 3.8 a_3 and Figure 3.9 b_2).

Taking together, our data show that irrespective to the time of infiltration, LDS mutant lines are able to develop SAR upon infiltration of different strains of *P. syringae*. As shown here, *Psm AvrRpt2* induces an overall weaker SAR in all lines. We conclude that there is no contribution of MeSA, AzA, G3P and, DIR1 to SAR establishment upon infiltration of different pathogen strains in the morning and in the evening. The bacterial suppression in *gli1-1* is less pronounced compared to Col-0. We hypothesize that cytosolic G3P is required for full activation of defense responses against different strains of *P. syringae* bacteria. GLI1 is likely to be required for a full SAR establishment in the morning. Moreover, MeSA possibly involves in light-independent defense mechanisms against *Psm AvrRpt2*.

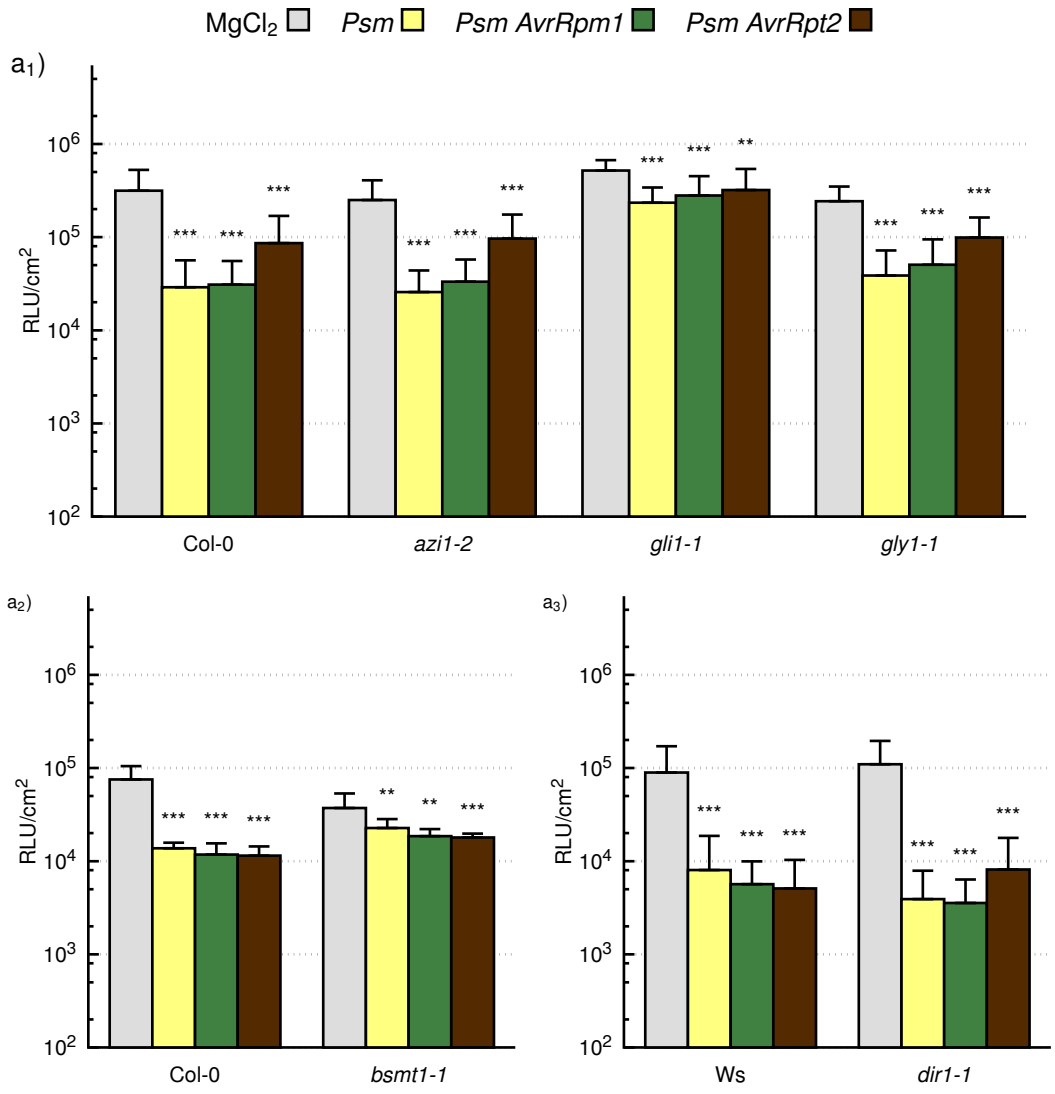

Figure 3.8: a_1, a_2, a_3: SAR establishment in Arabidopsis LDS-mutant lines upon *Psm*, *Psm AvrRpm1* and *Psm AvrRpt2* infiltration in the morning. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or one of the pathogen strains (*Psm* OD_{600}=0.005, *Psm AvrRpm1* OD_{600}=0.005, and *Psm AvrRpt2* OD_{600}=0.01), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between mock- and pathogen-infiltrated sample (*P < 0.05, **P < 0.01, ***P < 0.001). a_1 and a_3 were done by Katrin Gruner.

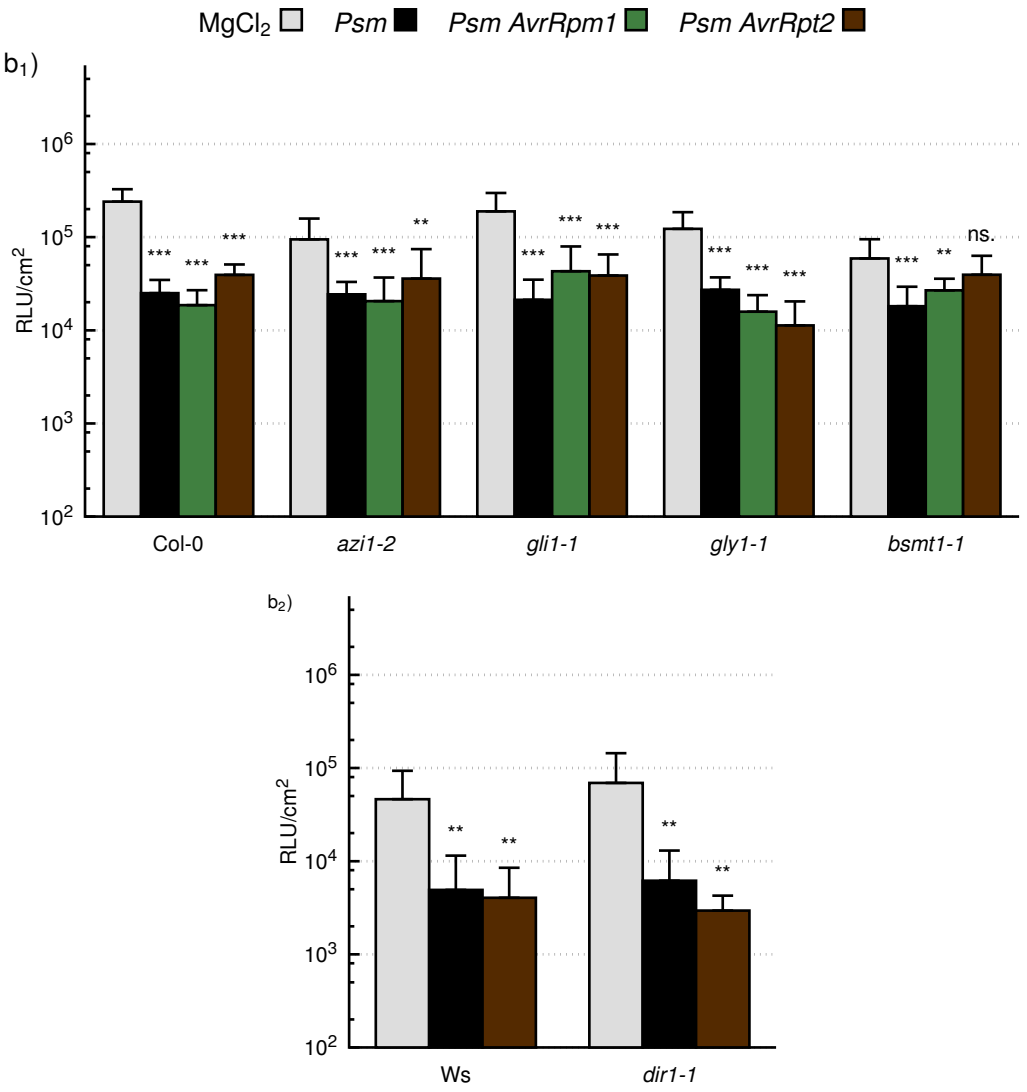

Figure 3.9: b_1, b_2: SAR establishment in Arabidopsis LDS-mutant lines upon *Psm*, *Psm AvrRpm1* and *Psm AvrRpt2* infiltration in the evening. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or one of the pathogen strains (*Psm* OD_{600}=0.005, *Psm AvrRpm1* OD_{600}=0.005, and *Psm AvrRpt2* OD_{600}=0.01), and three upper, 2° leaves were challenged-infected with *Psm lux* (OD_{600}=0.001) 2 d later. The growth of *Psm lux* in 2° leaves was assessed 2.5 d after 2º infiltration by luminescence measurements. Data represent means ± SD of the growth values of at least 18 leaf replicates. In all SAR graphs, a logarithmic scale is used for the y-axis. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between mock- and pathogen-infiltrated sample (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

3.6 Arabidopsis plants defective in production of AZI1, DIR1, G3P, and MeSA accumulate salicylic acid (SA) in local and systemic leaves upon morning and evening pathogen infiltrations

Plants synthesize a variety of defense metabolites to respond to a pathogen attack. One of the main regulatory defense metabolites is SA. SA is a defense hormone which is associated with plants resistance against biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). In the context of Arabidopsis thaliana and *Pseudomonas syringae* interaction, SA production will be induced by pathogens at the site of pathogen inoculation and leaves distal to the site of pathogen attack (Malamy et al., 1990; Návarová et al., 2012). To uncover the principles behind SAR development in long-distant-signaling mutants, upon morning and evening infiltrations, we tested whether the LDS mutant lines (*gli1-1*, *gly1-1*, *azi1-2*, *bsmt1-1*, and *dir1-1*) are able to accumulate defense metabolites such as SA. As explained in Zeier et al., 2004, light conditions influence a set of defense responses including SA accumulation and SA-induced PR1 expression. Therefore, we conducted an experiment in which plants were infiltrated either with $MgCl_2$ or virulent strain of *Pseudomonas syringae* (OD_{600}=0.005) in two different time points of a day: at 9 AM (morning-infiltration) and at 7 PM (evening-infiltration). In order to evaluate the pathogen-induced level of free SA, leave materials were collected at 24 and 48 hpi for further metabolite analysis using GC/MS. We repeated the experiment at least two times for each line.

Morning-infiltration of Col-0 with *Psm* induced strong accumulation of SA at the inoculation site (local leaves) at 24 hpi and in systemic leaves at 48 hpi (Figure 3.10, 3.11). Quantification of free SA at this time point in LDS mutant lines exhibited an increased level of *Psm*-induced SA in all lines as well. Although, local accumulation of SA in LDS mutant lines varies between independent experiments and either a higher or a lower values of SA compared to respective wild-type plants was observed, however, SA was accumulated in local and systemic leaves of all SAR signaling mutants.

Upon morning-infiltration of *Psm*, *gli1-1* mutant lines showed a slightly lower level of local SA compared to Col-0 in both experiments (Figure 3.10 a_1-a_2) whereas, upon evening-infiltration of *Psm* lower level of SA compared to Col-0 was observed in *gly1-1* lines (Figure 3.12 b_1-b_2). This suggests that participation of GLI1 and GLY1 in SA-biosynthesis pathway might differ in the presence and absence of light. It is likely that there is an interconnection between cytosolic G3P and local SA biosynthesis pathway upon morning-infiltration, on the other hand, plastidal G3P seems to participate in the biosynthesis of local SA in the absence of light. Moreover, when *Psm* attacks in the evening, the systemic level of SA in *gli1-1*, *azi1-2*, and *gly1-1* was higher (slightly higher in *gly1-1*) than that of Col-0 (Figure 3.13 a_1-a_2, b_1-b_2, c_1-c_2). Since these lines are proved to be SAR-competent in the evening, it is possible that for the establishment of SAR in the evening, a higher level of SA is required at the absence of G3P or AZI1.

BSMT1 catalyzes methylation of carboxyl group of SA to produce MeSA in Arabidopsis (Chen et al., 2003; Attaran et al., 2009). Lack of a functional BSMT1 did not block accumulation of SA in local and systemic leaves, in both morning and evening infiltrations (Figures 3.10 d_1-d_2, 3.11 e_1-e_3, 3.12 d_1-d_2, 3.13 d_1-d_2). Since *bsmt1-1* did not fail to accumulate systemic SA upon morning and evening infiltration, it is obvious that the weaker SAR establishment in the morning in this line (Figure 3.2 b) is due to the lower level of MeSA but not SA.

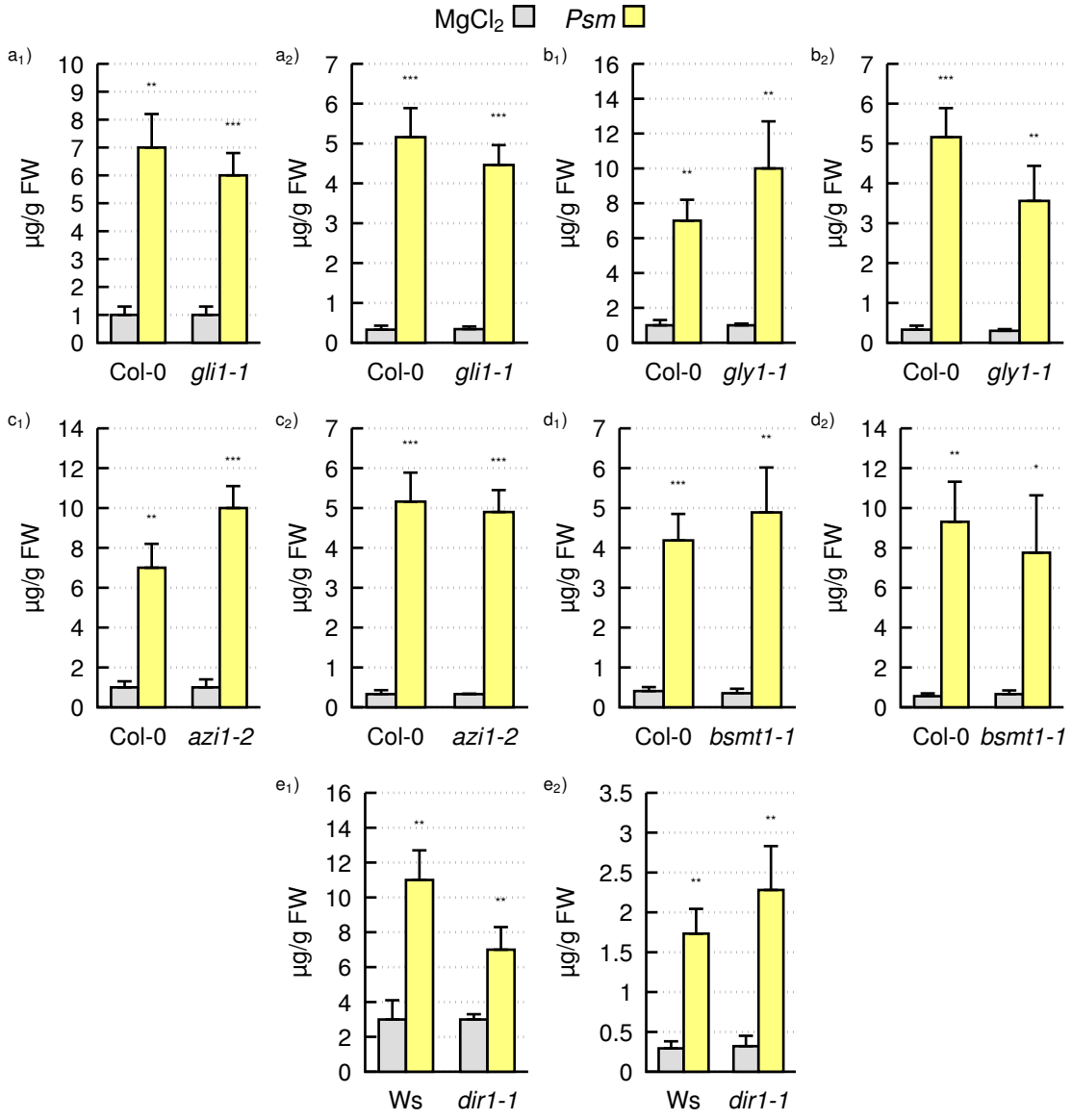

Figure 3.10: Free SA accumulation in infiltrated (local) leaves at 24 hpi after morning infiltration. a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e_1-e_2: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and infiltrated leaves were collected at 24 hpi. Two independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of SA in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). a_1 and b_1, c_1, e_1 and, e_2 were done by Katrin Gruner.

Increased level of SA in mock-treated leaves of Ws and *dir1-1* (after morning and evening infiltration) exhibited an induced basal resistance in these two lines compared to other Col-0 and other LDS mutant lines (Figures 3.10 e_1-e_2, 3.12 e_1-e_2). Upon morning-infiltration of *Psm*, a slightly higher level of systemic SA compared to Ws was observed in *dir1-1* (Figure 3.11 d_1-d_2). *Psm* also triggers a higher level of local SA in *dir1-1* when it attacks in the evening (Figure 3.12 e_1-e_2).

Together, morning and evening evaluation of *Psm*-induced SA in LDS mutant lines shows that these lines do not fail to accumulate a significant amount of SA required for SAR, suggesting that there is no contribution of MeSA, G3P, AzA, and DIR1 to the production of *Psm*-induced SA locally and systemically.

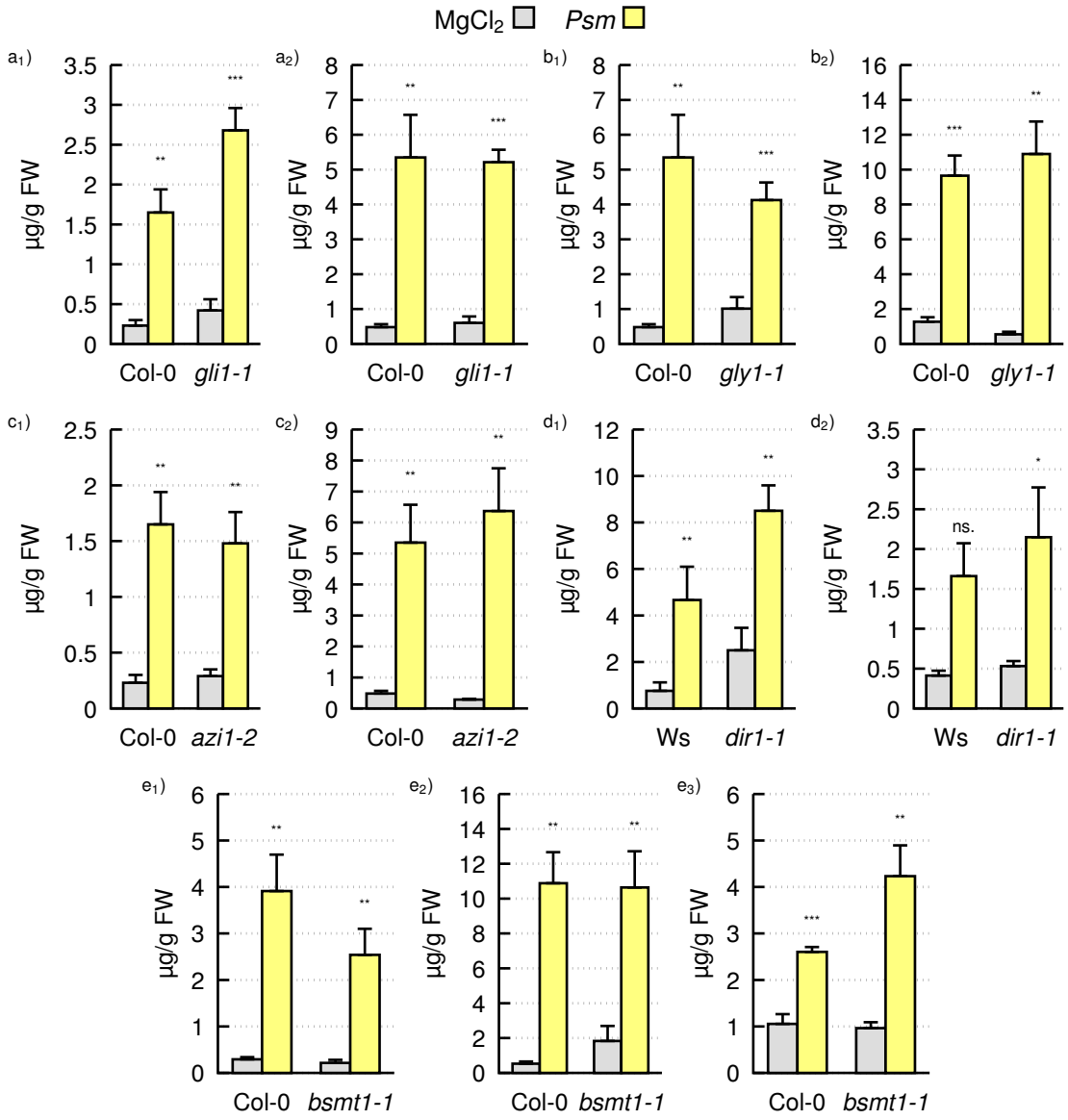

Figure 3.11: Free SA accumulation in systemic leaves at 48 hpi after morning infiltration. a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *dir1-1*, e_1-e_3: *bsmt1-1*. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and systemic 2° leaves were collected at 48 hpi. Two-three independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between lthe evel of SA in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). a_1 and c_1 were done by Katrin Gruner.

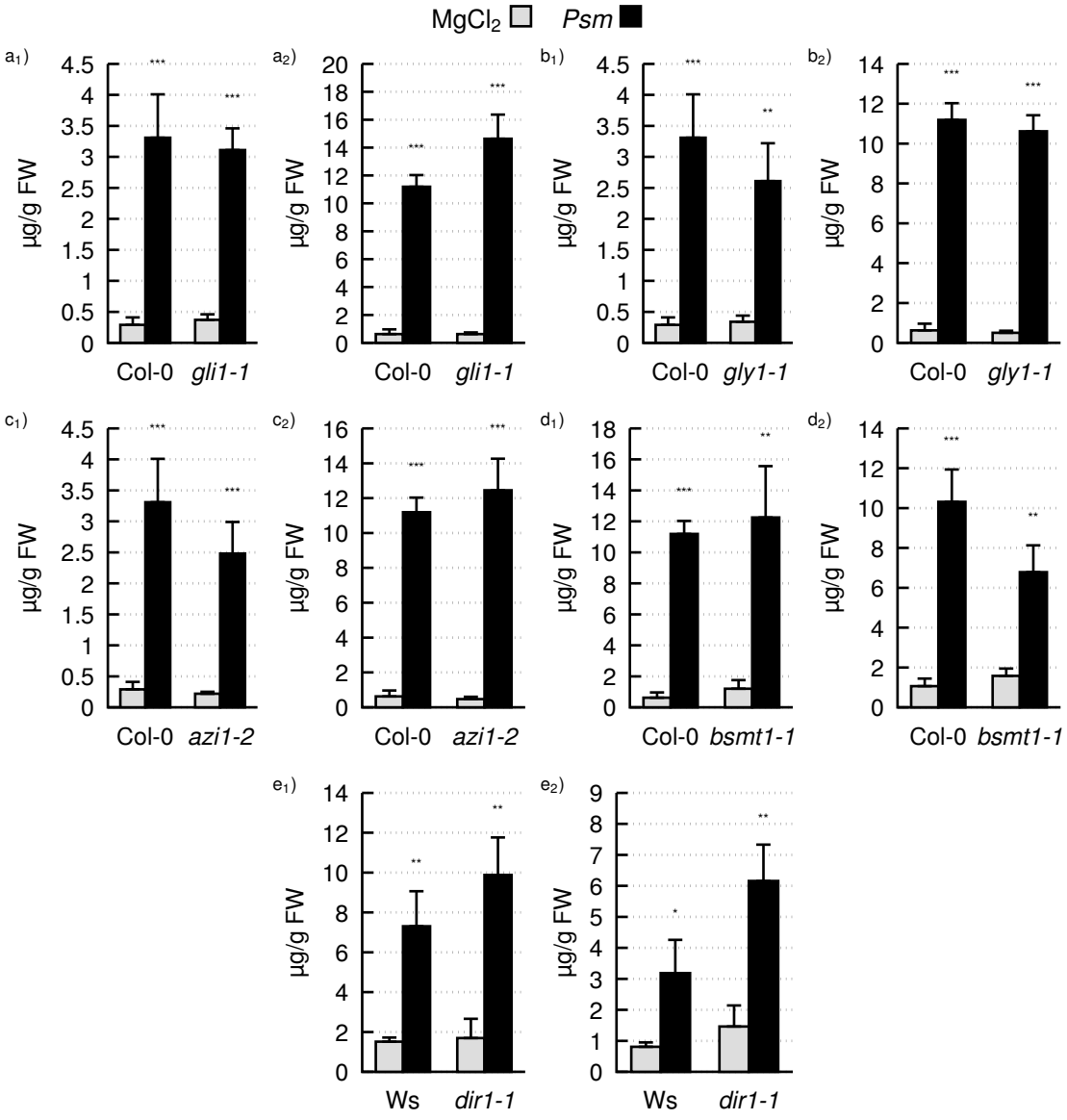

Figure 3.12: Free SA accumulation in local leaves at 24 hpi after evening infiltration. a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e_1-e_2: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and local 1° leaves were collected at 24 hpi. Two independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of SA in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

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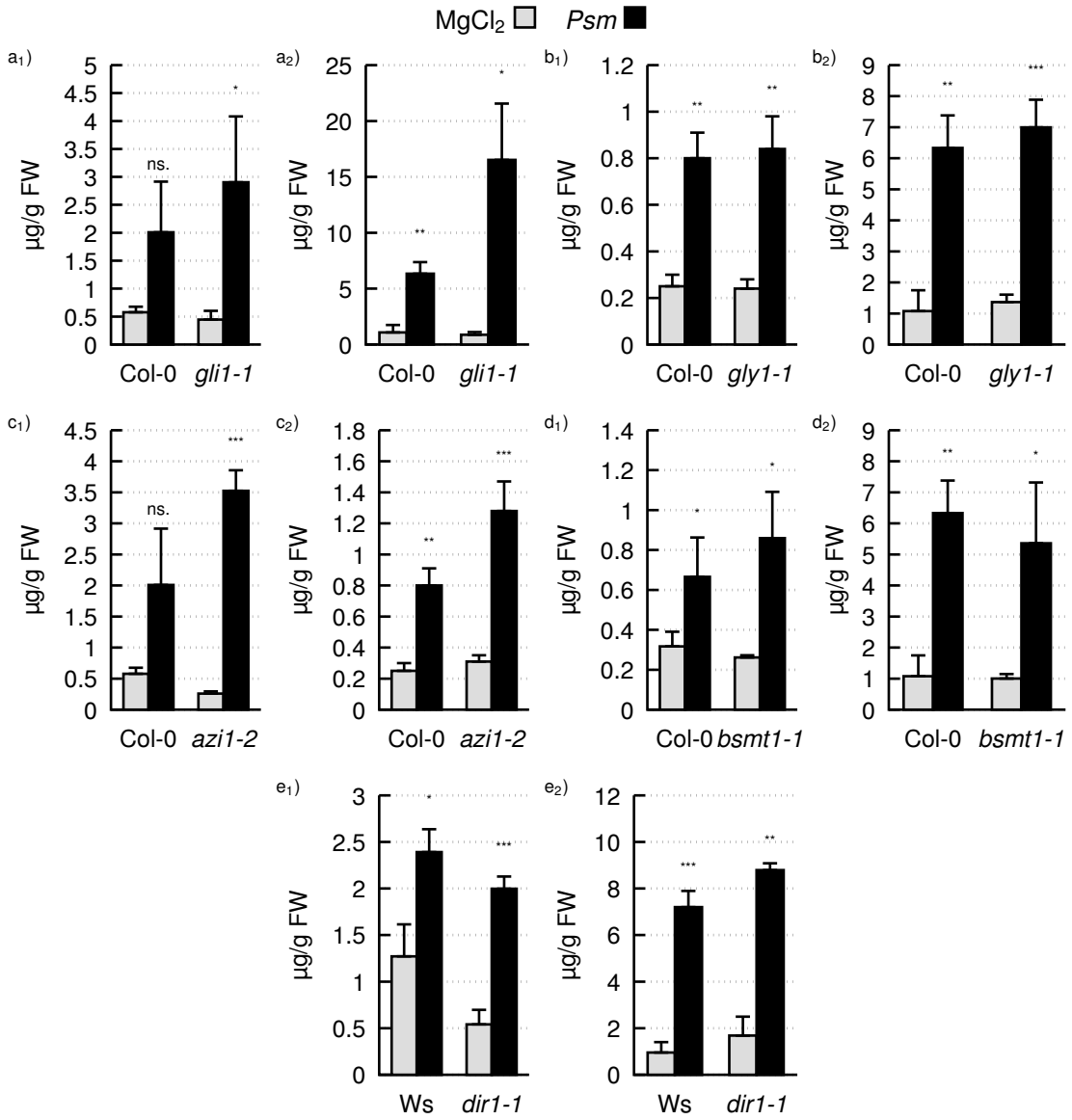

Figure 3.13: Free SA accumulation in systemic leaves at 48 hpi after evening infiltration. a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e_1-e_2: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and systemic 2° leaves were collected at 48 hpi. Two independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of SA in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). b_1 and c_2 were done by Katrin Gruner.

3.7 Arabidopsis mutants are able to accumulate Pip locally and systemically upon morning and evening infiltration.

It is known that following a pathogen attack, there is an increased level of amino acids such as Trp, Phe, Leu, and Lys and particularly pipecolic acid (Pip) and α-aminoadipic acid (Aad), which are highly accumulated in inoculated leaves. Also, Pip was found in leaves distal to the inoculation site and in petiole exudate of infiltrated leaves (Návarová et al., 2012). Moreover, Bernsdorff et al., 2016 showed that SA and Pip act both independently from each other and synergistically in Arabidopsis thaliana basal immunity to *Pseudomonas syringae*. Bernsdorff et al., 2016 highlighted the significant role of Pip as the main regulator of systemic immunity, which orchestrate SAR establishment via SA-dependent and independent signaling pathways. Thus, we predicted that long-distant signal mutants, which are SAR competent, must be capable of accumulating Pip as well.

Time course evaluation of Pip accumulation upon *Psm* and *Psm AvrRpm1* showed that pathogen triggers Pip accumulation 24 hpi in local leaves and 36 hpi in systemic leaves which reaches the highest level at 48 hpi (Návarová et al., 2012). To evaluate the level of Pip in LDS mutant lines, three local (1°) leaves of plants were infiltrated either with $MgCl_2$ or virulent strain of *Pseudomonas syringae* (OD_{600}=0.005) in two different time points of a day: at 9 AM (morning-infiltration) and at 7 PM (evening-infiltration). Pip was evaluated in local and systemic leaves at 24 and 48 hpi respectively. We repeated the experiment at least two times for each line.

As shown by our data, upon morning-infiltration of *Psm*, the level of Pip in inoculated leaves, significantly increased in all LDS lines (at least $P < 0.01$ for all LDS lines and $P < 0.001$ for most of the lines) (Figure 3.14). Even in *bsmt1-1* and *dir1-1* a higher level of local-Pip was observed when compared to their respective wild-type lines (Figure 3.14 d_1, d_2, e).

gli1-1 and *azi1-2* plants accumulated higher levels of Pip in systemic leaves upon morning and evening infiltrations (Figures 3.15 a_1 , a_2,c_1 , c_2 3.17 a_1 , a_2, c_1 , c_2). Accumulation of higher level of systemic-Pip compared to Ws was also observed in *dir1-1* when *Psm* attacks in the evening (Figure 3.17 e_1, e_2). Surprisingly, among all LDS lines, *gly1-1* plants accumulated the lower level of local and systemic Pip compared to Col-0 during morning and evening infiltration of *Psm*, indicating that there might be a connection between plastidal G3P and Pip production (Figures 3.14 b_1, b_2, 3.15 b_1- b_3 and 3.16 b_1 , b_2, 3.17 b_1 , b_2). Interestingly, compared to Col-0, local and systemic levels of pathogen-induced Pip is highly reduced in *gly1-1* plants irrespective of the time of infiltration. Although the level of Pip is highly suppressed in this line, this amount (1 to 5 µg/g FW) is quite sufficient to regulate SAR establishment. Interactions between the pathogen-inducible Pip and the G3P signaling pathways seem to exist, but the mechanisms underlying this interplay still need to be elucidated.

Evaluation of the level of Pip shown in the Figures 3.14, 3.15, 3.16, and 3.17 indicate, that although with different variation in the level of Pip among experiments, LDS mutants are able to significantly accumulate locally and systemically Pip (as main SAR regulator) upon pathogen infiltration in the morning and evening. Accumulation of Pip in these lines is certainly contributed to positive SAR observed in all LDS mutant lines upon morning and evening infiltration of *Psm*.

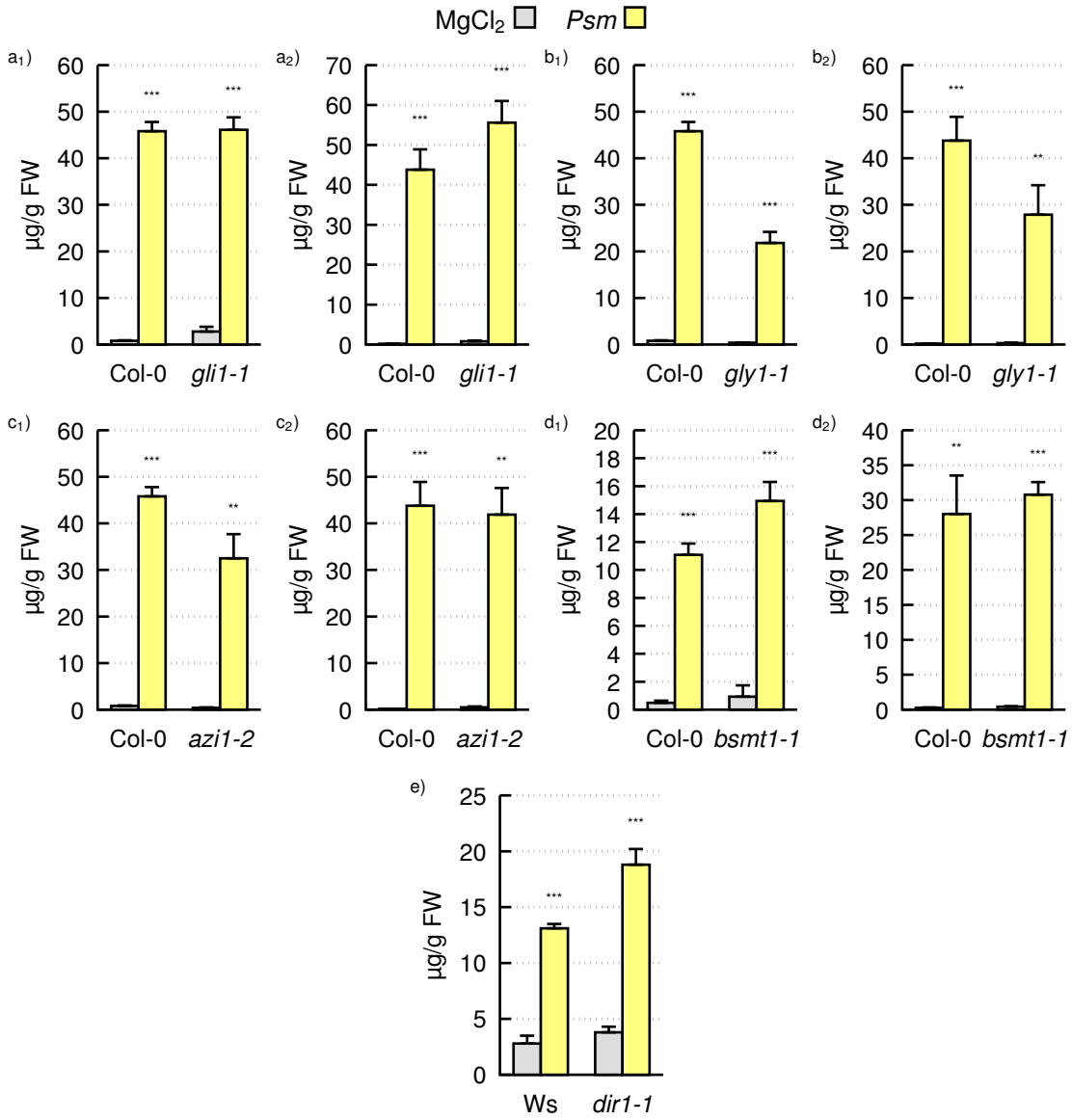

Figure 3.14: Pip accumulation in LDS mutants in local leaves at 24 hpi after morning infiltration a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and local 1° leaves were collected at 24 hpi. Two independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of Pip in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). All graphs except d_1 and d_2 were done by Katrin Gruner.

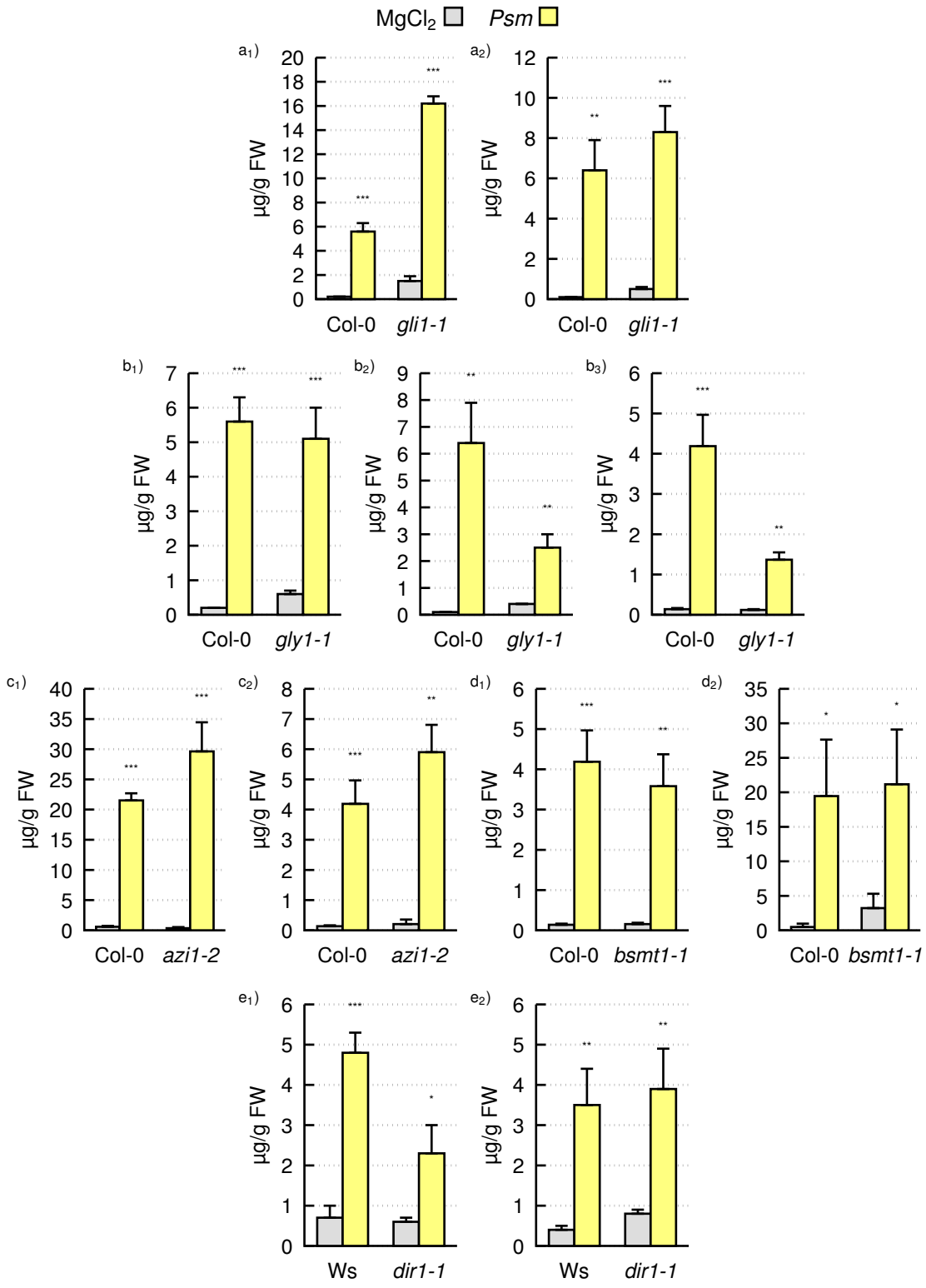

Figure 3.15: Pip accumulation in LDS mutants in systemic leaves at 48 hpi after morning infiltration. a_1-a_2: *gli1-1*, b_1-b_3: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e_1-e_2: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and systemic 2° leaves were collected at 48 hpi. Two-three independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of Pip in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant.) a_1, a_2, b_1, b_2, e_1 and, e_2 were done by Katrin Gruner.

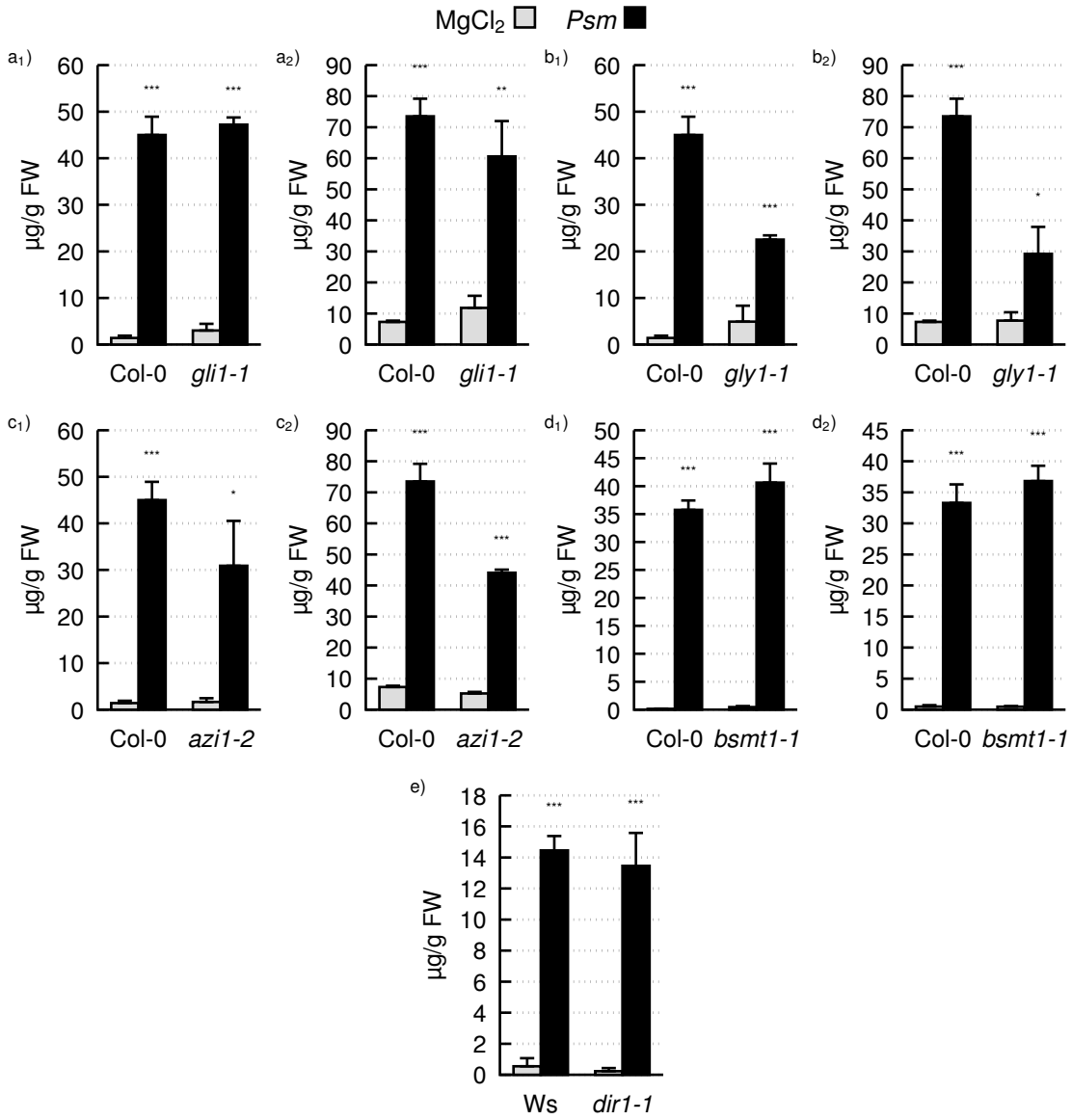

Figure 3.16: Pip accumulation in LDS mutants in local leaves at 24 hpi after evening infiltration a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and local 1° leaves were collected at 24 hpi. Two independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of Pip in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). Experiments a_2, b_2, and c_2 were done by Katrin Gruner.

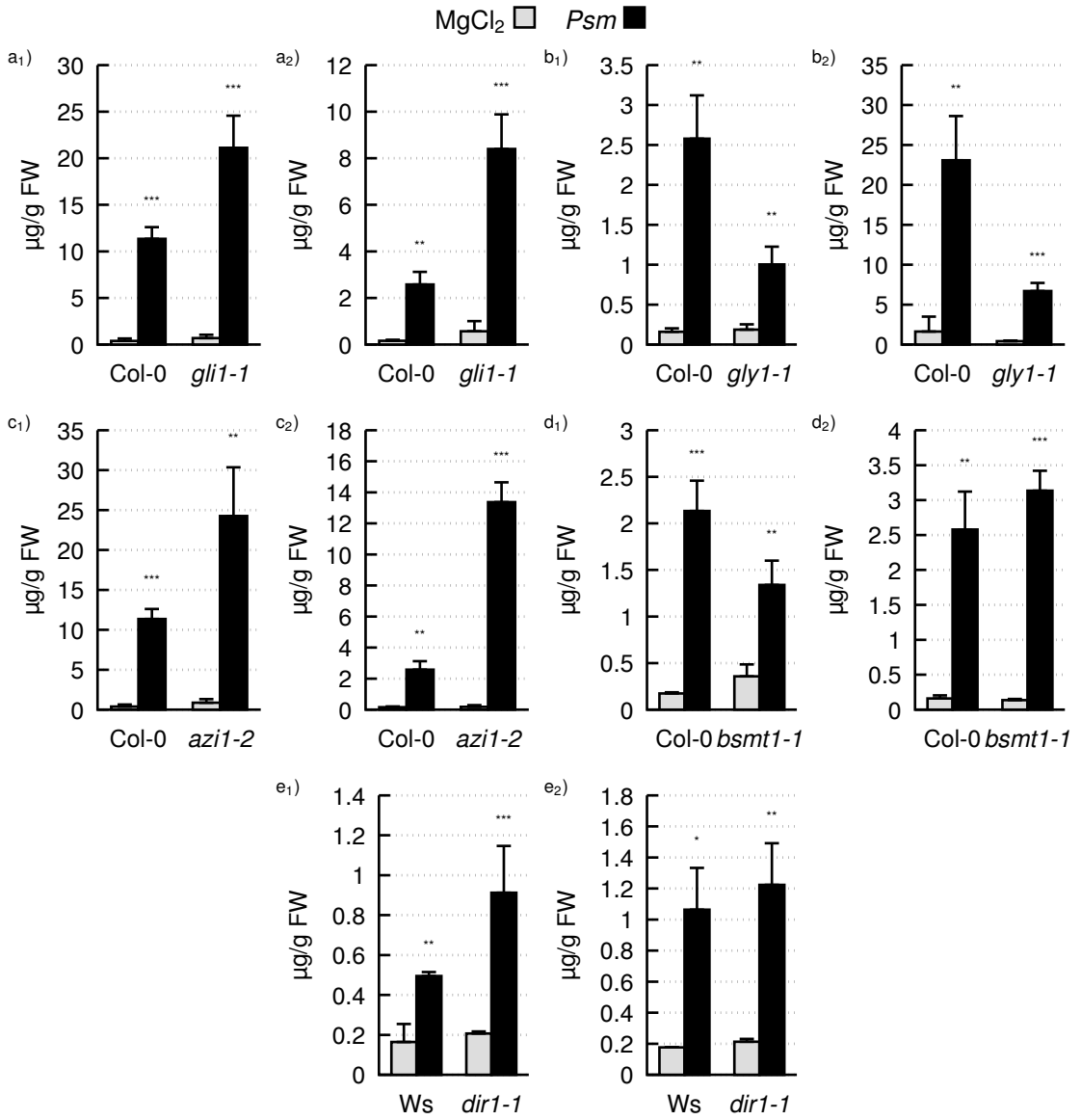

Figure 3.17: Pip accumulation in LDS mutants in systemic leaves at 48 hpi after evening infiltration. a_1-a_2: *gli1-1*, b_1-b_2: *gly1-1*, c_1-c_2: *azi1-2*, d_1-d_2: *bsmt1-1*, e_1-e_2: *dir1-1*. Three lower, 1° leaves per plant were infiltrated at 7 PM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and systemic 2° leaves were collected at 48 hpi. Two independent experiments were performed for each mutant lines. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of Pip in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

3.8 Time course of G3P accumulation in local and systemic leaves of Col-0

Glycerol-3-phosphate (G3P) was considered as a critical inducer of SAR and plants defective in production of G3P were shown to be SAR compromised (Chanda et al., 2011). Inconsistent with these findings, our results show that G3P is not required for SAR, although its contribution to SAR is not neglected. To investigate the accumulation of G3P in response to the pathogen, we analyzed the local and systemic level of G3P at different time points in Col-0 plants infiltrated with *Pseudomonas syringae* at 9 AM. Three local leaves of 5-week-old Col-0 plants were infiltrated with either 10 mM $MgCl_2$ (control buffer) or *Psm* OD_{600}=0.005 at 9 AM. Local infiltrated and systemic leaves were collected every 8 hours and the G3P level was quantified by LC/MS. The G3P evaluation results show that G3P accumulates significantly at 24 and 48 h after pathogen infiltration in local leaves (Figure 3.18 a), whereas Chanda et al., 2011 found the increased level of G3P at 6 hpi in local leaves. In systemic leaves, the level of G3P shows a reduction at 8 hpi and it increases significantly at 24 hpi in systemic leaves (Figure 3.18 b). Therefore, a link between G3P and Pip in long-distance signaling might exist.

Considering the daytime, accumulation of G3P by the pathogen is more induced during morning time in local leaves since we observe an increase in the level of local and systemic G3P after 48 hpi at 9 AM (Figure 3.18 a, b). On the other hand, the level of induced/ non-induced DHAP, as the G3P precursor, is varied during the different time of the day and its lowest amount is detected in the afternoon at night (1 AM) (Figure 3.19 a, b). Moreover, *Psm*-induced glycerol is detected in early time points of pathogen attack at 8 hpi and at 32 hpi in infiltrated leaves and both induced level are depicted at 5 PM (Figure 3.20 a). It is likely that the level of G3P and its precursors (DHAP, glycerol) are regulated by the circadian clock as well and this consequently might affect the amount of induced G3P.

To find out whether the time of pathogen infiltration affects the contribution of G3P to SAR, three leaves of five-week-old Col-0 plants were infiltrated with either 10 mM $MgCl_2$ or *Psm* OD_{600}=0.005 at 9 AM (morning) and at 7 PM (evening) and local and systemic leaves were collected at 24 hpi and 48 hpi respectively. As our results show, the higher level of basal and *Psm*-induced G3P is detected when plants are infiltrated in the evening (Figure 3.21) Moreover, G3P is not induced in systemic leaves of infiltrated plants in both morning and evening time. Therefore, we conclude that G3P is not an active defense inducer in systemic leaves and it is likely that G3P participates in defense mechanism activating in local leaves, especially when pathogen attacks during the evening.

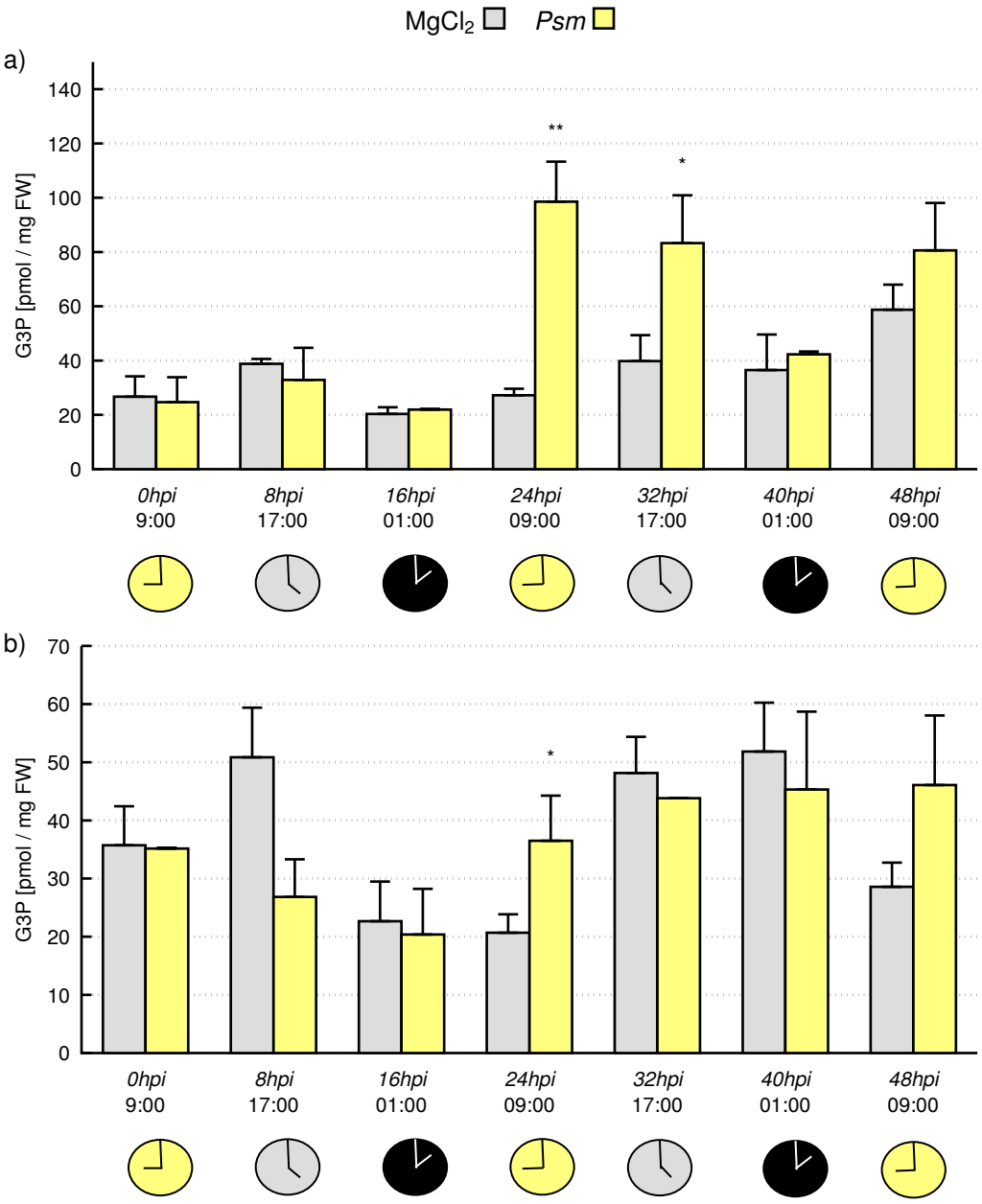

Figure 3.18: Time course accumulation of *Psm*-induced G3P in local (a) and systemic (b) leaves of Col-0. Three lower (local) leaves per plant were infiltrated at 9 AM with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and local and systemic leaves were collected every 8 hours as depicted by clock symbols. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of G3P in mock- and pathogen-infiltrated samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

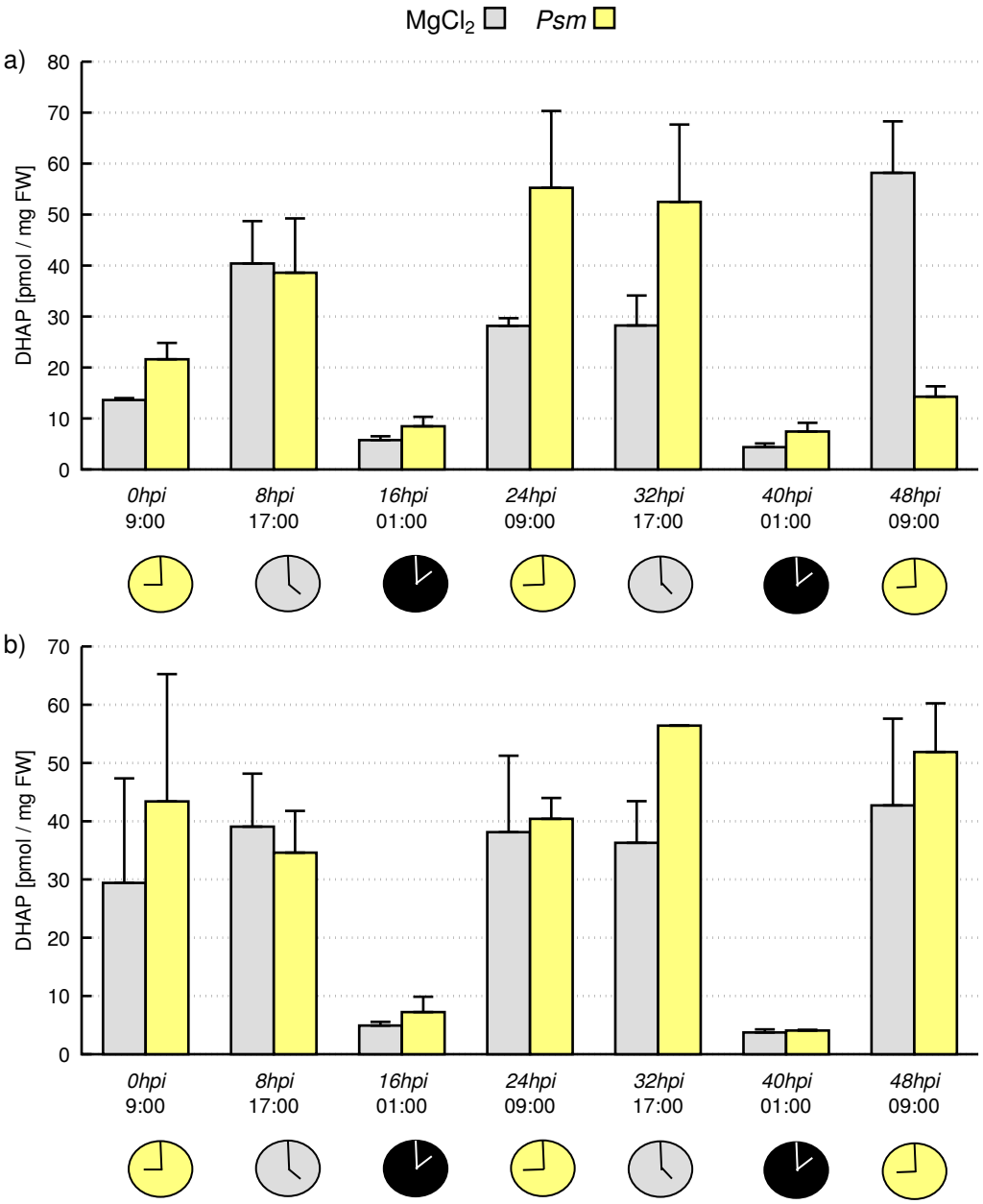

Figure 3.19: Time course accumulation of *Psm*-induced DHAP in local (a) and systemic (b) leaves of Col-0. The experiment and the statistical analysis are explained in the legend of Figure 3.18. The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

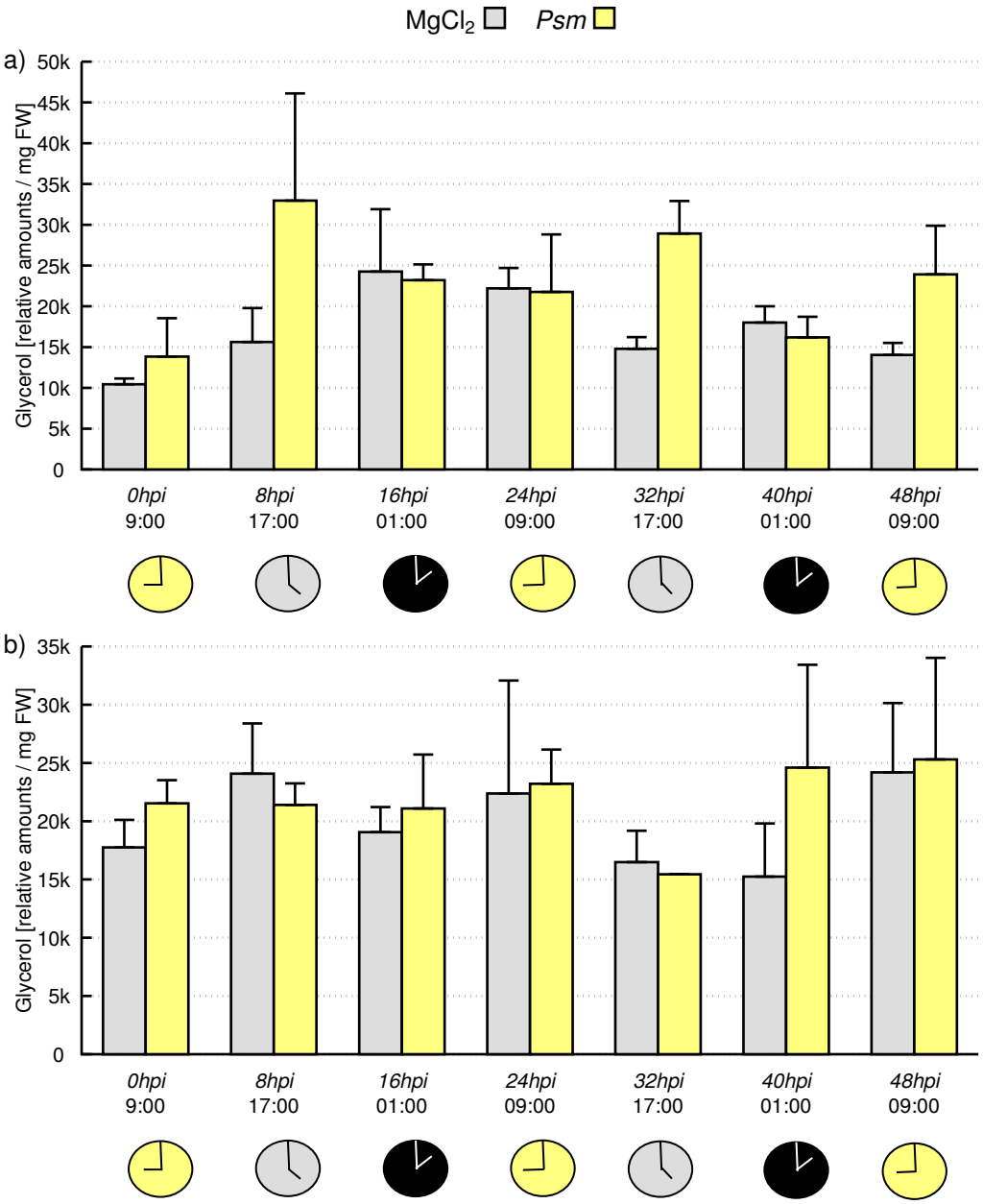

Figure 3.20: Time course accumulation of *Psm*-induced glycerol in local (a) and systemic (b) leaves of Col-0. The experiment and the statistical analysis are explained in the legend of Figure 3.18. The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

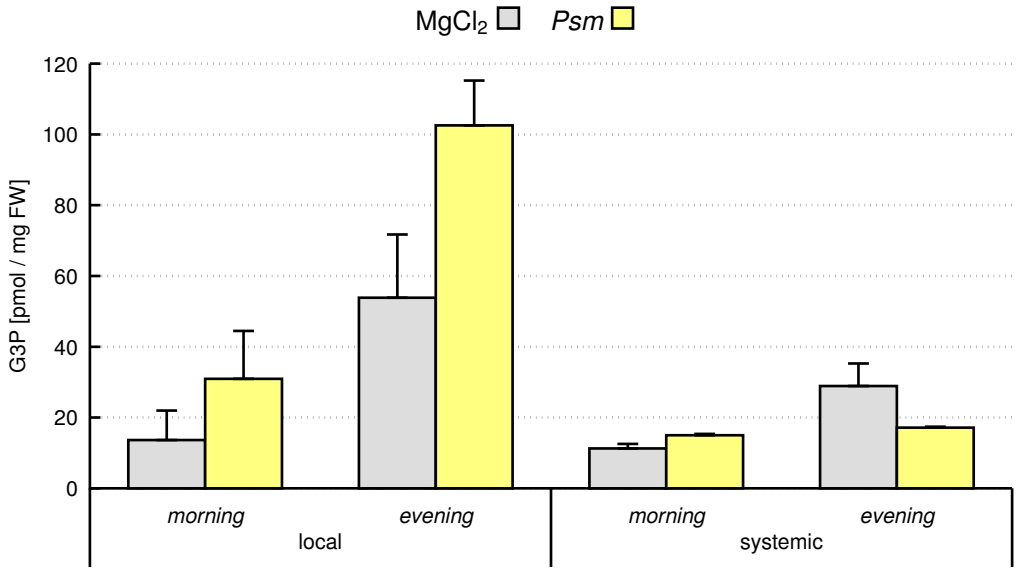

Figure 3.21: Accumulation of G3P in local and systemic leaves of Col-0 after morning and evening infiltration with *Psm*. Three lower (local) leaves per plant were infiltrated at 9 AM (morning) or at 7 PM (evening) with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005) and local and systemic leaves were collected at 24 hpi and 48 hpi respectively. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

3.9 Local and systemic accumulation of G3P upon *Psm* infiltration in the morning and evening

We further evaluated the level of G3P and its precursors (DHAP and glycerol) in local and systemic leaves of Col-0, *gli1-1*, *gly1-1*, and *azi1-2* after *Psm* infiltration in the morning and evening. Three local (1°) leaves of plants were infiltrated either with $MgCl_2$ or virulent strain of *Pseudomonas syringae* (OD_{600}=0.005) in two different time points of a day: at 9 AM (morning-infiltration) and at 7 PM (evening-infiltration). The level of G3P, DHAP, and glycerol was quantified by LC/MS in local and systemic leaves at 24 and 48 hpi respectively.

Arabidopsis *gli1-1* and *gly1-1* mutant lines are not totally blocked in biosynthesis of G3P and morning infiltration of *Psm* induces G3P accumulation local leaves of Col-0, *gly1-1*, and *azi1-2*, but not in *gli1-1* (Figure 3.22 a_1,a_2.). Leaves contain higher level of G3P in the evening than in the morning. Evening-infiltration of *Psm* induces local accumulation of G3P in all lines (Figure 3.22 a_1). It is likely that GLI1 is required for G3P accumulation in the local leaves when pathogen attacks in the morning but not during evening infiltration (Figure 3.22 a_1).

In systemic leaves, morning infiltration of pathogen induces slight accumulation of G3P in Col-0, *gli1-1*, and *azi1-2* but not in *gly1-1* (Figure 3.22 a_2). However, evening infiltration of *Psm* failed to induce systemic G3P accumulation in Col-0, *gli1-1*, and *gly1-1*, but not in *azi1-2*, showing that AZI1 might not be required for systemic G3P accumulation upon *Psm* attack in the evening and GLY1 might be required for systemic accumulation of G3P upon morning- and evening-infiltration of *Psm* (Figure 3.22 a_2). The pattern of *Psm*-induced DHAP accumulation in the local leaves was similar in both morning and evening infiltrations (Figure 3.23 b_1, b_2). *Psm* was not able to induce DHAP in local leaves of *gli1-1* and upon morning and evening infiltrations (Figure 3.23 b_1). Moreover, irrespective to the time of infiltration (morning/evening), no significant increase in the systemic level of DHAP was observed in Col-0, *gli1-1*, *gly1-1*, and *azi1-2* (Figure 3.23 b_2).

Expectedly, *gli1-1* plants contain a higher local and systemic level of glycerol compared to Col-0 (Figure 3.24 c_1, c_2). *Psm* did not induce local accumulation of glycerol upon morning infiltration, however, *Psm* slightly induced glycerol accumulation in local leaves of Col-0 and *gli1-1* during evening infiltration (Figure 3.24 c_1). In systemic leaves of plants infiltrated in the evening, *Psm* induced glycerol accumulation in Col-0, *gli1-1*, and *gly1-1* but not in *azi1-2* (Figure 3.24 c_2).

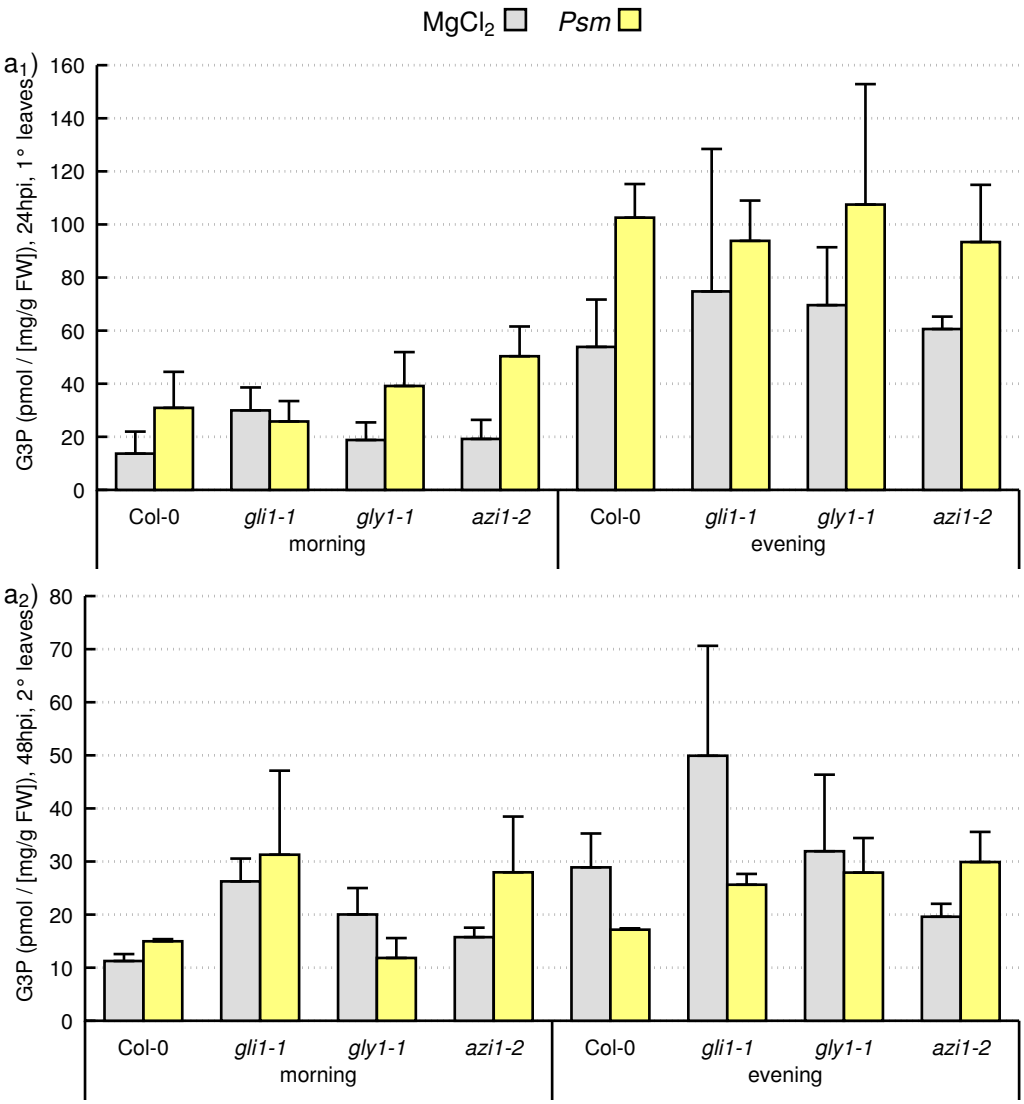

Figure 3.22: accumulation of *Psm*-induced G3P in local (1°) leaves at 24 hpi (a_1) and in systemic (2°) leaves at 48 hpi (a_2) in Col-0, *gli1-1*, *gly1-1* and *azi1-2*. Three local leaves of plants were infiltrated with either $MgCl_2$ (as control buffer) or with *Psm* in two different time points (9 AM for morning and 7 PM for evening infiltration). Infiltrated (1°) and systemic (2°) leaves were collected after 24 and 48 hpi respectively and the level of G3P was quantified using LC/MS. The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

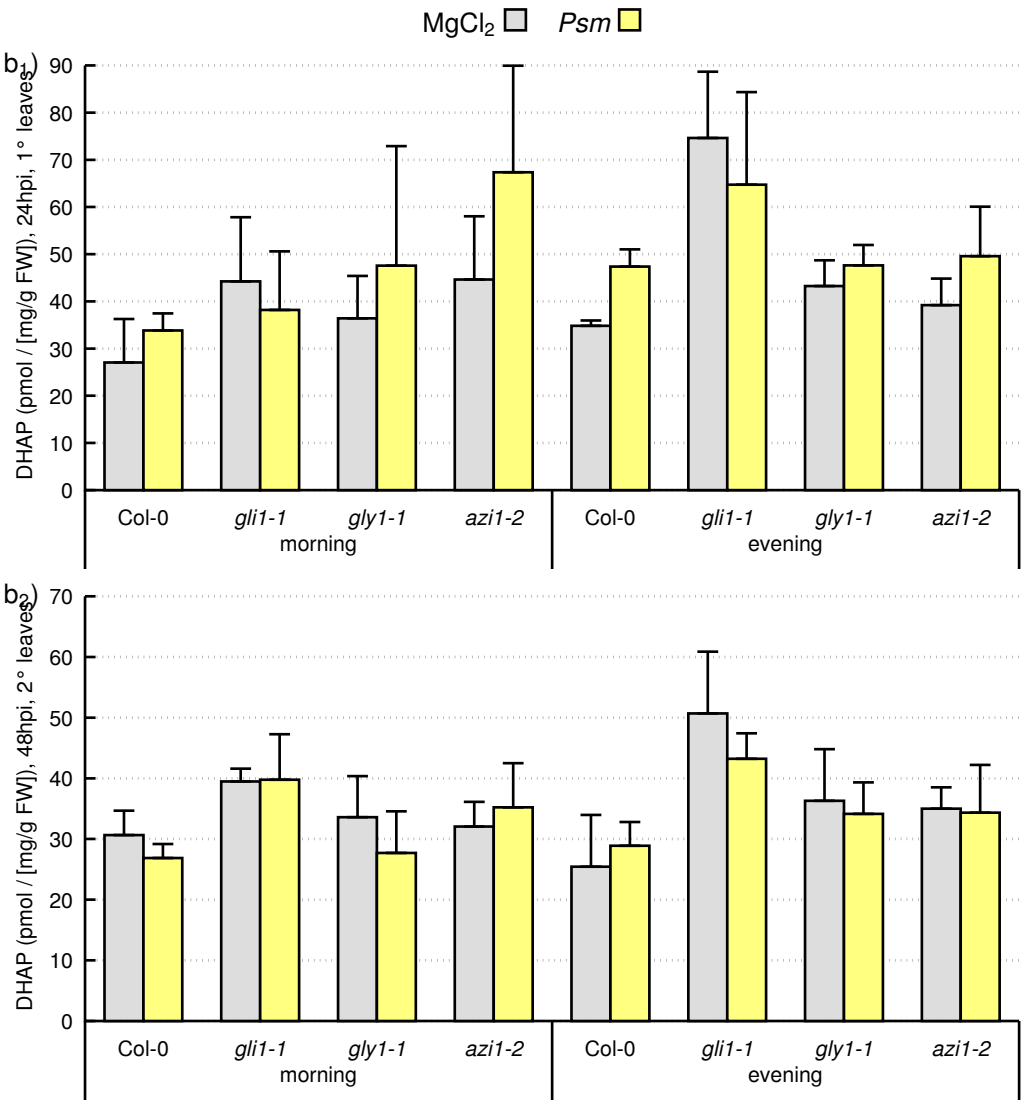

Figure 3.23: accumulation of *Psm*-induced dihydroxyacetone phosphate (DHAP) in local (1°) leaves at 24 hpi (b_1) and in systemic (2°) leaves at 48 hpi (b_2) in Col-0, *gli1-1*, *gly1-1* and *azi1-2*. The experimental procedure is the same as the one in Figure 3.22. The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

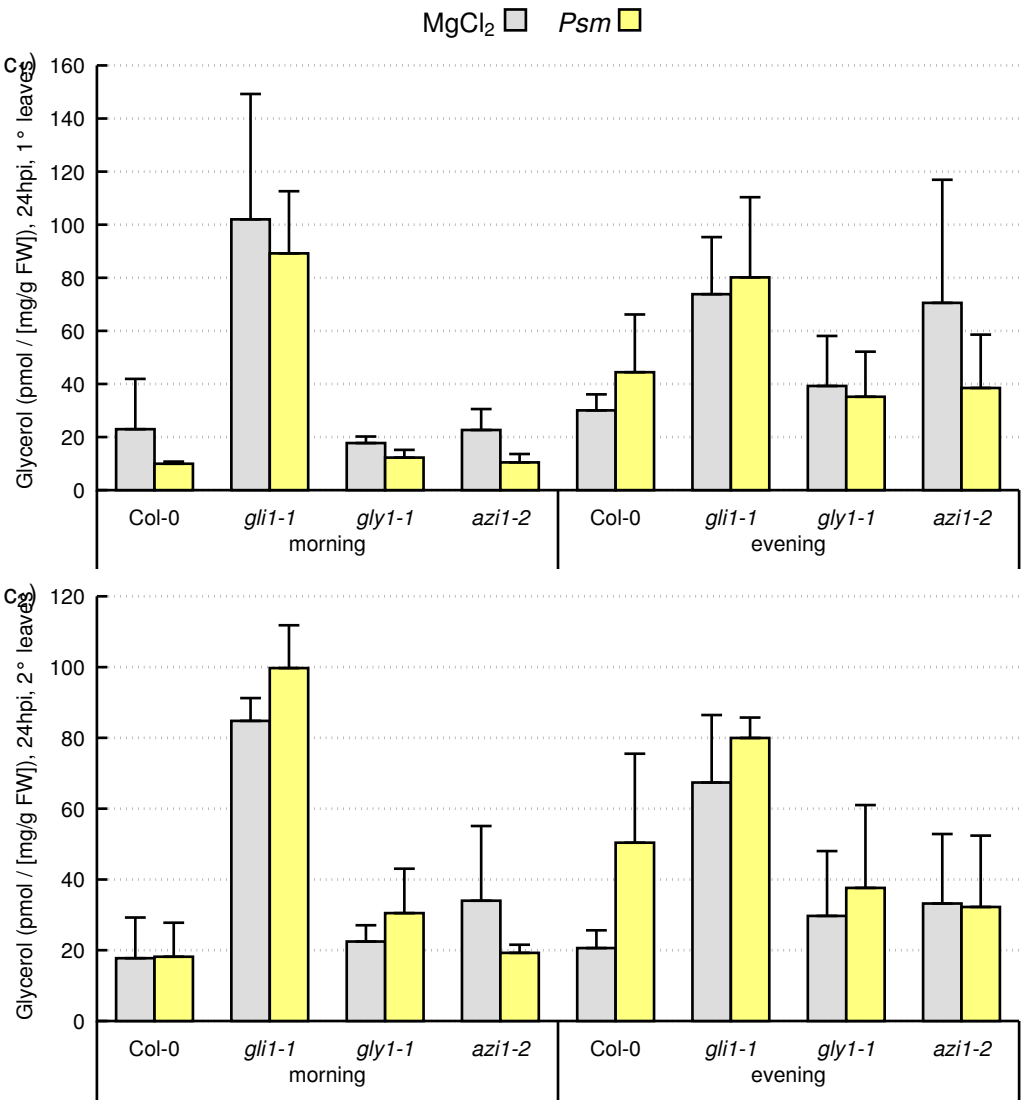

Figure 3.24: accumulation of *Psm*-induced glycerol in local (1°) leaves at 24 hpi (c_1) and in systemic (2°) leaves at 48 hpi (c_2) in Col-0, *gli1-1*, *gly1-1* and *azi1-2*. The experimental procedure is the same as the one in Figure 3.22. The experiment was done in collaboration with Dr. Tabea Mettler at the metabolic profiling center, HHU.

3.10 Pip-induced resistance develops similarly in *gli1-1*, *gly1-1*, *bsmt1-1*, *azi1-2*, *dir1-1* and wild-type plants

According to Návarová et al., 2012, exogenous application of Pip promotes a primed, SAR-like state in plants. It has been shown that feeding the plants with 10 ml of 10 µmol exogenous Pip restores SAR in SAR-defective mutant *ald1*. Moreover, applied exogenous Pip is sufficient to enhance resistance to *Psm* and induces defense priming to the similar extent as in biological SAR (Návarová et al., 2012). To examine whether there is a crosstalk between Pip and long-distant signals, Arabidopsis LDS mutants were exogenously supplied (through root system) with 10ml of 1 mM (10 µmol) D,L-Pip and 10 ml of water as the control. Three leaves in each plant were challenged with *Psm lux* (OD_{600}=0.001) one day after water and Pip treatment. Bacterial growth in all lines was scored 2.5 days after pathogen inoculation. We repeated the experiment three times independently.

Figures 3.25 and 3.26 indicate that exogenous Pip significantly suppresses the manipulation of bacteria in all lines and obviously in *ald1* as well. A lower basal resistance compared to Col-0 was observed in *gli1-1* lines in at least two out of three experiments, however this line positively responded to exogenous Pip (Figures 3.25 a_1 and a_3). *bsmt1-1*, *azi1-2*, and *dir1-1* lines showed a wild-type like Pip-IR in at least two out of three individual experiments (Figures 3.25 and 3.26). As proved previously in Návarová et al., 2012, *fmo1* is not capable to induce resistance upon Pip feeding and Pip-priming requires a functional FMO1. Our data prove that there is no contribution of MeSA, AzA, DIR1, and G3P to Pip-IR.

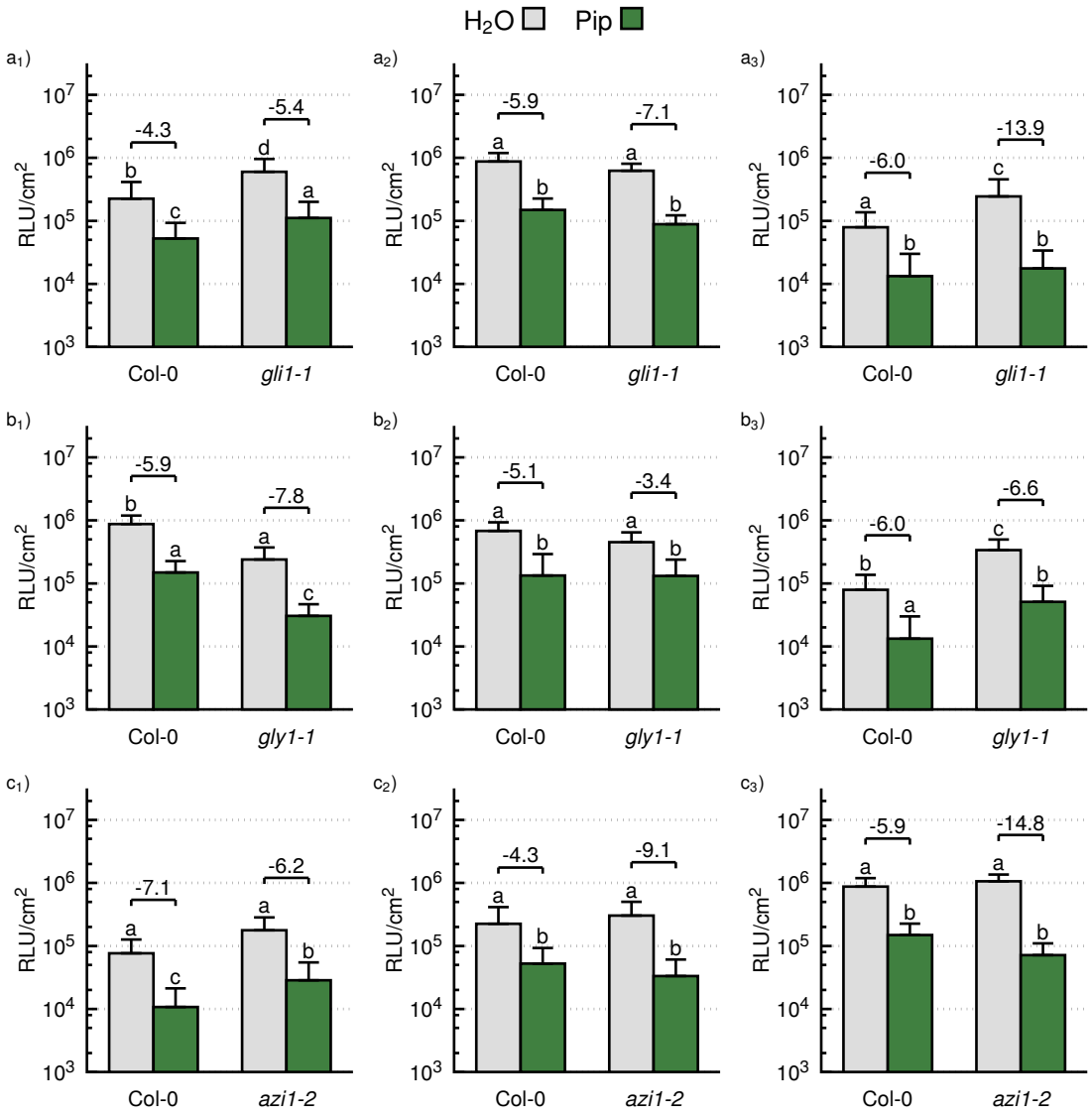

Figure 3.25: Pip induced-resistance (Pip-IR) in *gli1-1*, *gly1-1*, and, *azi1-2*. a_1-a_3: *gli1-1*, b_1-b_3: *gly1-1*, c_1-c_3: *azi1-2*. Plants were supplied with 10 ml of 1 mM D,L Pip (= dose of 10 µmol) or with 10 ml of H_2O (control treatment) via the root system, and three leaves of each plant were inoculated with *Psm lux* OD_{600}=0.001 one day later. The growth of *Psm lux* was assessed after 2.5 d by luminescence measurements. Three independent experiments were performed for each LDS mutant lines. Data represent mean ± SD of the growth value of at least 18 leaf replicates. A logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between water- and Pip-treated samples which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.) a_1, c_1, and c_2 were done by Katrin Gruner.

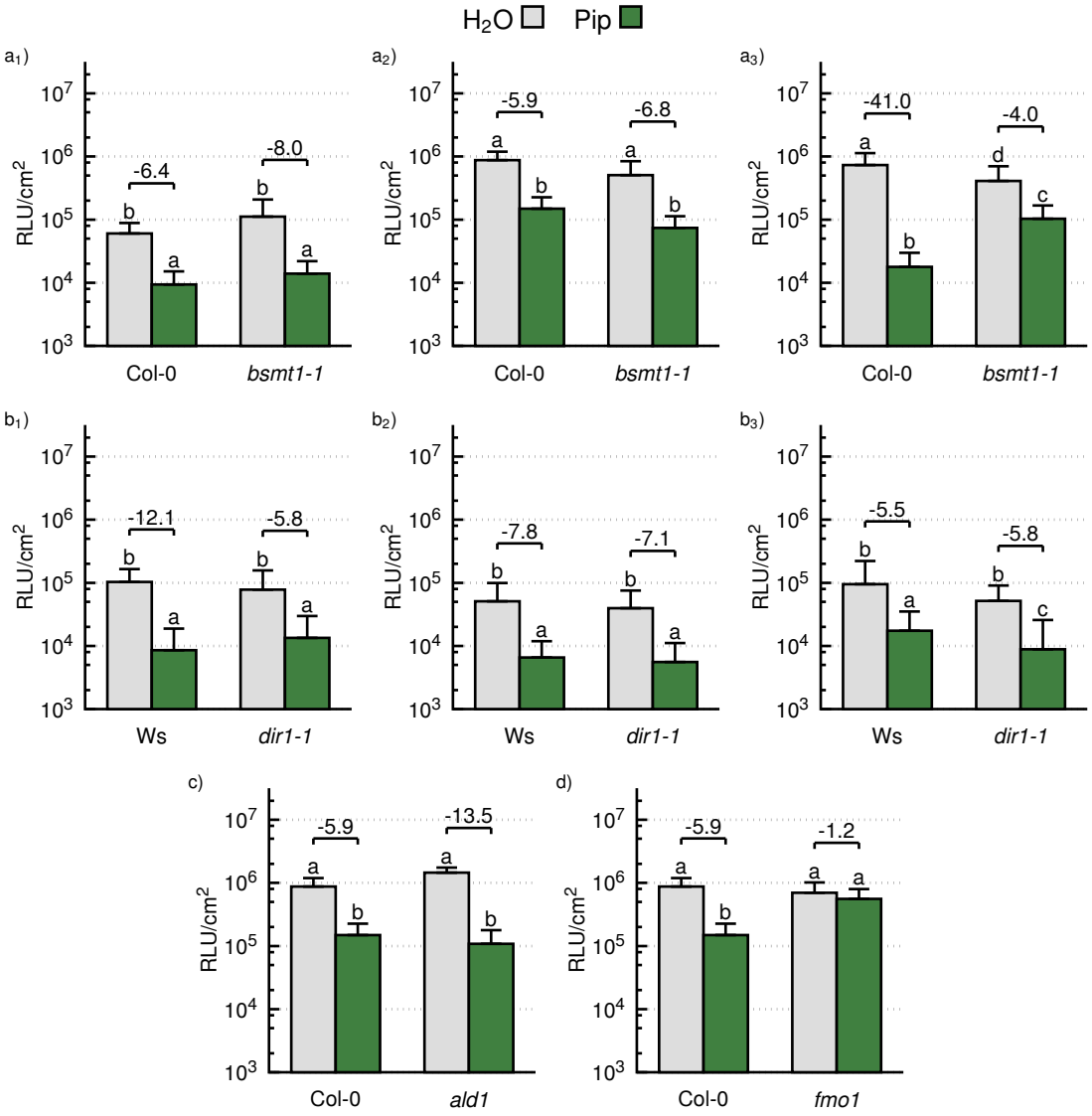

Figure 3.26: Pip induced-resistance (Pip-IR) in *bsmt1-1*, *dir1-1*, *ald1* and, *fmo1-1*. a_1-a_3: *bsmt1-1*, b_1-b_3: *dir1-1*, c: *ald1*, d: *fmo1-1*. Plants were supplied with 10 ml of 1 mM D,L Pip (= dose of 10 µmol) or with 10 ml of H_2O (control treatment) via the root system, and three leaves of each plant were inoculated with *Psm lux* OD_{600}=0.001 one day later. Growth of *Psm lux* was assessed after 2.5 d by luminescence measurements. Three independent experiments were performed for each LDS mutant lines. Data represent mean ± SD of the growth value of at least 18 leaf replicates. A logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between water- and Pip-treated samples which are calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.) b_1 and b_2 was done by Katrin Gruner.

3.11 SA acts downstream of MeSA, AzA, DIR1, and G3P for induction of basal resistance

In addition to Pip, exogenous SA is shown to induce expression of a set of defense-related genes and confer plant disease resistance to hemibiotrophic and biotrophic pathogens (Delaney et al., 1995). We already know that exogenous SA can induce resistance independently of Pip/FMO signaling pathway (Bernsdorff et al., 2016). To examine whether there is an interplay between individual SAR signals and SA, three leaves of plants were directly infiltrated either with water (as control) or with 0.5 mM SA (pH=7). The same leaves were challenged with *Psm lux* (OD_{600}=0.001) four hours later. Bacterial growth was measured 2.5 days after *Psm lux* infiltration. We repeated this experiment two times for each line (Figures 3.27 a_1,a_2 and Figure 3.28 a, b). According to our ANOVA analysis, upon SA infiltration, bacterial growth was significantly reduced in wild-type lines (Col-0 and Ws) and also in all LDS mutant lines *gli1-1*, *gly1-1*, *azi1-2*, *bsmt1-1*, and *dir1-1* ($P < 0.05$).

NPR1 functions downstream of Pip and SA in defense signaling pathways and is essential for SA-IR (Bernsdorff et al., 2016). Therefore *npr1* plants did not show defense responses upon exogenous SA, indicating that SA-IR requires a functional NPR1. Moreover, *fmo1-1* and *ald1* showed a significant bacterial reduction upon SA infiltration and this lines were not defective in SA-IR. Our data show that there is no contribution of MeSA, AzA, DIR1, and G3P for activation of SA-induced defense responses and SA acts downstream of these signals.

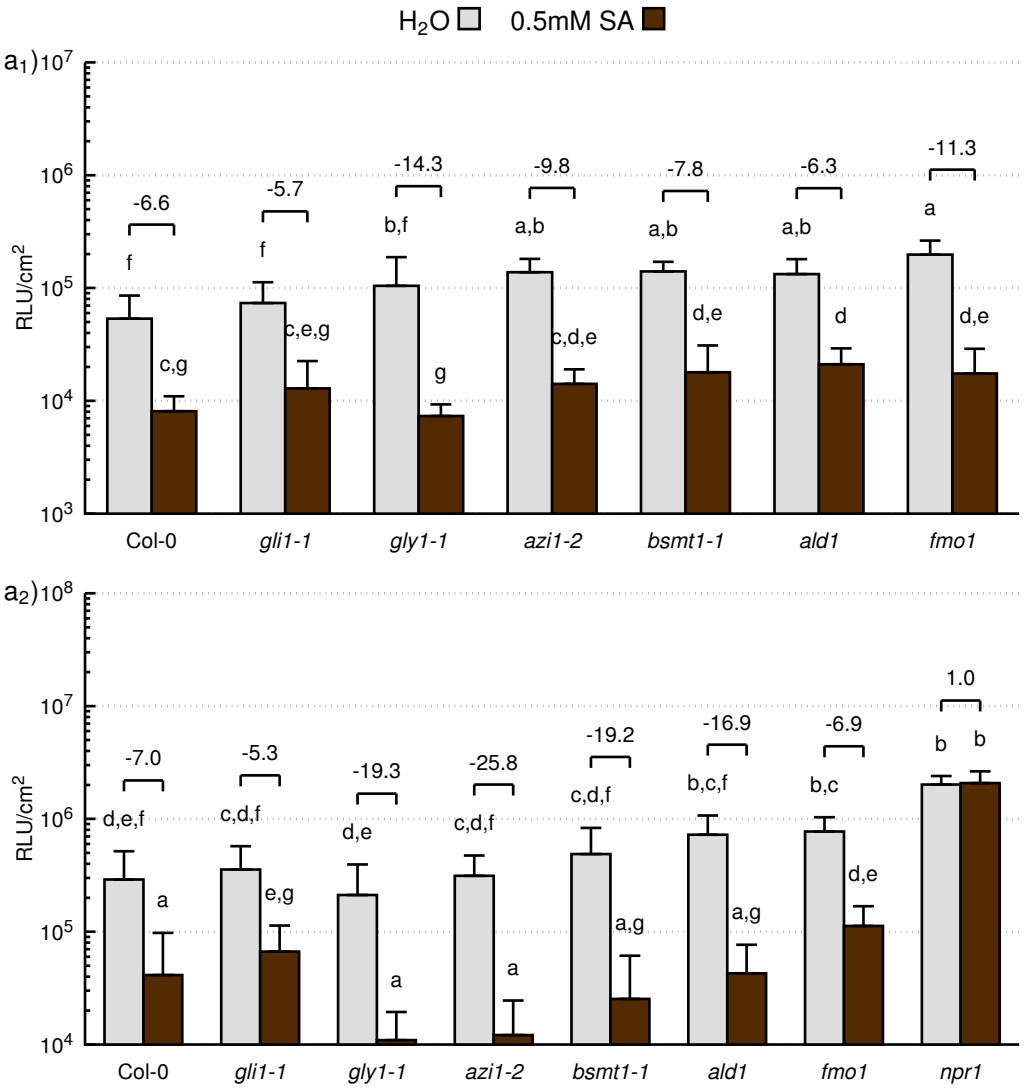

Figure 3.27: SA induced-resistance (SA-IR) in LDS mutant lines. a_1: SA-IR repetition experiment 1. a_2: SA-IR repetition experiment 2. Three leaves of each plant were infiltrated with either with water (as control) or with 0.5 mM SA (pH=7) and the same leaves of each plant were inoculated with *Psm lux* OD_{600}=0.001 4 h later. The growth of *Psm lux* was assessed after 2.5 d by luminescence measurements. Two independent experiments were performed for each LDS mutant lines. Data represent mean ± SD of the growth value of at least 18 leaf replicates. A logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between water- and SA- infiltrated samples which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.)

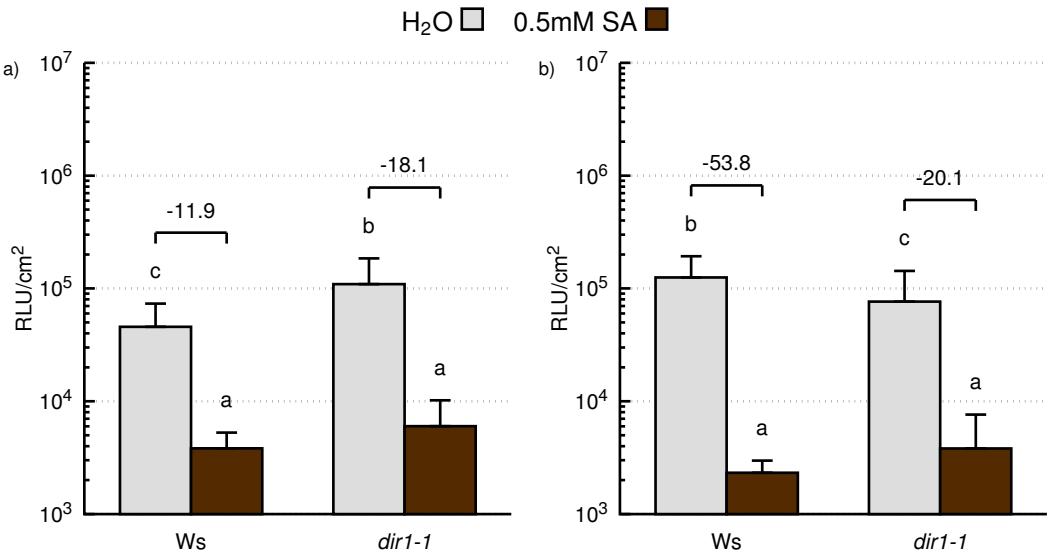

Figure 3.28: SA induced-resistance (SA-IR) in Ws and *dir1-1*. a: SA-IR repetition experiment 1. b: SA-IR repetition experiment 2. Three leaves of each plant were infiltrated with either with water (as control) or with 0.5 mM SA (pH=7) and the same leaves of each plant were inoculated with *Psm lux* OD_{600}=0.001 4 h later. The growth of *Psm lux* was assessed after 2.5 d by luminescence measurements. Two independent experiments were performed for each lines. Data represent mean ± SD of the growth value of at least 18 leaf replicates. A logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between water- and SA- infiltrated samples which is calculated based on total average values. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.)

4 Results: Effect of light on SAR and long-distance signaling

4.1 SAR is a light-dependent process

Light has been demonstrated to affect local defense responses and SAR in Arabidopsis (Zeier et al., 2004). Infection of Arabidopsis plants with an avirulent strain of *Pseudomonas syringae* (*Psm AvrRpm1*) in darkness, resulted in higher bacterial growth and reduced basal resistance as compared to bacterial infection in the light (Zeier et al., 2004). Induction of several defense responses such as activation of phenylalanine ammonia-lyase (PAL), accumulation of SA, expression of PR1, and the development of hypersensitive response (HR) are proved to be light dependent, whereas pathogen-induced accumulation of jasmonic acid, production of camalexin and transcriptional induction of a pathogen- inducible myrosinase are even more pronounced in the dark (Zeier et al., 2004).

In addition light influences the establishment SAR and SAR development was totally lost when the primary inoculation of Arabidopsis with *Psm AvrRpm1*, was performed in the absence of light (Zeier et al., 2004). When plants infiltrated with *Psm AvrRpm1*, were able to establish SAR under medium (70 µmol photons m^{-2} s^{-1}) and strong (500 µmol photons m^{-2} s^{-1}) light conditions but not under constant darkness (Zeier et al., 2004). Development of SAR under strong light was independent on SA and PR1 expression (Zeier et al., 2004). In order to confirm the light dependency of SAR, we conducted SAR assay under light and dark conditions. Therefore, we challenged 5-week-old Arabidopsis Col-0 plants either with 10 mM $MgCl_2$ as control buffer or with bacterial pathogen *Pseudomonas syringae* (OD_{600}=0.005) at 9 AM at the first day of the experiment (day 0) as shown in Figure 4.1 a. Then we divided the plants into two groups (light- and dark-plants). Half of the plants remained in the same growth condition in which they received 10 hours light per day from 9 AM till 7 PM (light plants) (Figure 4.1 a, top). The second half of the plants were adjusted to dark condition in which the plants were exposed to darkness during SAR induction which means during first two days after inoculation of pathogen (day 0-2) (dark plants) (Figure 4.1 a, below). Two days after first pathogen inoculation (on day 2), dark plants were also located in normal growth conditions as same as light plants and the systemic leaves of both groups were challenged with *Psm lux* (OD_{600}=0.001) at 9 AM on this day. The systemic bacterial level was scored 2.5 days later (on day 4) (Figure 4.1 a).

Regardless of light or dark condition, infiltration of primary leaves with $MgCl_2$ as control resulted in massive growth of bacteria in systemic leaves . Expectedly, primary infection with *Psm* under normal light condition reduced the bacterial growth (8.6 fold reduction), indicating a successful SAR establishment

(Figure 4.1 b). However, when primary inoculation process occurred in dark, no significant difference was observed in the level of systemic bacteria in $MgCl_2$- and *Psm*-infiltrated plants (Figure 4.1 b). Plants failed to establish SAR when there was no light during the induction of SAR. In comparison to light condition, increased level of bacteria growth in mock-treated leaves under constant darkness indicated reduced basal resistance in dark-adapted plants. This results demonstrated that the establishment of SAR is light dependent.

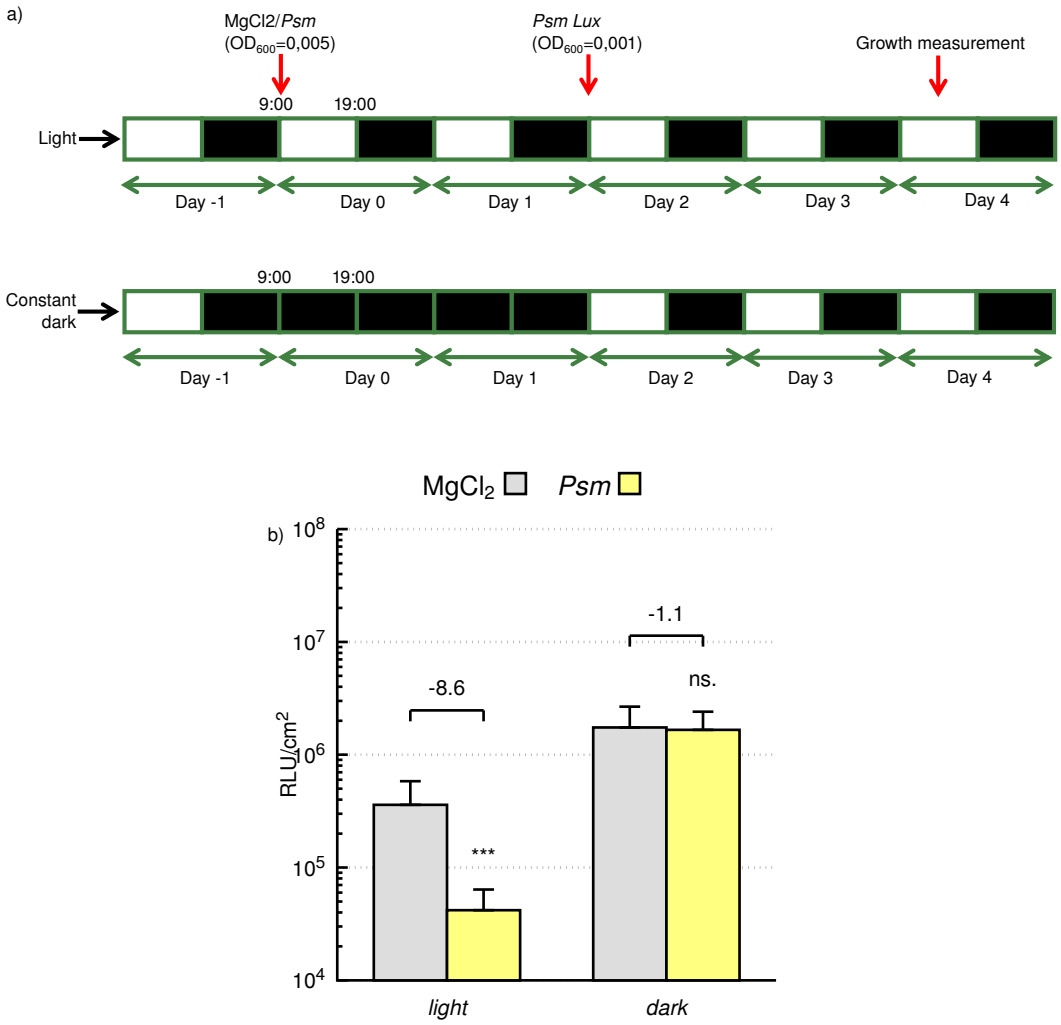

Figure 4.1: SAR assay in Arabidopsis Col-0 under light and constant dark condition. a: Light (top) and constant dark (below) regime in the plant growth chamber. Black and white boxes correspond to dark and light periods, respectively. Plants were grown under normal light (top) condition for four weeks and half of the plants were adjusted to constant dark (below) condition as shown. 5-week-old Col-0 plants were infiltrated with 10 mM $MgCl_2$ (control buffer) or *Psm* (OD_{600}=0.005) at 9 AM on day 0. Three systemic leaves of all plants were infiltrated with *Psm lux* (OD_{600}=0.001) two days later (on day 2). The growth of *Psm lux* was assessed after 2.5 d by luminescence measurements. b: Comparison of SAR establishment in Col-0 in light and constant dark conditions. Data represent the mean ± SD of at least 20 leaf replicates from 6 to 7 different plants. A logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Two-tailed *t* -test was used to determine the statistically significant difference between bacterial growth in mock- and pathogen -infiltrated plants (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

4.2 Accumulation of SAR regulators SA and Pip is dependent on light

4.2.1 Pathogen-induced SA accumulation in local and systemic leaves, is light dependent

Morning and midday inoculation of Arabidopsis plants with *Psm* resulted in higher accumulation of defense regulator salicylic acid (SA) and faster expression of PR1 compared to evening or night inoculation (Griebel and Zeier, 2008). SAR was also developed in plants under strong light condition (PDF = 500 µmol photons m^{-2} s^{-1}), however, the SAR development was not associated with activation of SAR markers (SA and PR1) in systemic tissue (Zeier et al., 2004). To test whether loss of SAR in dark-adapted plants is associated with the lower amount of free SA and its conjugated form (SAG) in systemic leaves, two sets of Col-0 plants adjusted to light conditions following the experimental setup described in Figure 4.2. We inoculated three local leaves of each plant with either avirulent or virulent strain of *Pseudomonas syringae* (OD_{600}=0.005) on day 0 at 9 AM and local and systemic leaves were collected in three time points (10, 24, and 48 hours post infiltration (hpi)) for metabolite analysis.

When Col-0 plants remains in their grown-light condition, following *Psm AvrRpm1* inoculation, SA increases significantly ($P < 0.01$) in early hours after pathogen attack at 10 hpi in local leaves (Figure 4.3 a_1) and at 48 hpi in systemic leaves (Figure 4.3 a_2). When plants were translocated in darkness, *Psm AvrRpm1*-induction of local SA was following the same pattern as in light-grown plants and free SA was significantly induced at 10 hpi ($P < 0.001$). Nevertheless, a highly reduced level of SA compared to light-grown plants was observed in dark-adjusted plants (Figure 4.3 a_1). Systemic leaves of *Psm AvrRpm1*-infiltrated plants did show an increased level of SA at 24 hpi, however, lower values of SA were observed in these leaves when compared to light-grown leaves (Figure 4.3 a_2).

In local and systemic leaves of light-grown plants, *Psm*-induced SA accumulation begins at 24 hpi in both local and systemic leaves (Figure 4.3 b_1-b_2). The local level of SA increased to the highest amount (~ 40 µg/g FW) after 48 hpi which is an almost double amount of SA collected after 24 hpi. Dark-adapted plants followed the same pattern, however, the highest level of SA was almost 10 µg/g FW which was significantly ($P < 0.05$) induced in local leaves (Figure 4.3 b_1). In plants grown under the normal light/dark condition, the pathogen induced the systemic accumulation of SA after 24 hpi (~ 4 µg/g FW) and similar to local leaves, this level was increased to its highest amount (~ 8 µg/g FW) after 48 hpi (Figure 4.3 b_2). Under the constant dark condition, we detected an increased *Psm*-induced level of SA after 24 hpi, however, this level was low when compared to that of SA accumulated 24 hpi in light-grown plants (increased up to 1 µg/g FW) (Figure 4.3 b_2). Dark-adapted plants failed to increase this level after 48 hpi, suggesting the requirements of light to maintain and to increase the *Psm*-induced SA level especially in systemic leaves (Figure 4.3 b_2).

To sum up, lower SA levels comparable with those accumulating in leaves of plants experiencing normal light/dark condition was detected at 10, 24, and 48 hpi in both local and systemic tissues upon *Psm* and *Psm AvrRpm1* inoculation. Differences in level of SA accumulation observed in light and dark conditions, resulted from the length of the light period after pathogen inoculation. Obviously, absence of light did not

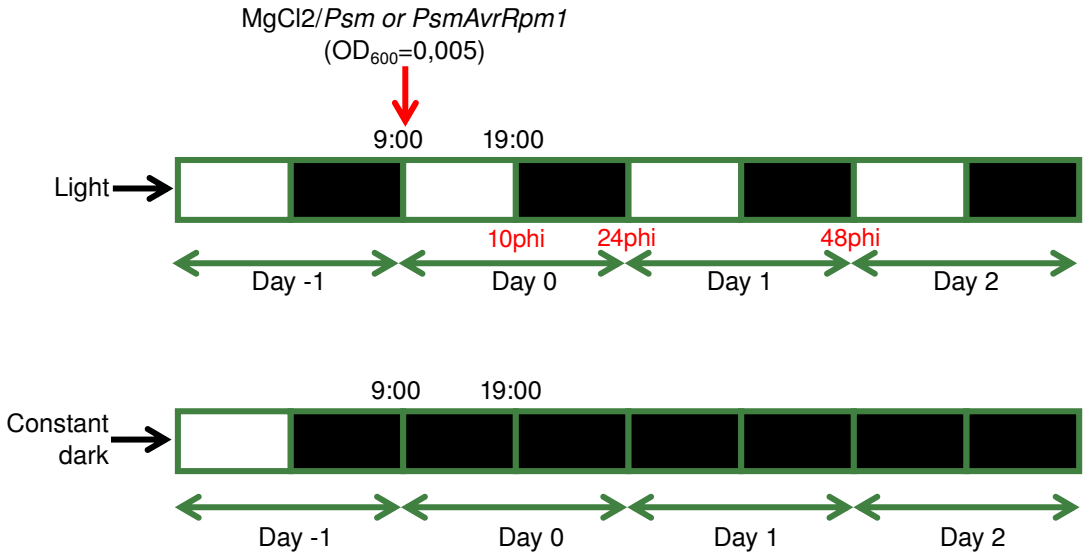

Figure 4.2: Experimental setup for SA and Pip quantification in Light (top) and constant dark (bottom) regimes in the plant growth chambers. Black and white boxes correspond to dark and light periods, respectively. The experiment is more explained in Figure 4.3.

abolish the SA production, however, the level of total SA is reduced to a significant amount. This results confirmed the previous work by Griebel and Zeier, 2008 and demonstrated that the amount of total SA strongly depends on the light applied after bacterial inoculation (Figure 4.3).

4.2.2 Pip biosynthesis is a light-dependent process

In the course of SAR, Pip is accumulated in inoculated and distal leaves from the inoculation site, and petiole exudate of inoculated leaves and the accumulation of Pip is necessary for SAR (Návarová et al., 2012). To examine whether the production of pathogen-induced Pip is influenced by light, we compared the level of Pip in response to *Psm* inoculation, in two sets of 5-week-old Col-0 plants. All plants were grown under conventional 10 h light/14 h dark photoperiodic conditions (referred as a light-grown condition) and half of these plants were transferred to continuous darkness one day before pathogen inoculation (as described in Figure 4.2). We inoculated three local leaves of each plant with *Psm* (OD_{600}=0.005) on day 0 at 9 AM. The level of Pip was quantified in local and systemic leaves after 10, 24, and 48 hours post infiltration (hpi). Under the normal light condition, a strong increase in *Psm*-induced Pip level of 20 µg/g FW was observed at 24 hpi in local leaves which this amount was increased by the factor of 6 (120 µg/g FW) after 48 hpi (Figure 4.4 a_1). We next checked for the level of Pip in systemic leaves in light-grown plants. A low but highly significant increase in the level of Pip (4 µg/g FW) was observed in leaves distal systemic leaves when compared to mock-treated leaves (Figure 4.4 a_2). .

Although plants which were grown under constant darkness did not totally block the induction of *Psm*-induced Pip biosynthesis in both local and systemic leaves, the level of Pip was strongly reduced in all three time points when compared to light condition (Figure 4.4 a_2). To confirm our data, we repeated the same experiment twice, and to analyze the Pip level, local and systemic leaves were collected at 24 hpi and 48 hpi respectively (Figure 4.4 b_1, b_2, c_1, c_2). In both experiments, the pathogen was able

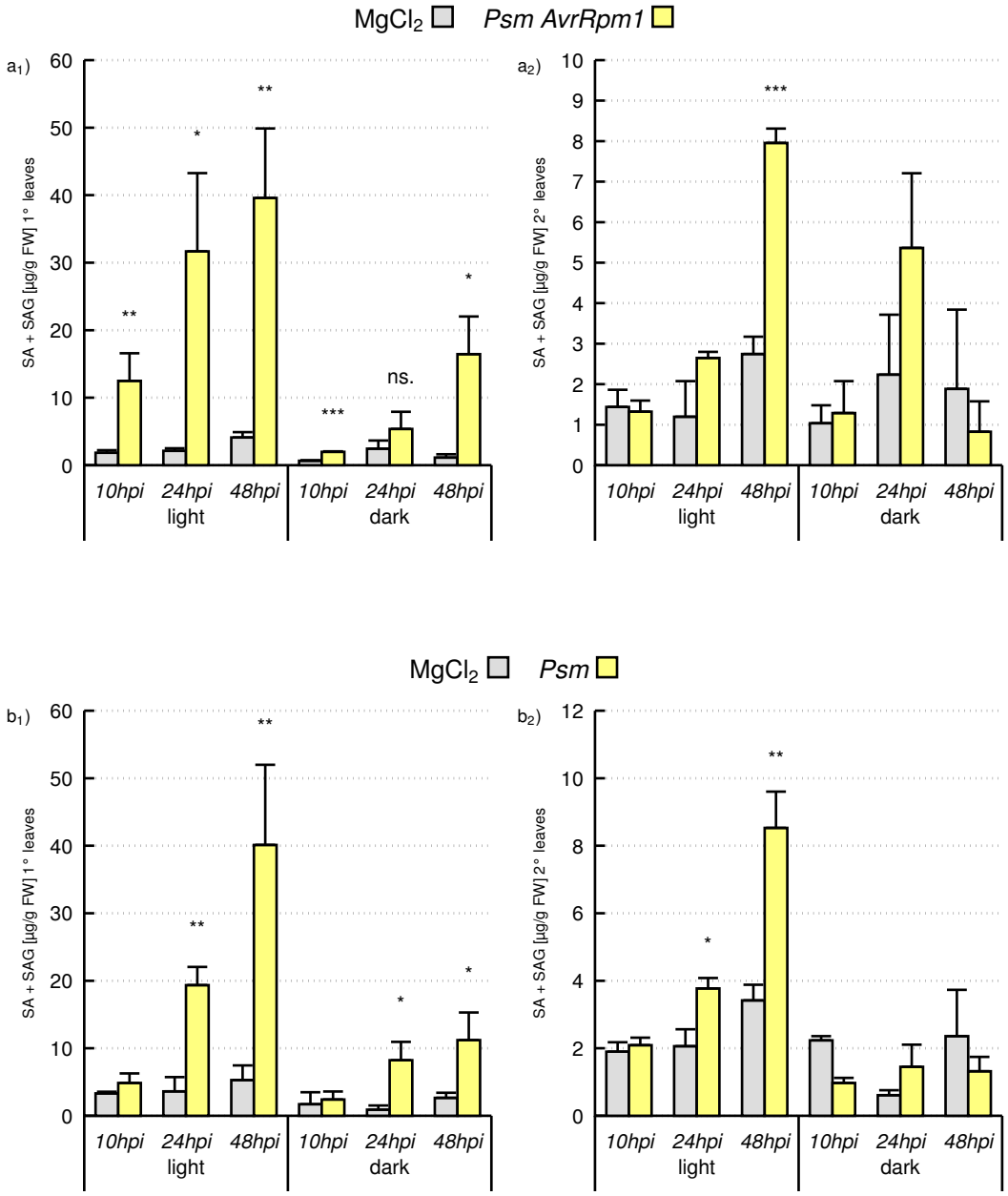

Figure 4.3: Accumulation of total SA (sum of free SA and conjugated SA) in local (a_1 and b_1) and systemic (a_2 and b_2) leaves of Arabidopsis Col-0 infiltrated with avirulent (top) or virulent strains (bottom) of *Pseudomonas syringae* under light and constant dark conditions as illustrated in Figure 4.2. In both groups of light- and dark-adapted plants, three local leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or with *Psm AvrRpm1* OD_{600}=0.005 (a_1, a_2) or *Psm* OD_{600}=0.005 (b_1, b_2) at 9 AM on day 0. Local (1°) and systemic (2°) leaves were collected after 10, 24, 48 hpi for total SA quantification. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of SA in mock- and pathogen-infiltrated samples in each time point (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

to induce Pip accumulation in local leaves under light and constant dark conditions, nevertheless, in dark-adapted plants, the lower level of Pip (8 fold less) was observed when compared to that of light-grown plants (Figure 4.4 b_1, c_1). Systemic leaves accumulated about 15 µg/g FW Pip upon pathogen attack, but these leaves were failed to accumulate Pip when exposed to darkness (Figure 4.4 b_2, c_2). In addition, comparative analysis of the level of *Psm*-induced amino acids to mock-treated in local and systemic leaves showed that there are several amino acids which are induced in dark, however, among all amino acids pipecolic acid is the only amino acid which is not induced in dark and pathogen-induced Pip accumulation is weaker in darkness (Supplemental tables S.2, S.3). We found that the amount of Pip produced in both local and systemic leaves is highly dependent on light conditions in which *Psm*-inoculated plants are exposed. The low level of Pip accumulated in systemic leaves under dark conditions is associated with attenuated SAR in dark-adapted plants.

4.2.3 Dark-adapted plants accumulate less pathogen-induced Pip in petiole exudate compared to light-grown plants

Pip was also detected in petiole exudate (PEX) collected from inoculated leaves with *Psm* (Návarová et al., 2012). Next, We tested whether light and dark applications after pathogen inoculation, impact the level of Pip in petiole exudate. Therefore, 5-week-old Arabidopsis Col-0 plants were grown under 10 h light /14 h dark cycle (referred as light-grown) and half of the plants were transferred and kept in constant darkness one day before *Psm* inoculation. Six leaves of each plant were inoculated with *Psm* (OD_{600}=0.005) or with 10 mM $MgCl_2$ (as control buffer) and petiole exudate of inoculated-leaves was collected from 6 to 48 hpi while plants were still located in the light and constant dark conditions.

Under normal light condition (10 h light /14 h dark cycle), the levels of Pip in exudate from *Psm*-treated leaves was significantly higher than the levels in exudate from mock-treated leaves (Figure 4.5). *Psm*-inoculated leaves which were located in darkness failed to accumulate Pip in PEX and there was no significant difference between level of Pip in mock- and *Psm*-treated leaves. In addition, there was a significant difference in the basal level of Pip accumulating upon mock treatment in both sets of plants and the PEX of light-grown plants was enriched by three fold higher level of Pip compared to that of dark-adjusted plants. Our data shows that Pip enrichment in petiole exudate is highly dependent on the light condition in which the infected plants are exposed. Whether light directly affects the flow of Pip into PEX, remains to be investigated.

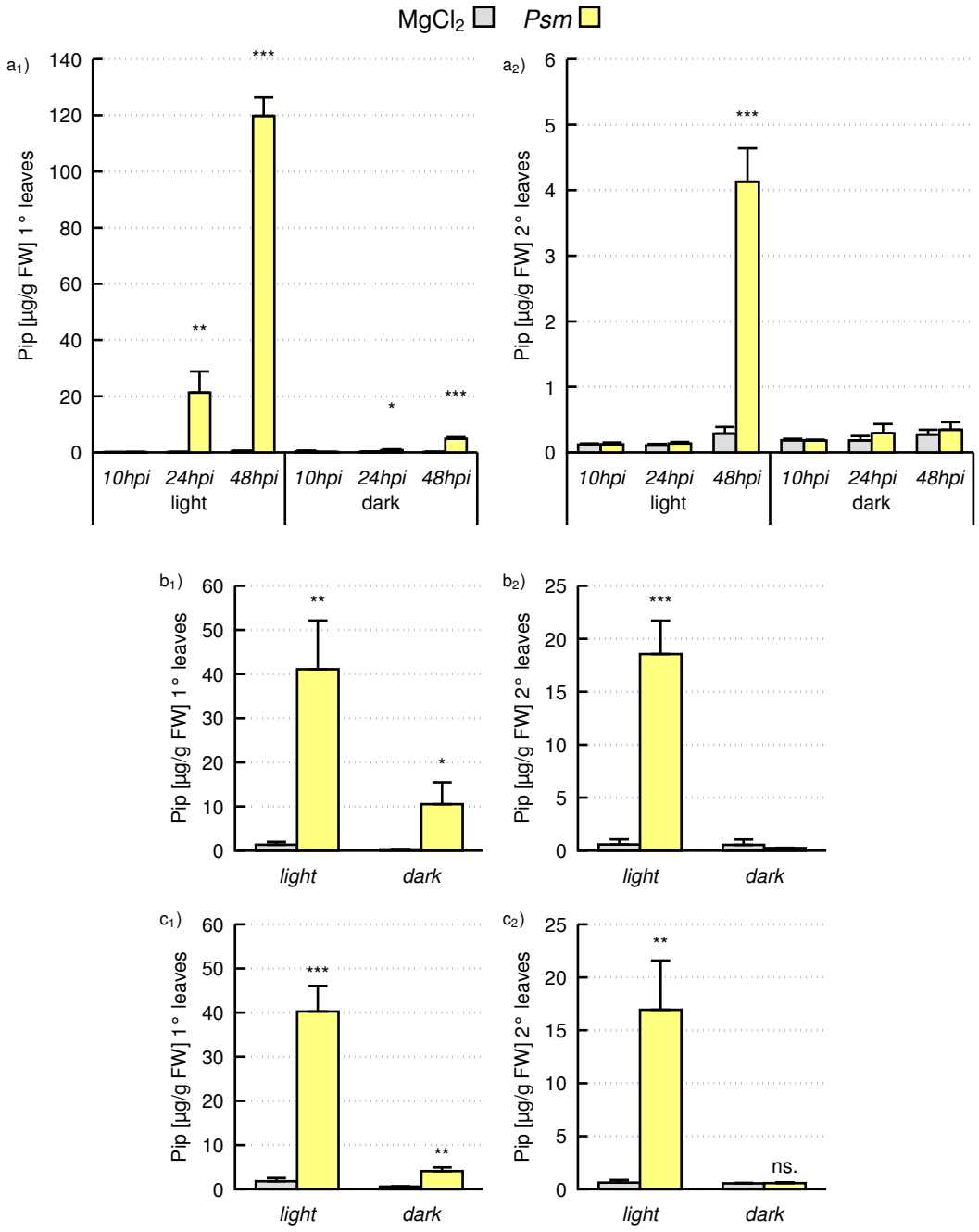

Figure 4.4: Accumulation of Pip in local (1°) and systemic (2°) leaves of Arabidopsis Col-0 infiltrated with *Psm* OD_{600}=0.005 in light and constant dark conditions as illustrated in Figure 4.2. To confirm our results, three independent experiments were performed. a_1-a_2: Exp. 1 b_1-b_2: Exp. 2 c_1-c_2: Exp. 3 Three local (1°) leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or *Psm* (OD_{600}=0.005) at 9 AM on day 0. In both groups of light- and dark-adapted plants, three local leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or with *Psm* OD_{600}=0.005 at 9 AM on day 0. Local (1°) and systemic (2°) leaves were collected after 10, 24, 48 hpi for Pip quantification. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of Pip in mock- and pathogen-infiltrated samples in each time point (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

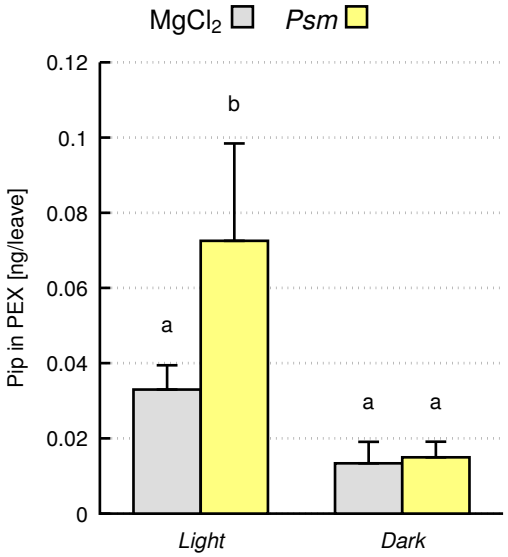

Figure 4.5: Comparison analysis of Pip levels in petiole exudate (PEX) of *Psm*-infiltrated leaves of Arabidopsis Col-0 under normal light (10 h light/ 14 h dark cycle) and constant dark conditions (As depicted in Figure 4.2). Six leaves of 5-week-old plants (grown under 10 h light/ 14 h dark cycle) were infiltrated with either 10 mM $MgCl_2$ or with *Psm* OD_{600}=0.005 at 9 AM and plants were either kept grown light condition or translocated to constant darkness (Figure 4.2). PEX of inoculated leaves was collected between 6 to 48 h after inoculations using EDTA. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of twelve leaves from two plants. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.) This result was confirmed in another independent experiment shown in supplemental Figure S.1.

4.3 The priming responses mediated by pipecolic acid are light dependent

Exogenous Pip is known to promote plants to a primed state in which they are able to more quickly and vigorously induce defense responses after pathogen attack. Exogenous pipecolic acid boosts induction of total SA biosynthesis and potentiates accumulation of phytoalexin camalexin at the early stages of the pathogen attack. (Návarová, 2012). To examine whether Pip-induced priming responses are light dependent, 5-week-old Col-0 plants, grown under 10 h light/ 14 h dark condition, were treated with either 10 ml of water (as control) or 10 ml of 1 mM D,L-Pip applied at 9 AM in the soil (Figure 4.6). Due to obtain similar Pip absorption in both light and dark conditions, plants were kept in normal growth condition (10 h light/ 14 h dark condition). Three leaves of each plant were infiltrated with *Psm* (OD_{600}=0.005) or 10 mM $MgCl_2$ (as control) one day after water/Pip treatment and plants were separated into two groups. One set of plants remained in their growth light condition (10 h light/ 14 h dark) and the second group was transferred to constant darkness as shown in Figure 4.6. To assess the priming effect of Pip on total SA and camalexin levels, infiltrated leaves were collected at 10 hpi.

Under normal light condition, a strong potentiation of *Psm*-induced total SA (free and glucosidic SA) biosynthesis was observed upon Pip-treatment when compared to water-treatment (Figure 4.7 a, b, c). Priming effect of Pip on free SA, SAG, and total SA accumulation was also observed in $MgCl_2$-infiltrated leaves when plants are grown in normal light condition.

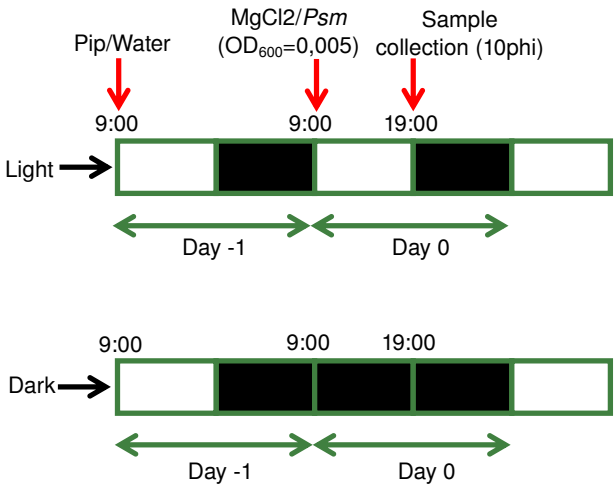

Figure 4.6: Pip priming

In dark-adjusted plants, applied Pip did not enhance the level of free SA, SAG, and consequently total SA (4.7 a, b, c), indicating that Pip priming effect on SA accumulation requires light. In addition, *Psm*-inoculated plants (transferred to darkness) failed to increase free SA, SAG, and total SA level upon either water or Pip treatment (4.7 a, b, c). Defense priming mediated by applying exogenous Pip was highly pronounced in those plants which were exposed to at least 9 hours light after pathogen-inoculation.

Next, we monitored the level of camalexin which is a phytoalexin accumulated in local leaves upon pathogen-inoculation. Based on our ANOVA analysis, application of Pip resulted in a highly significant accumulation of camalexin (compared to water-treated) and this priming effect happened only when plants exposed to at least 9 hours light after pathogen inoculation (Figure 4.7 d). The priming effect on camalexin biosynthesis was completely absent when plants were exposed to darkness after *Psm* or $MgCl_2$ infiltration.

To examine whether Pip has a direct effect on SA and camalexin accumulation, we also applied exogenous Pip to untreated plants (which were not treated with either$MgCl_2$ or *Psm*) (Figure S.3). Our ANOVA analysis showed that Pip application to plants directly stimulated total SA but not camalexin biosynthesis in untreated plants. Untreated plants did not respond to exogenous Pip when they were located in darkness. Therefore, the direct Pip priming effect on SA biosynthesis is light dependent (Figure S.3).

Our data indicate that plants absorbing the same amount of exogenous Pip, are only capable to execute the Pip-mediated priming effect when they expose to at least 9 hours light after pathogen inoculation. The priming effect of Pip on the accumulation of defense metabolites such as total SA (Free and glucosidic forms) and camalexin is therefore a light-dependent process. Moreover, the direct effect (Pip-induced biosynthesis of SA in the absence of a pathogen) is light dependent (Figure S.3).

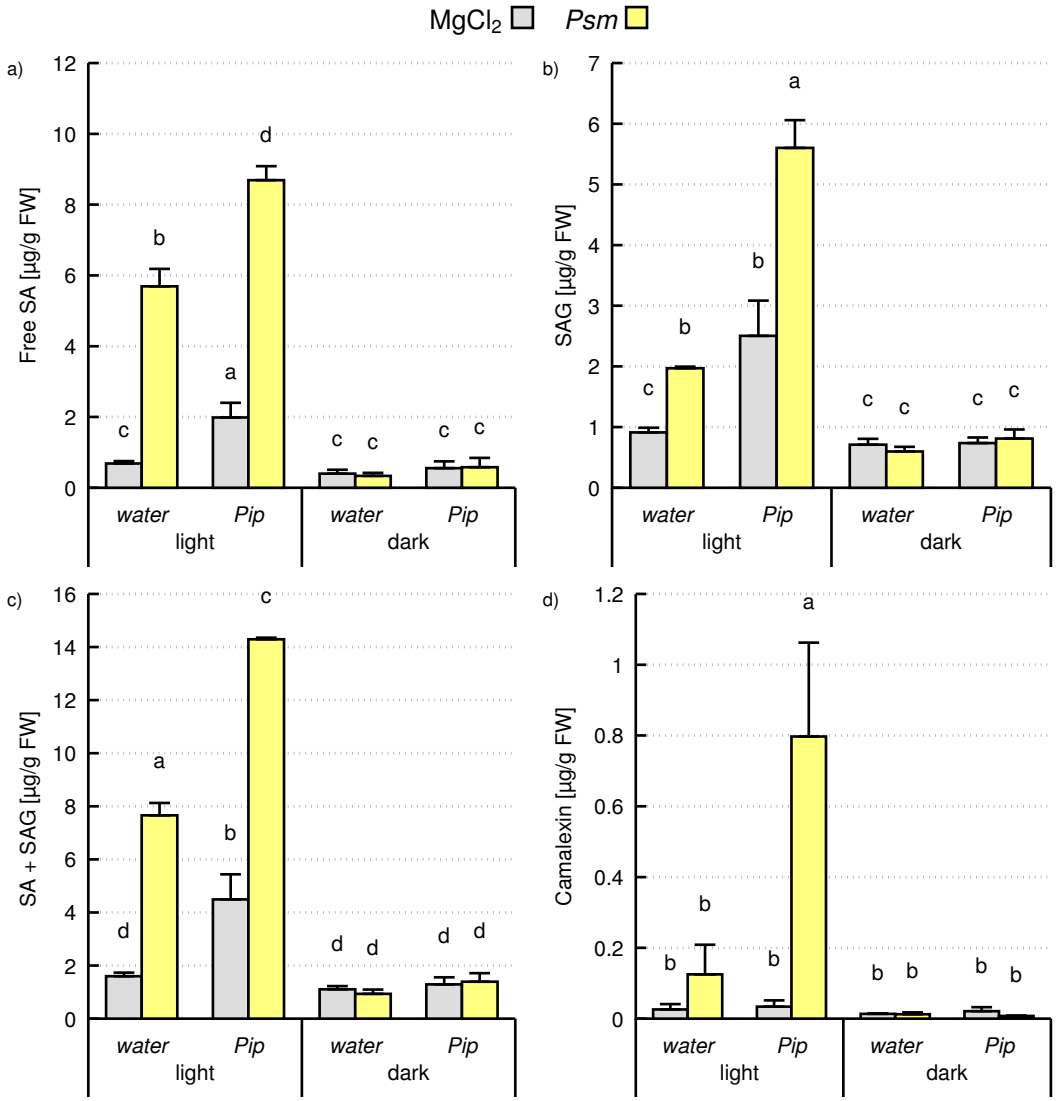

Figure 4.7: Priming effect of exogenous Pip on defense metabolites SA and camalexin under light and constant darkness. 5-week-old Col-0 plants (grown under 10 h light/ 14 h dark cycle) were supplied with 10 ml of 1 mM D,L Pip (= dose of 10 µmol) or with 10 ml of H_2O (control treatment) via the root system one day before pathogen inoculation. Plants were either kept in their grown-light condition (control group) or translocated to constant darkness (as shown in Figure 4.2). Three leaves of water/Pip treated plants in both light and dark conditions, were inoculated with either 10 mM $MgCl_2$ or *Psm* OD_{600}=0.005 and infiltrated leaves were collected at 10 hpi for quantification of free SA (a), glucosidic SA (b), total SA (c), and camalexin (d). Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test).
This result was confirmed in another independent experiment shown in supplemental data Figure S.3.

4.4 SA- and Pip-induced PR1 expression is less induced in dark-adapted compared to light-grown plants

SA-induced PR1 expression is light dependent (Zeier et al., 2004). It was previously demonstrated that Pip and SA regulate PR1 expression in a synergistic and independent manner (Bernsdorff et al., 2016). To examine the light dependency of priming function of SA and Pip at gene expression level, we watered Col-0 plants with 10 µmol D,L Pip via root system, subsequently infiltrated three leaves of plants with 0.5 mM SA one day after Pip treatment. Single Pip and SA applications as well as a control treatment with water, were included.

To assess the light dependency of the regulating function of SA and Pip on PR1 expression, different light and dark regimes were applied after each individual treatment (as shown in Figure 4.8). Therefore, plants were located either into light or dark conditions after each individual treatment (water/Pip treatment and/or after water/SA infiltration). Transcript levels of SA- or Pip-inducible PR1 gene was determined 4 hours after SA infiltration for each light/dark regimes.

Under a normal light condition, when 10 hours of light is applied after each treatment, SA alone induced strong expression of PR1, showing that elevated SA is sufficient to trigger PR1 expression independent from Pip. Pip alone primed expression of PR1 gene as well, though not as strong as SA. However, application of both Pip and SA markedly strengthened PR1 expression showing a synergism between SA and Pip to trigger PR1 expression under normal light condition. When the dark condition was applied after every single treatment, SA- and Pip-induced PR1 expression was not totally abolished, however, the transcription level of PR1 showed the lowest intensity (DD condition). Application of dark only after SA infiltration illustrated the similar expression pattern observed under normal light condition (LL: when the light was applied after both Pip and SA treatments), though the pattern was less pronounced (LD conditions). This data shows that induction of PR1 expression is potentiated by light and it happens more strongly when light is present during SA-induced gene expression (LD condition). Obviously, light exposure one day before SA treatment influences the SA-induced expression of PR1 and it shows a positive effect (Comparing DL with DD).

For Pip to induce PR1 expression, a normal light/dark cycle (LL condition) seems to be required since dark application either after Pip treatment or after subsequent water infiltration, strongly inhibited PR1 expression (Figure 4.9 PWLL, PWLD, and PWDL). [Note: Due to confirm the Pip-mediated priming of SA response, this experiment needs to be repeated.]

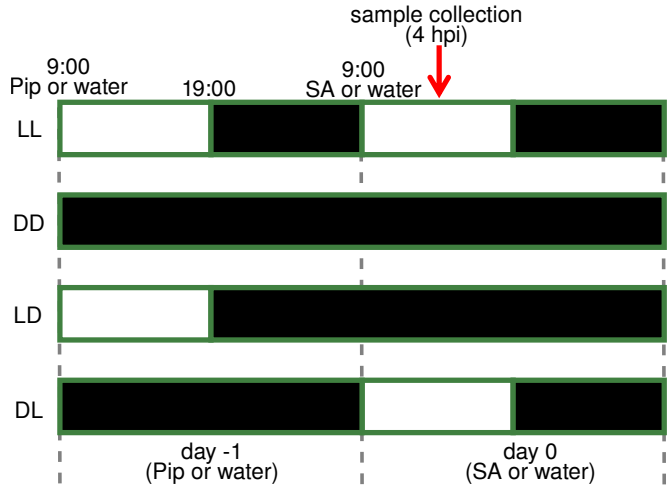

Figure 4.8: Daytime of Pip/water treatment and SA/water infiltration in light/dark regime. Black and white boxes correspond to dark and light periods, respectively.

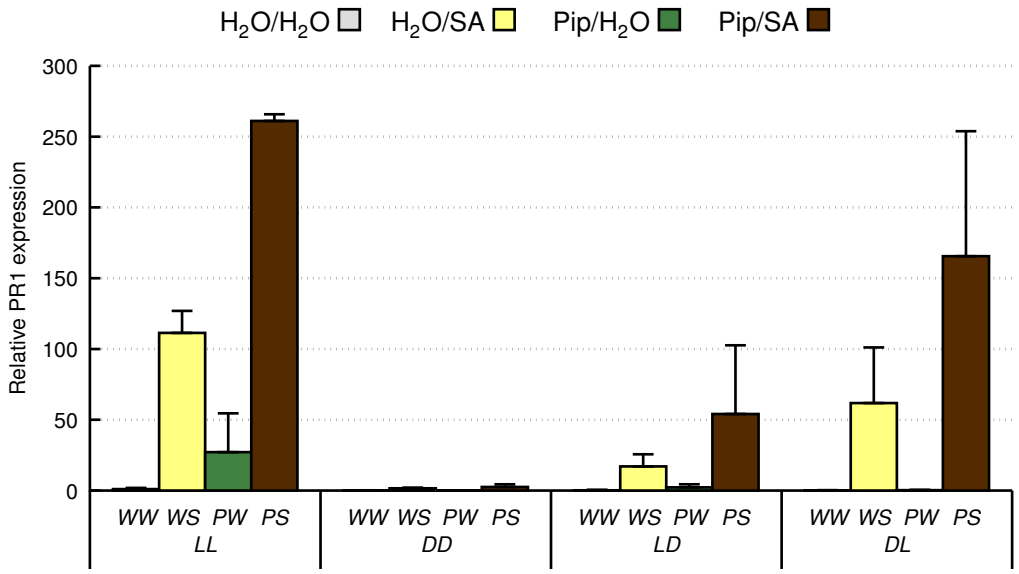

Figure 4.9: Pip- and SA-induced priming of PR1 expression in different light regimes. plants were pre-treated with 10 ml of 1 mM D,L Pip (= dose of 10 µmol) or with 10 ml of H_2O and one day later 0.5 mM SA or water was infiltrated into leaves. PR1 expression in leaves was monitored 4 h after the SAH_2O infiltration by qPCR analysis (experimental setup is shown in Figure 4.8. Values represent the mean ± SD of three biological replicates from different plants. Each biological replicate consists of two leaves from one plant and involves two technical replicates. PR1 transcript levels are expressed relative to the H_2O/ H_2O value of Col-0.

4.5 Effect of the length of the light period on basal resistance and the accumulation of defense-related metabolites

A day-time dependency of Arabidopsis defense responses was previously demonstrated by (Griebel and Zeier, 2008). It was shown that the availability of long light period after pathogen inoculation, resulted in increased sets of plant defense responses towards pathogen attack (Griebel and Zeier, 2008). To test whether the duration of light after pathogen attack affects the plant resistance, 5-week-old Col-0 plants (grown under conventional 10 h light/14 h dark photoperiodic conditions) were situated in variable light length (5, 10, 15 hours and continuous light) after *Psm lux* inoculation for only one day (Figure 4.10 a). The light regime had the same light intensity of 70 µmol photons m^{-2} s^{-1} in all four conditions. We inoculated three leaves of Col-0 plants with *Psm lux* (OD_{600}=0.001) at 9 AM and local bacterial growth was scored 2.5 days post-inoculation for each condition.

Our ANOVA analysis showed a significant reduction ($P < 0.05$) in bacterial growth after 5 h light-exposure compared to normal 10 h light condition (Figure 4.10 b). Increasing the length of light to 15 h and even applying constant light after pathogen inoculation, induced higher bacterial growth at the site of infection (Figure 4.10 b). Exposure of plants to 15 hours light after *Psm lux* inoculation, showed a higher tendency of bacterial growth comparing to normal 10 h light period however, it was not significantly different. Plants under constant light after pathogen inoculation, showed a statistically significant increase in the bacterial growth and consequently a reduced basal resistance compared to 10 h light condition. Unexpectedly, the length of light period applied after pathogen inoculation is correlated with the bacterial growth, indicating a direct effect of light on defense mechanisms against the pathogen in inoculated leaves, and light length negatively correlates with basal resistance.

We next assessed whether light would directly induce the production of defense-related metabolites such as SA, camalexin, and Pip. We challenged three leaves of Arabidopsis Col-0 plants (grown in 10 h light /14 h dark cycle) with either *Psm* (OD_{600}=0.005) or 10mM $MgCl_2$ (as control) at 9 AM and plants were located in different light-length regime exactly after pathogen inoculation for one day (Figure 4.10 a). Local (infiltrated) and systemic leaves were collected at 24 hpi and 48 hpi for further metabolite analysis.

We found that the amount of total (sum of free and glucosidic) SA produced within the first 24 hpi in inoculated leaves, depends on the length of light applied after pathogen inoculation, with SA accumulating to 5.2 µg/g FW, 17.3 µg/g FW, 24.5 µg/g FW, and 23.9 µg/g FW after 5 h, 10 h, 15 h and constant light respectively (Figure 4.11 c). Comparing to control 10 h light condition, exposure of plants to 15 h light after *Psm* infiltration, resulted in higher accumulation of total SA (free SA and SAG) at the site of pathogen attack however, this level was not increased upon constant light exposure (Figure 4.11 c).

The trend for total SA depicted in Figure 4.11 was similarly observed in systemic leaves. The total amount of SA produced after 48 hpi in systemic leaves rose from 0.74 µg/g FW in plants adjusted to 5 h light period to 2.61 µg/g FW, and 10.42 µg/g FW in plants grown in 10 h and 15 h light periods respectively (Figure 4.12 c). This amount reduced to 7.2 µg/g FW under constant light. This result shows that applying of five hours more light leads to increase in total SA production by approximately a

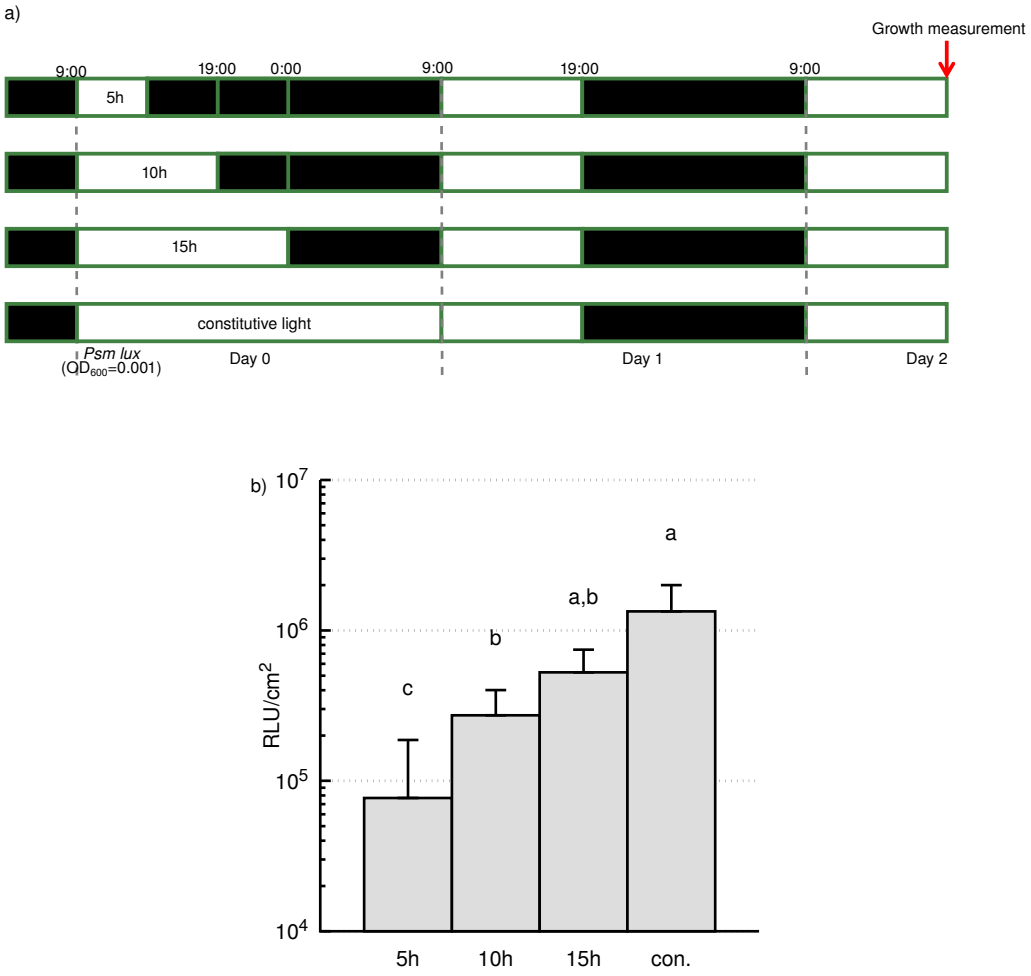

Figure 4.10: Basal resistance of Arabidopsis Col-0 in different light-length regime applied after pathogen infiltration. Three leaves of 5-week-old Col-0 plants (grown under conventional 10 h light/14 h dark photoperiodic conditions) were infiltrated with *Psm lux* OD_{600}=0.001 at 9 AM. A group of plants was kept in growth light condition (10 h) as control and the rest of plants were situated in variable light length (5 h,15 h, and continuous light) exactly after infiltration foor only one day (as shown in Figure 4.10 a). The growth of *Psm lux* was assessed after 2.5 d by luminescence measurements (Figure 4.10 b). A logarithmic scale is used for the y-axis. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.)

factor of five and length of the light period after pathogen inoculation directly affect the accumulation of SA in both local and systemic leaves.

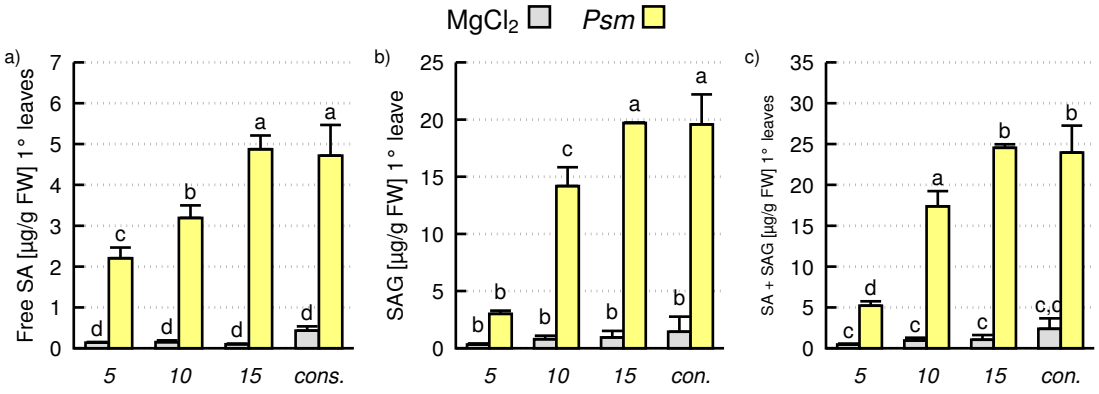

Figure 4.11: *Psm*-induced accumulation of free SA (a), SAG (b), and total SA (c) in local leaves of Arabidopsis Col-0 in a light-length regime. Three local leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or with *Psm* OD_{600}=0.005 at 9 AM. Local infiltrated leaves were collected for SA quantification at 24 hpi. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. The statistical analyses were performed as described in legend of Figure 4.10.

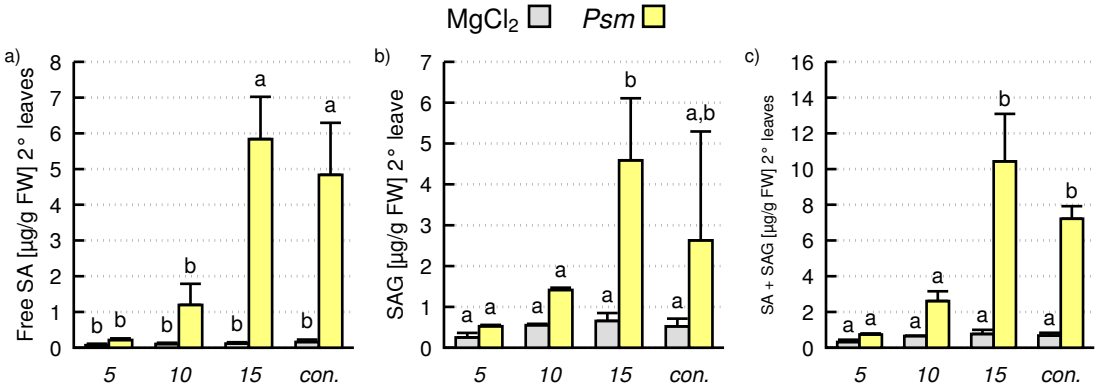

Figure 4.12: *Psm*-induced accumulation of free SA (a), SAG (b), and total SA (c) in systemic leaves of Arabidopsis Col-0 in a light-length regime. Three local leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or with *Psm* OD_{600}=0.005 at 9 AM. systemic leaves were collected for SA quantification at 48 hpi. The statistical analyses were performed as described in legend of Figure 4.10.

Duration of a light period after bacterial inoculation also directly influenced the accumulation of phytoalexin camalexin in infiltrated leaves at 24 hpi. Under normal condition (10 h light), the level of *Psm*-induced camalexin is 2.3 µg/g FW which this amount was increased to almost 3 µg/g FW after application of five hours more light (15 h light period) (Figure 4.13). Under normal 10 h light period, the level of camalexin increased up to five fold in comparison to short application of light length (5 h light period) with a low level of 0.5 µg/g FW (Figure 4.13). Our ANOVA analysis shows that applying 5 h light after pathogen infiltration, is not sufficient to induce camalexin accumulation, since the level of camalexin does not differ in mock- and *Psm*-infiltrated leaves. It seems that there is a positive correlation between light length and the level of *Psm*-induced camalexin accumulation. However, our ANOVA analysis does not confirm a significant difference in the level of camalexin in this light regime (Figure 4.13).

We next tested whether light regimes would influence the magnitude of Pip accumulation in local and systemic leaves. Therefore, local and systemic level of Pip in Col-0 plants situated in conventional 10 h

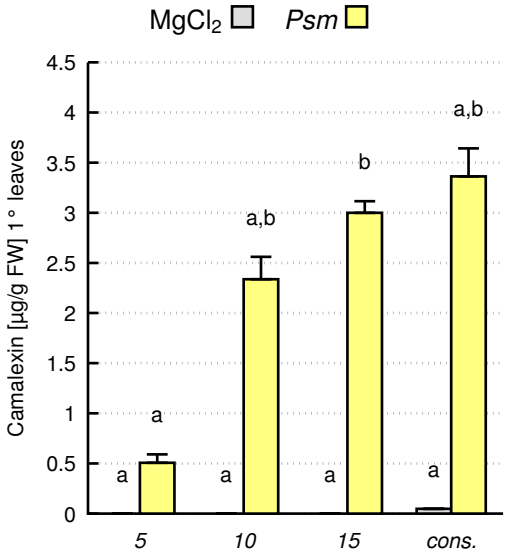

Figure 4.13: *Psm*-induced camalexin accumulation in infiltrated leaves of Col-0 plants in light-length regime. Three local leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or with *Psm* OD_{600}=0.005 at 9 AM. Level of camalexin was quantified in infiltrated leaves at 24 hpi. The statistical analyses were performed as described in legend of Figure 4.10.

light/ 14 h dark, was compared with those of plants adjusted to 5 h, 15 h, and continuous light period. Interestingly, the highest level of local Pip (14.65 µg/g FW) collected from inoculated leaves after 24 hpi, was observed in plants grown in normal 10 h light / 14 h dark condition (Figure 4.14 a). This amount was reduced to 12.88 µg/g FW under 15 h light period and was constant under continuous light (10.58 µg/g FW). Our ANOVA analysis did not show any significant differences in the level of local Pip among plants situated in different light-length conditions, although plants showed a slight tendency to accumulate a higher level of local-Pip in longer light-period applied after *Psm* attack (Figure 4.14 a). Although a low level of *Psm*-induced Pip (7.35 µg/g FW) was observed in local leaves exposed to 5 h light, our ANOVA analysis showed that exposure of only 5 h light after *Psm*, was not sufficient for significant Pip accumulation in infiltrated leaves upon *Psm* infiltration (Figure 4.14 a).

The amount of systemic Pip produced after 48 hpi, positively correlated with the length of light applied after pathogen inoculation. Like SA accumulation, light directly influenced the *Psm*-induced Pip level in an upward manner, accumulating to 0.7 µg/g FW, 3.12 µg/g FW, 7.72 µg/g FW, and 20.26 µg/g FW under 5 h, 10 h, 15 h, and constant light conditions, respectively (Figure 4.14 b). According to our ANOVA analysis, application of only 5 h light, did not block the production of systemic Pip but this level was not significantly increased upon *Psm* infiltration. In addition, no significant difference was observed in the *Psm*-induced level of Pip at 10 and 15 h light-length conditions. Applying constant light after *Psm*, resulted in an increased level of Pip (Figure 4.14 b). In summary, these results illustrate a direct effect of light length after pathogen inoculation on plant defense-related metabolites such as SA, camalexin, and particularly systemic Pip. Defense metabolite production are positively regulated with the length of light period. How this direct interaction takes place remains to be elucidated.

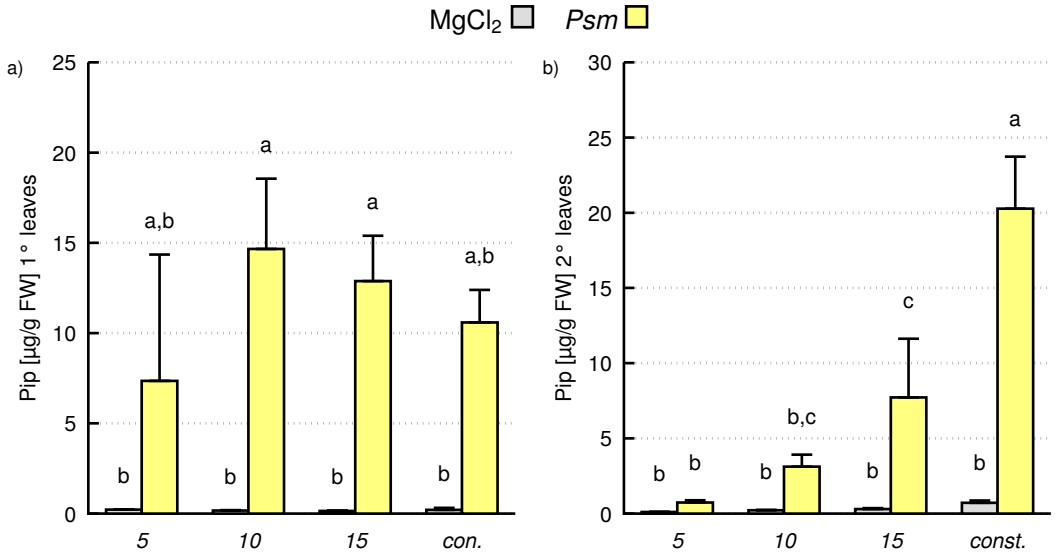

Figure 4.14: *Psm*-induced accumulation of local Pip (a) and systemic Pip (b) in Col-0 exposed to a light-length regime after pathogen inoculation. Three local leaves were infiltrated with 10 mM $MgCl_2$ (control buffer) or with *Psm* OD_{600}=0.005 at 9 AM. Local and systemic leaves were collected for Pip quantification at 24 and 48 hpi respectively. The statistical analyses were performed as described in legend of Figure 4.10.

4.6 Effect of dynamic light on defense-related metabolites

The previously described results indicated that changing the duration of light affected the plant defense responses. Since plants live in a dynamic nature with fluctuating light, we next tested whether changes in light intensity could influence plant-pathogen interaction. Since acclimation responses toward fluctuating light (such as growth, photosynthesis, metabolites, and gene transcription) were shown to be differently expressed in young and mature leaves (data not shown), thus in this experiment age of the leaves (mature or young) was also considered as a factor which may influence the production of defense-related metabolites.

Arabidopsis Col-0 plants were grown under constant light (CL) with 75 µmol photons m^{-2} s^{-1} intensity for four weeks. Three days before pathogen infiltration half of the plant population was transferred to a fluctuating light (FL) where 20 s pulses of high light (1000 µmol photons m^{-2} s^{-1}) were applied every 6 min under the background light intensity of 75 µmol photons m^{-2} s^{-1} during the day. Half of the plant population remained in constant light (CL) as a control group.

To uncover the effect of high light intensity on accumulation of *Psm*-induced SA, we conducted two experiments. In the first experiment, we considered the mature leaves of Arabidopsis as targets of the pathogen and therefore, we infiltrated three lower (mature) leaves (under CL or FL conditions), as local leaves (or 1° leaves) with either 10 mM $MgCl_2$ (as control buffer) or *Psm* (OD_{600}=0.005) at 10 AM. Levels of *Psm*-induced SA were measured in the 1° infiltrated leaves (mature) and also in younger leaves above the infiltrated-mature leaves (which are considered as 2° or systemic leaves). We collected both mature (infiltrated/local) and young (non-infiltrated/systemic) leaves at 24 and 48 hpi.

Analysis of SA level in mature-infiltrated leaves under FL, showed a slight but not significant reduction in the level of free SA, SAG, and consequently total SA after 24 and 48 hpi when compared to CL (Figure 4.15). Interestingly, most of the SA produced in mature-infiltrated leaves was in storage-form of SA (glucosidic SA) rather than free SA. However, there was no accumulation of SA in the systemic, non-infiltrated young leaves (above the mature-infiltrated leaves) after 24 hpi (Figure 4.15 a_1-a_3). An increased level of free SA about 1.72 µg/g FW was detected in CL at 48 hpi, whereas a reduced level of free SA (0.5 µg/g FW) was observed under FL at this time point (Figure 4.15 b_1). The level of total systemic SA in young leaves was also significantly reduced compared to CL (Figure 4.15 b_3). Thus, the systemic accumulation of SA (total) was reduced in FL compared to CL which probably due to an inhibiting factor for the induction of systemic SA biosynthesis under FL.

In the second experiment, we considered the young leaves above the mature leaves of Arabidopsis as our local leaves and the targets of the pathogen. Therefor, we repeated the first experiment (explained above) and this time we infiltrated three young leaves (as local or 1° leaves) by either $MgCl_2$ or *Psm* (OD_{600}=0.005) and we collected the samples from young (infiltrated/local leaves) and mature leaves below the young leaves (as non-infiltrated/ systemic leaves). Both infiltrated-young and non-infiltrated mature leaves were collected at 24 and 48 hpi.

Here, when young leaves were infiltrated, we could only detect SA (free or SAG) in the infiltrated young leaves under both CL and FL, however no SA was detected in mature leaves (which were here considered as systemic leaves to infiltrated-young leaves) (Figure 4.16). The total level of SA was accumulated to a significantly lower level in FL compared to CL after 24 hpi (Figure 4.16 a_3). Although, the level of *Psm*-induced free SA in FL compared to CL after 48 hpi was significantly higher ($P < 0.05$) (Figure 4.16 b_1), nevertheless the total level of SA in young-infiltrated leaves under FL, was not significantly different from that of CL (Figure 4.16 b_3).

Taking together, these results indicate that dynamic fluctuations of light affect the accumulation of defense metabolite SA in both mature and young leaves to a moderate extent. Under our experimental setup, when young leaves are inoculated, no systemic response in mature leaves is observed. Moreover, when mature leaves are infiltrated, the systemic accumulation of total SA is reduced under FL compared to CL.

To check whether fluctuating light affects the production of local and systemic Pip, mature leaves of plants under CL or FL conditions were infiltrated with either *Psm* (OD_{600}=0.005) or 10 mM $MgCl_2$. Level of Pip was evaluated after 24 hpi in mature-infiltrated leaves (as local leaves) and 48 hpi in young leaves distal to mature leaves (as systemic leaves). The local level of *Psm*-induced Pip in both CL and FL conditions, increased similarly up to 18 µg/g FW (Figure 4.17 a). Although in systemic leaves, Pip production was less induced under FL compared to CL, this difference was not statistically significant (Figure 4.17 b). Taken as a whole, our results show that there is no contribution of FL to the production of local and systemic Pip upon pathogen inoculation. We assume that fluctuation of light intensity is not a major factor affecting the pathogen-induced Pip level. However, our data indicate that FL impacts the production of SA, in particular in the systemic leaves.

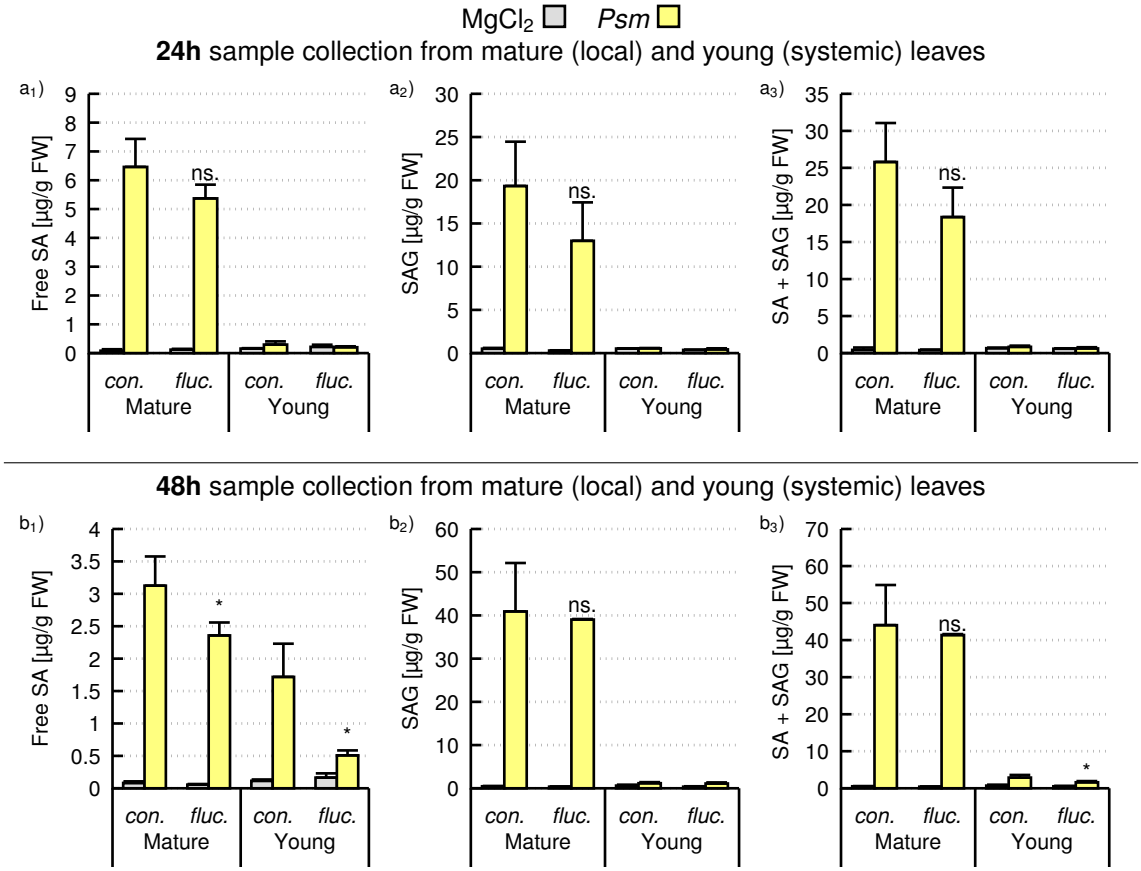

Figure 4.15: Level of *Psm*-induced free SA (a_1, b_1), SAG (a_2, b_2), and total SA (a_3, b_3) in young and mature leaves of Col-0 plants under CL and FL at 24 and 48 h after infiltration of **mature leaves**. Four-week-old Col-0 plants were adjusted to CL and FL one week before physiological experiment. Three mature leaves of plants were infiltrated with either 10 mM $MgCl_2$ or *Psm* OD_{600}=0.005. Infiltrated-mature and non-infiltrated young leaves were collected after 24 and 48 hpi from both CL- and FL-adjusted plants. Data represent means ± SD of at least three biological replicates from different plants, each replicates consisting of six leaves from two plants. In each experiment, two-tailed *t* -test was used to determine the statistically significant difference between the level of SA in mock- and pathogen-infiltrated samples in each time point (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

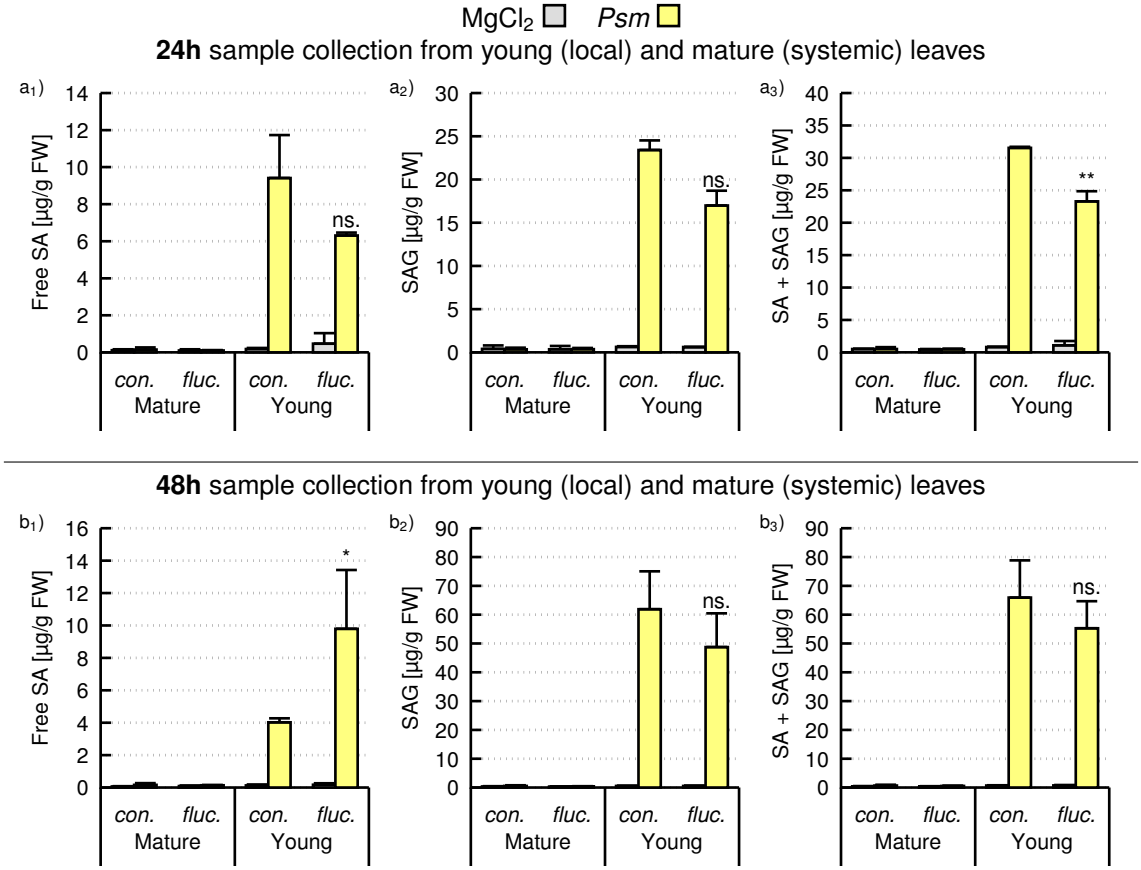

Figure 4.16: Level of *Psm*-induced free SA (a_1, b_1), SAG (a_2, b_2), and total SA (a_3, b_3) in young and mature leaves of Col-0 plants under CL and FL at 24 and 48 h after infiltration of **young leaves**. Four-week-old Col-0 plants were adjusted to CL and FL one week before the physiological experiment. Three young leaves of plants were infiltrated with either 10 mM $MgCl_2$ or *Psm* OD_{600}=0.005. Infiltrated young and non-infiltrated mature leaves were collected after 24 and 48 hpi from both CL- and FL-adjusted plants. The statistical analyses were performed as described in the legend of Figure 4.15.

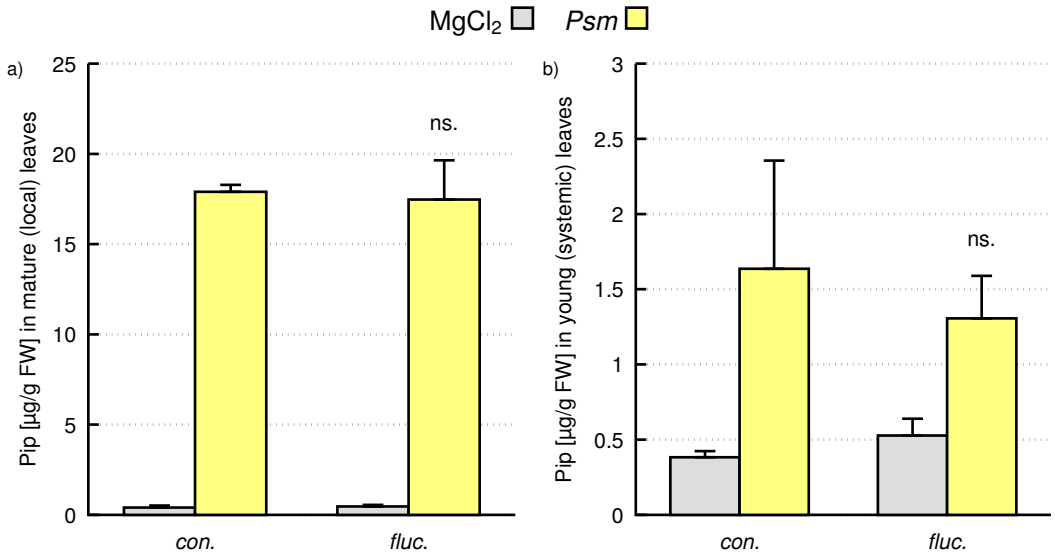

Figure 4.17: *Psm*-induced Pip accumulation in mature and young leaves of plants grown under CL and FL. Four-week-old Col-0 plants were adjusted to CL and FL one week before the physiological experiment. Three **mature leaves** of plants were infiltrated with either 10 mM $MgCl_2$ or *Psm* OD_{600}=0.005. Infiltrated mature (local)(a) and non-infiltrated young (systemic)(b) leaves were collected at 24 and 48 hpi respectively. The statistical analyses were performed as described in the legend of Figure 4.15.

5 Effect of nitrogen supply on SAR and defense metabolites

5.1 Adequate nitrogen supply is required for SAR establishment

Nitrogen and nitrogen-based compounds are essential for plant physiology and development. Many studies have shown that not only the amount of nitrogen but also the form of nitrogen supply affect the outcome of plant-pathogen interaction (Gupta et al., 2013). To uncover the role of nitrogen supply on SAR, we conducted comparative systemic resistance assay in Arabidopsis Col-0 plants which were hydroponically grown in gibeaut nutrient solution (full buffer containing 4.7 mM N) under standard conditions for 4 weeks, and they adjusted to different solutions with distinct nitrate concentrations and identical ion strength for another 5 days till the start of physiological experiments.

Nitrate concentration was changed from full buffer (N = 4.7 mM) to its half amount (½ N = 2.35 mM), ¼ N amount =1.18 mM N, and nitrate deficient buffer (0 N). For SAR assay, we first inoculated three local leaves of each plant with either 10 mM $MgCl_2$ or *Psm* (OD_{600}=0.005). The second inoculation of *Psm lux* (OD_{600}=0.001) followed two days later in distal leaves and bacterial growth was measured after an additional 2.5 days. Bacterial growth after *Psm* treatment was reduced in control plants grown in full buffer (N= 4.7 mM) showing a successful establishment of SAR (Figure 5.1). Hydroponically-grown plants are still capable to establish a significant SAR ($P < 0.005$) when they adjusted to lower N concentration buffer (1.18 and 2.35 mM N). Interestingly, plants grown in hydroponic buffer containing 2.35 mM N, induced a stronger SAR with 19.9 fold-change reductions of bacteria than full medium. However, no significant difference in the level of bacterial growth was observed between mock and *Psm*-treated plants which grown in nitrate deficient buffer (Figure 5.1). These plants failed to establish SAR. According to our data, plants require nitrate or a nitrate-dependent factor as an essential element to induce resistance and establish a full SAR.

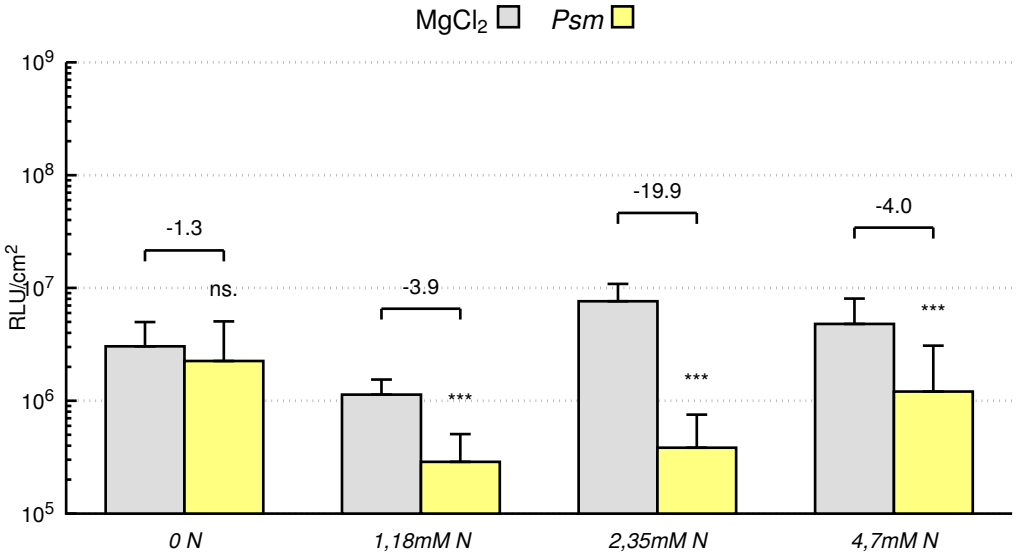

Figure 5.1: Establishment of SAR in Col-0 plants grown under different nitrogen concentrations. Col-0 plants were grown in control hydroponic buffer (consisting of 4.7 mM nitrate (N)) for 4 weeks and plants were transferred to different solutions with distinct N concentrations (0 N, ¼ N = 1.18 mM, ½ N = 2.35 mM) 5 d before the physiological experiment. Three local leaves of plants were infiltrated with 10 mM $MgCl_2$ (control buffer) or *Psm* (OD_{600}=0.005) at 9 AM. Three systemic leaves of all plants were infiltrated with *Psm lux* (OD_{600}=0.001) two days later. The growth of *Psm lux* was assessed after 2.5 d by luminescence measurements. Data represent the mean ± SD of at least 18 leaf replicates from 6 different plants. A logarithmic scale is used for the y-axis. Numbers above each bar represent the fold change growth reduction (-) between mock- and pathogen-infiltrated samples for each condition which is calculated based on total average values. Two-tailed *t* -test was used to determine the statistically significant difference between bacterial growth in mock- and pathogen -infiltrated plants in the same condition (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant). The experiment was done by Holger Hallmann.

5.2 Nitrogen affects local and systemic salicylic acid accumulation upon pathogen inoculation

We next examined whether nitrate supply affects the accumulation of local and systemic SA as one of the main SAR regulators. To do this, Col-0 plants were grown in a hydroponic solution (2,8 mM N as a control solution) for 5 weeks. Plants were translocated in different nitrate-containing solutions 5 days before physiological experiments. [Note: We decided to use 2.28 mM N as the control buffer in which plants grow for five weeks before physiological experiment starts, This is because the highest induction of SAR is observed at a close concentration to this amount (Figure 5.1) and later on, we change the concentration of nitrate to ½ N =1.4 mM N and ¼ N =0.7 mM N, and deficient solution 0 N]. Three leaves of each plant were infiltrated with either 10 mM $MgCl_2$ (control buffer) or with *Psm* (OD_{600}=0.005), and the level of total SA (free SA + SAG) in infiltrated leaves was quantified after 48 hpi. As shown in Figure 5.2 a, increasing of nitrate concentration did not correlate with the level of local free SA. Plants did not fail to accumulate free SA when there is no nitrate available in their growth solution (Figure 5.2 a). The level of *Psm*-induced free SA and SAG are even higher than that of plants grown in solutions containing nitrate (Figure 5.2 a, b). Quantification of the total level of SA, showed more induced SA in N-deficient plants Plants (Figure 5.2 c) compared to other conditions.

In contrast, nitrate concentration positively correlates with the level of *Psm*-induced free SA in systemic leaves. A low but still significant systemic level (P < 0.01) of free SA was detected in plants grown under

0 N (1 µg/g FW) (Figure 5.2 d). Plants grown in a solution containing only a small amount of nitrate (0.7 mM), showed 2 fold higher level of SA compared to 0 N condition. The highest level of SA (6 µg/g FW) was detected when we raised the concentration of nitrate to 1.4 mM, and this level was 6 fold higher than 0 N and even 3 fold higher than that of plants grown under control buffer (N = 2.8 mM) (Figure 5.2 d). Together this data shows that there is an association between nitrate supply and accumulation of plant defense regulators such as SA. It is more likely that nitrate amount positively affects the systemic accumulation of SA, in contrast, a higher concentration of nitrate results in the reduced level of SA in local inoculated leaves.

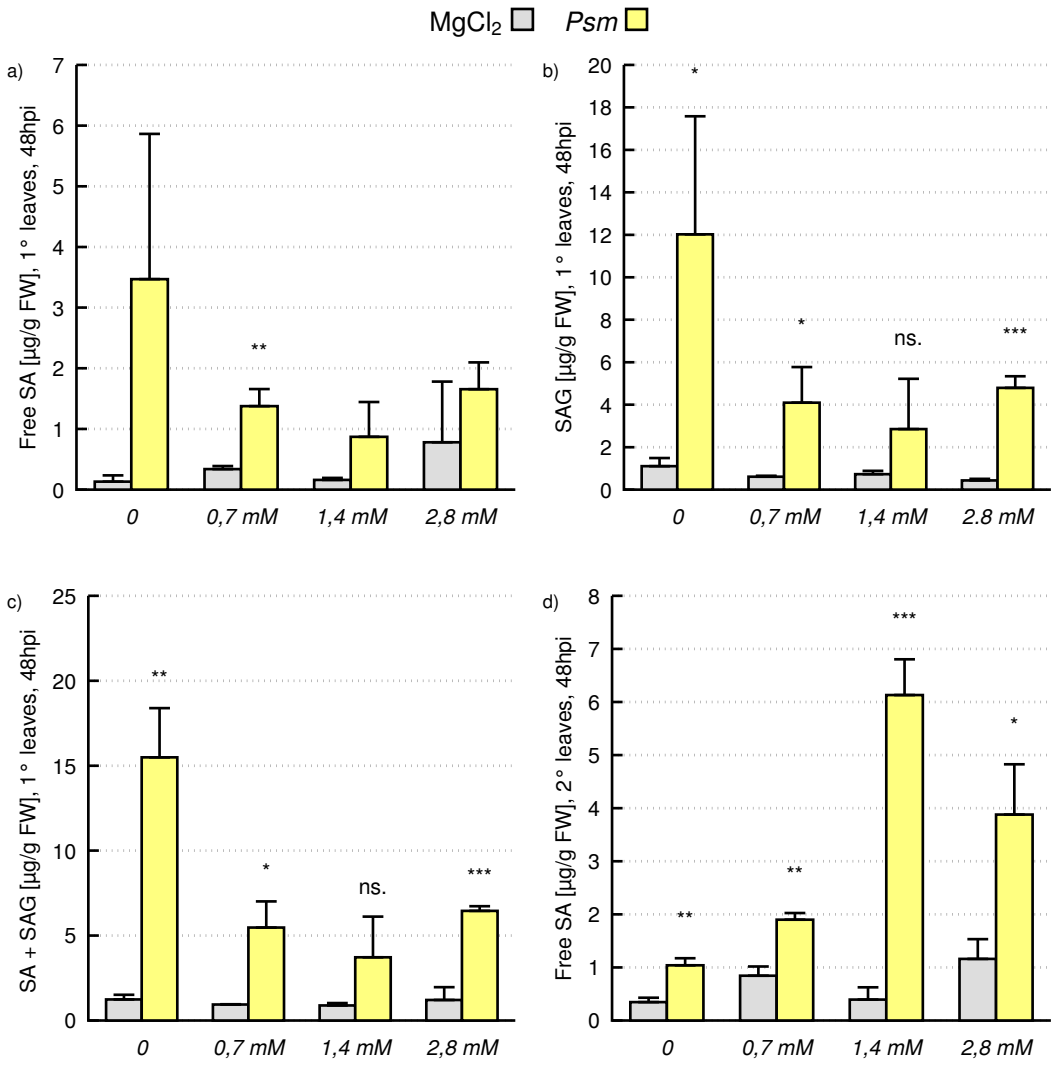

Figure 5.2: Accumulation of *Psm*-induced SA in Col-0 plants grown under different nitrogen concentrations. Col-0 plants were grown in control hydroponic buffer (consisting of 2.8 mM nitrate (N)) for 4 weeks and plants were transferred to different solutions with distinct N concentrations (0 N, ¼ N = 0.7 mM, ½ N = 1.4 mM) 5 d before the physiological experiment. Three local leaves of plants were infiltrated with 10 mM $MgCl_2$ (control buffer) or *Psm* (OD_{600}=0.005) at 9 AM. Local-infiltrated leaves were collected at 48 hpi for quantification of free SA (a), SAG (b), and total SA (c) and free SA was quantified in systemic leaves at 48 hpi (d). The statistical analyses were performed as described in the legend of Figure 5.1. a,b, and c experiments were done by Holger Hallmann.

5.3 Pip biosynthesis is dependent on nitrate concentration

The previous section has shown that level of *Psm*-induced SA is affected by nitrate supply. Moreover, no SAR response was observed in Arabidopsis plants grown in nitrate deficient (0 N) buffer. To determine whether nitrogen has an impact on the level of Pip as a crucial regulator of SAR, changes in the level of *Psm*-induced Pip was compared in Col-0 plants grown in solutions with different N concentrations. 5-week-old Col-0, which were grown in control full buffer (N = 2.8 mM N), were transferred to different solutions with distinct nitrate concentrations (0 N, ½ N = 1.4 mM, and ¼ N = 0.7 mM N) five days before inoculation. Three leaves of each plant were infiltrated with either *Psm* (OD_{600}=0.005) or $MgCl_2$ as control buffer, and local Pip values were measured at 24 hpi and 48 hpi and systemic values at 48 hpi.

We observed a significant positive correlation between nitrate supply and Pip values in both local and systemic leaves (Figure 5.3 a, b, c). After 24 hpi, *Psm* induced an increase in the level of Pip up to 20 µg/g FW in local leaves of plants grown in full buffer (Figure 5.3 a). Applying lower concentration of nitrate in solution, resulted in a reduced level of Pip accumulating to 17.71 µg/g FW, 16.17 µg/g FW, and 7.21 in 1.4 mM N, 0.7 mM N, and 0 N buffer respectively (Figure 5.3 a). To determine the impact of nitrate deficient buffer on Pip biosynthesis, Pip was also measured in local leaves after 48 hpi. We noticed that nitrate deficiency did not completely prevent the production of Pip and *Psm* induced the Pip level up to 48.18 µg/g FW, nevertheless this level is significantly low compared to other solutions containing a small amount of nitrate (Figure 5.3 b). Local Pip reaches its highest value (160 µg/g FW) in plants grown in buffer containing 1.4 mM N (Figure 5.3 b). This significant higher level of *Psm*-induced Pip in comparison to a higher concentrated buffer containing 2.8 mM nitrate, suggested the existence of a limiting factor for Pip production in a higher concentration of N in inoculated leaves.

Similar to local leaves, the level of Pip in the systemic leaves, is positively regulated by th amount of nitrate in growth buffer. In contrast to local leaves, systemic leaves, which were collected from plants grown in the N-deficient buffer, failed to accumulate Pip upon pathogen inoculation (Figure 5.3 c). This suggests a nitrogen-dependent factor is likely to be required for systemic signaling and Pip production in distal leaves. In addition, loss of SAR in plants grown in deficient buffer, is likely to be attributed to low level of systemic Pip in these plants. By the slight increase of nitrate to 0.7 mM N in growth solution, level of Pip was markedly elevated ($P < 0.001$) to 45.98 µg/g FW. The highest *Psm*-induced Pip level was detected in plants grown in 1.4 mM nitrate buffer, and expectedly this level was reduced to 41.38 µg/g FW in plants grown in high N concentrated buffer with 2.8 mM N. The positive correlation between nitrogen and Pip emphasizes the role of nitrogen on Pip production in a concentration-dependent manner. Our study provides additional support for the crucial role of nitrate supply on the plasticity of SAR and regulation of defense metabolites.

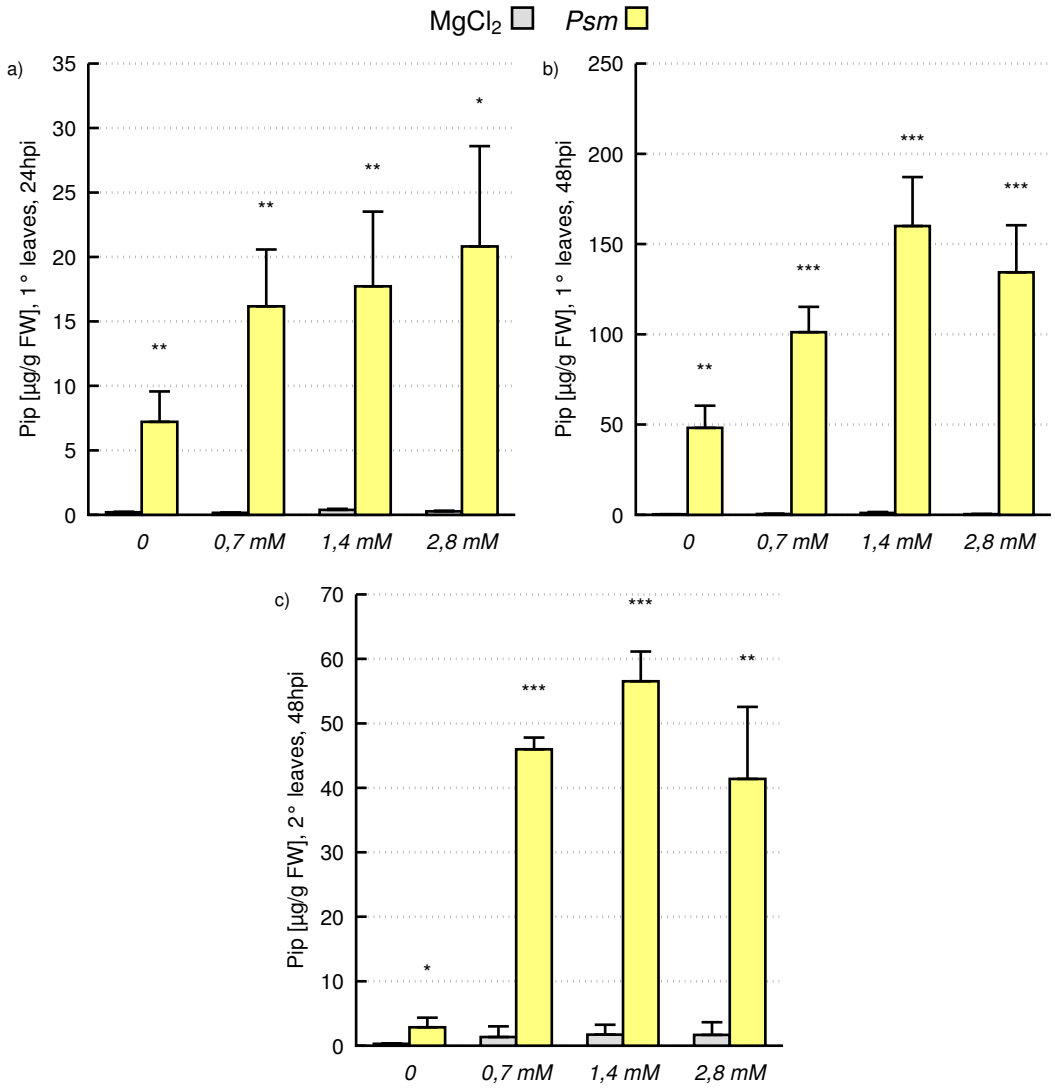

Figure 5.3: Accumulation of *Psm*-induced Pip in Col-0 plants grown under different nitrogen concentrations. Col-0 plants were grown in control hydroponic buffer (consisting of 2.8 mM nitrate (N)) for 4 weeks and plants were transferred to different solutions with distinct N concentrations (0 N, ¼ N = 0.7 mM, ½ N = 1.4 mM) 5 d before the physiological experiment. Three local leaves of plants were infiltrated with 10 mM $MgCl_2$ (control buffer) or *Psm* (OD_{600}=0.005) at 9 AM. Local-infiltrated leaves were collected at 24 hpi (a), 48 hpi (b) and systemic leaves were collected at 48 hpi (c). The statistical analyses were performed as described in the legend of Figure 5.1. The experiment was done by Holger Hallmann.

6 Discussion

Systemic acquired resistance in plants is a form of long-lasting and broad-spectrum protection in the whole plant foliage against subsequent pathogen attack. SAR involves generation of a signal (s) at the site of pathogen attack, translocation of the signal (s) through phloem and its perception in distal uninfected leaves. Although the identity of SAR signal(s) has not been understood yet, several chemically-different SAR inducers have been proposed as SAR signals in the past. This includes methyl salicylate (MeSA), glycerol-3-phosphate (G3P), azelaic acid (AzA), pipecolic acid (Pip), dihyroabetinal (DA), defective in induced resistance 1 (DIR1), and Azelaic acid Induced 1 (AZI1). Among these putative signals, Pip has been shown to play a crucial role in SAR and genetic and biochemical evidence demonstrate that Pip accumulation is necessary for SAR (Návarová, 2012; Bernsdorff et al., 2016; Ding et al., 2016; Hartmann et al., 2017). Pip is hydroxylated to NHP, which has been shown to be the SAR-active Pip-derivative (Hartmann et al., 2018; Chen et al., 2018). NHP is recently discovered as the main regulatory metabolite which mediates SAR against bacterial and oomycete pathogens (Hartmann et al., 2018). NHP induces expression of plant defense genes to increase the defense readiness of plants and it primes the plants for effective defense activation towards pathogens (defense priming) (Hartmann et al., 2018).

Pathogen infection induces multiple defense responses in plants and changes several processes at metabolic and transcriptional levels as well. One of this major metabolites is phenolic compound salicylic acid (SA) which was previously thought to be a SAR signal, however, its role as a SAR signal was rejected (Vernooij et al., 1994). Systemic accumulation of SA has been shown to be crucial for SAR establishment. A recent study from our group suggested that Pip operates in a SA-dependent and SA-independent manner to orchestrate SAR (Bernsdorff et al., 2016). In the present work, we aimed to clarify whether and to which extend the putative SAR signals G3P, AZA, DIR1, and MeSA interact with Pip in resistance induction and contribute to SAR establishment in the *Pseudomonas syringae pv. maculicola*-Arabidopsis interaction. We furthermore intended to obtain new insights into mechanisms underlying Arabidopsis systemic distance resistance induced by various strains of *Pseudomonas syringae* in different daytime.

6.1 Contribution of Glycerol-3-phosphate (G3P) to SAR

G3P is synthesized either from glycerol by the glycerol-kinase activity of GLI1 or by reduction of DHAP by G3P-dehydrogenase activity of GLY1. G3P was shown to contribute to basal resistance against the hemibiotrophic pathogen *Colletotrichum higginsianum* and G3P was considered as a novel regulator of

plant defense signaling (Chanda et al., 2008; Venugopal et al., 2009). Moreover, it was reported by Chanda et al., 2011 that mutant defective in G3P biosynthesis (*gli1* and *gly1*) were fully compromised in SAR and exogenous G3P was able to compensate the SAR defect of these mutants. According to Nandi et al., 2004, Arabidopsis thaliana SUPPRESSOR OF FATY ACID DESATURASE DEFICIENCY 1 (SFD1; alias GLY1) mutation was reported to be compromised in systemic SA accumulation and PR1 expression and consequently in SAR establishment against *Pseudomonas syringae pv. maculicola*. In addition, it was hypothesized that the SAR defect of *sfd1* was attributed to changes in glycerolipid composition in this mutant line (Nandi et al., 2004). Moreover, Gao et al., 2014b explained the role of galactolipids (monogalactosyldiacyl-glycerol (MGDG) and digalactosyldiacyl-glycerol (DGDG) in SAR and it was reported that exogenous G3P complemented the SAR defect in galactolipid mutants (*mgd1* and *dgd1*). Beside Arabidopsis, G3P was also mentioned to contributes to wheat systemic acquired resistance against stripe rust (Yang et al., 2013). In the context of SAR, G3P is shown to be involved in a feedback regulatory loop in which AzA- and G3P-mediated SAR are dependent on lipid transfer proteins (AZI1 and DIR1) and on the other hand, G3P regulates the transcriptional stability of AZI1 and DIR1 (Yu et al., 2013).

In this study, our comparative analysis of SAR assay between Col-0 and G3P mutant lines (*gli1-1* and *gly1-1*) showed that GLI1 and GLY1 are not crucial for SAR establishment under our experimental system and our findings are significantly differ from previous results reported in above-mentioned literature. Our results indicate that irrespective to the time of infiltration (morning/evening), not only virulent strain of *Pseudomonas syringae* (*Psm*), but also two other avirulent strains of the pathogen (*Psm AvrRpm1* and *Psm AvrRpt2*) were able to induce SAR in *gly1-1* and to a lesser extent in *gli1-1* (Figures 3.8 a_1 and 3.9 b_1). Our results suggest that GLI1 and GLY1 are dispensable for long-lasting induction of resistance against compatible pathogen (*Pseudomonas syringae pv. maculicola*) in Arabidopsis thaliana. It is notable that our LC/MS quantification of pathogen-induced G3P and its precursors (DHAP and glycerol) in *gli1-1* and *gly1-1* lines showed that these lines are not totally blocked in the production of G3P. Thus, it appears that in a genetic but not metabolite level, GLI1 and GLY1 genes are not contributed to SAR (Figure 3.22).

Nevertheless, there is some evidence that G3P can influence the strength of inducible resistance responses. Morning-infiltration of pathogens into *gli1-1* mutants resulted in reduced basal resistance when compared to Col-0. In addition, morning- and evening-SAR induction in *gli1-1* was significantly weaker than Col-0, indicating that GLI1 contributing to G3P pool, is likely to play a role in basal resistance and in full SAR establishment (Table 6.1 on page 129). On the other hand, a modest, yet significant SAR compared to wild-type was also observed in *gly1-1* after *Psm*-infection in the evening, suggesting the requirement of GLY1 to amplify SAR in the evening against the virulent strain of *Pseudomonas syringae* (Figure 3.6 b). It is notable that a positive SAR in *gly1* mutants upon morning but not evening-infiltration of the pathogen was also previously reported by Liu et al., 2011a.

It is notable that the contribution of GLI1 and GLY1 to the generation of total G3P pool in Arabidopsis differs (Chanda et al., 2011). Since *gli1* mutants do not change the level of plastidal 16:3 lipid level, it is obvious that most of G3P produced by GLI1 remain in the cytosol and it does not contribute to lipid biosynthesis in plastids. In comparison, *gly1* mutants with reduced level of 16:3 lipid, highlighted the role of plastidal G3P in the lipid biosynthesis. It is likely that changes in the level of G3P pool in plants can affect the strengthens of SAR via disturbing the balance of lipid metabolism. More investigations are

required to reveal the mechanisms underlying the contribution of GLI1 to lipid biosynthesis and the role of cytosolic and plastidal G3P associated with lipid biosynthesis pathway to potentiate SAR.

The phytohormone SA is well known for its role in mediating plant resistance. SA is a phenolic compound which accumulates in pathogen-infected tissue. Although SA is probably not transported from locally inoculated to systemic leaf tissue, the systemic accumulation of SA in the foliage is required for SAR (Vernooij et al., 1994). Thus, we analyzed whether G3P-defective lines are able to accumulate local and systemic SA. Based on our metabolite analysis by GC/MS, *gli1-1* and *gly1-1* are not impaired in the accumulation of systemic SAR-associated SA upon morning and evening infiltrations with *Psm*. In comparison with wild-type lines, *gli1-1* and *azi1-2* lines showed even higher accumulation of systemic SA upon *Psm* infiltration in the evening, indicating a possible regulatory role of GLI1 and AZI1 in systemic SA levels in the evening (Table 6.1 on page 129).

Pipecolic acid (Pip) is a non-protein, Lys-derived amino acid which accumulates in local and systemic tissues of pathogen-infected plants (Návarová, 2012). Since a significant amount of Pip is also detected in petiole exudate of infected leaves, it is likely that Pip is transported to systemic leaves during SAR (Návarová, 2012). In plants, L-Pip is synthesized from lysin via the aminotransferase activity of ALD1 (AGD2 like defense response protein) (Návarová, 2012; Hartmann et al., 2017). Ald1 knockout plants are unable to accumulate Pip and NHP in response to pathogen attack and are fully compromised in SAR (Návarová, 2012). SAR-defect of *ald1* can be restored by applying Pip or NHP exogenously (Návarová, 2012; Hartmann et al., 2018). A recent study from our group suggested that Pip orchestrate SAR via SA-dependent and independent pathways and it is also required for SAR-associated transcriptional reprogramming in the systemic tissue (Bernsdorff et al., 2016; Gruner et al., 2013). The present study shows that upon morning and evening infiltration of *Psm*, *gli1* mutants accumulates Pip to almost the similar level as wild-type in local leaves but to a higher level than that of wild-type in the systemic leaves (Table 6.1 on page 129). Although a higher level of Pip is detected in systemic leaves of *gli1*, this line showed a weaker SAR induction compared to wild-type.

Pip quantification analysis in *gly1* mutants shows that irrespective to the time of pathogen infiltration (morning/evening), the *Psm*-induced level of Pip is highly attenuated in local and systemic leaves of *gly1* mutants (Table 6.1 on page 129). In comparison to wild-type, the lower systemic level of Pip is associated with a slightly weaker SAR observed in *gly1* upon evening infiltration. GLY1 has DHAP reductase activity (referred as G3P dehydrogenase) which this activity is required for plastid lipid metabolism contributing to plant defense mechanisms and SAR (Lorenc-Kukula et al., 2012; Nandi et al., 2004). On the other hand, localization of ALD1 in plastids (Cecchini et al., 2015), suggesting that Pip is also likely to be synthesized in plastids. Together this data raises the possibility that plastidal G3P produced via GLY1 interacts with Pip biosynthesis in both local and systemic leaves. Since Pip-derived NHP is recently discovered as the main regulator of SAR, research is also needed to determine whether there is an interaction between G3P-signaling and FMO1-mediated NHP production in future.

Our Pip and SA feeding experiments show that both *gli1-1* and *gly1-1* are responsive to exogenous Pip and SA (Table 6.1). Comparing to Col-0, SA-induced resistance seems to express weaker in *gli1-1* lines however, our ANOVA analysis does not show a significant difference in SA-IR between *gli1-1* and Col-0. Hence, GLI1 and GLY1 act upstream of Pip and SA in defense signaling pathway. In a recent paper by Wang et al., 2018, Pip is shown to confer SAR by increasing the levels of free radicals, nitric oxide (NO),

and reactive oxygen species (ROS) which act upstream of AzA and G3P. It is also reported that systemic level of G3P and SA are important for Pip production in distal leaves (Wang et al., 2018). However, our SAR assays, metabolite analysis, and Pip-/SA-IR results do not confirm their observations. According to our results, *gli1-1*, *gly1-1*, and *azi1* mutant lines are SAR-competent upon morning and evening infiltrations with *Psm*, *Psm AvrRpm1*, and *Psm AvrRpt2*. Despite the higher accumulation of systemic Pip level compared to Col-0, *gli1-1* induces a weaker but still significant SAR upon *Psm* infiltration in the morning and evening. Moreover, *gly1-1* mutants accumulates less *Psm*-induced Pip level in local and systemic leaves, but these plants are not SAR defective under our experimental system. It is likely that there is a connection between G3P and Pip production. *gli1-1*, *gly1-1* and *azi1* mutant lines respond positively to exogenous Pip and SA, therefore, in contrast with Wang et al., 2018, we show that Pip and SA seem to act downstream of these signals. Further research is necessary to find out the connection between G3P and Pip/NHP signaling pathway. Future work needs to be done to establish whether and to which extent, AzA and G3P play a role on production or function of Pip-derived NHP, as a potent inducer of SAR.

6.1.1 Time-course analysis of G3P accumulation in wild-type

Time-course evaluation of G3P level by LC/MS showed that when pathogen attacks in the morning, in Col-0, G3P accumulates in local leaves 24 and 32 h post pathogen infection which previously an earlier time point of 6 hpi was reported by (Chanda et al., 2011). At this time point (24 hpi) the level of DHAP as G3P precursor is concomitantly induced by the pathogen. We showed here that the level of DHAP is highly reduced during the night, this might be contributed to its role in the G3P synthesis which can be further utilized for lipid metabolism. This resulted in a higher level of basal G3P in leaves. Therefore, uninfected Col-0 produces more G3P during the night. *Psm*-induced G3P accumulation at 24 hpi coincides with the accumulation of Pip in local infiltrated leave at this time point. This result opens the possibility that a connection between G3P and Pip biosynthesis exists. Since accumulation of glycerol precedes the accumulation of G3P in local leaves (8 hpi) (Figure 3.21 a), it is obvious that glycerol is likely to be the major precursor for G3P in local leaves. This is consistent with our previous observation showing reduced basal resistance in *gli1-1* which is defective to produce G3P from glycerol (Figure 3.1 c).

Evening-infiltration of Col-0 resulted in higher basal and induced level of G3P in local leaves than that of the morning, showing that G3P is preferentially produced during the night (Figure 3.21). Since G3P might be derivatized to an unknown compound in the systemic leaves (Chanda et al., 2011), not so much G3P was detected in systemic leaves of Col-0 irrespective to the time of infiltration (morning or evening). However, despite the more basal level of G3P in systemic leaves in the evening, Col-0 plants failed to induce G3P upon *Psm*-infiltration in the evening. Nevertheless, Col-0 plants are SAR positive in the evening without accumulating systemic G3P supports our hypothesis that that G3P is not required for systemic resistance.

6.2 Contribution of DIR1 and AZI1 to SAR establishment in the morning and evening

Chemical SAR inducers such as G3P, AzA, and DA, require non-specific lipid transfer-like proteins DIR1 (defective in induced resistance 1) and AZI1 (AzA induced 1) for long-distance signaling (Yu et al., 2013; Chaturvedi et al., 2012). DIR1 and AZI1 are required for pathogen-induced biosynthesis of G3P and in turn, transcriptional stability of DIR1 and AZI1 are regulated by G3P. Thus, G3P operates in a feedback loop with DIR1 and AZI1 in which the C18 fatty acid-derived azelaic acid (AzA) functions upstream of this loop (Yu et al., 2013). DIR1 was proposed to be a key mobile component of SAR that was required for the generation and/or translocation of mobile SAR signals and *dir1* mutant lines in Arabidopsis Ws accession were reported to be compromised in SAR (Maldonado et al., 2002). The crystal structure of DIR1 demonstrates the existence of a center hydrophobic packet in this protein which accommodates the fatty acids in vitro and is suitable for binding to a lipid (or hydrophobic) signal(s) due to transfer the signal to distal leaves (Lascombe et al., 2006). DIR1 is required for SAR signaling activity of putative SAR signals dehydroabietinal (DA), AzA, and G3P and a potential regulatory role for DIR1 in MeSA signaling is also reported (Chaturvedi et al., 2012; Jung et al., 2009; Chanda et al., 2011; Liu et al., 2011a). According to our SAR-assay results, *dir1-1* mutants exhibit a slight increased basal resistance when compared to Ws upon morning-infiltration of *Psm*. In contradiction with earlier findings (Maldonado et al., 2002), we found that SAR-induced in *dir1-1* has significantly higher resistance than SAR-induced in Ws upon pathogen attack in the morning but not in the evening. A SAR-competent of *dir1* mutants in the morning but not in the evening infiltration was also previously reported by Liu et al., 2011a. Notably, irrespective to the time of infiltration (morning/evening), *dir1-1* mutants are able to accumulate SAR regulators SA and Pip in local and systemic leaves. Our results show that exogenous Pip or SA, has a positive influence on resistance induction in *dir1-1*, implying that DIR1 is not required for Pip- and SA-induced resistance and acts upstream of Pip and SA biosynthesis (Table 6.1).

In this study, we used *dir1-1* mutant lines in Arabidopsis Ws accession and we show that SAR establishment in Ws accession is independent on DIR1. An occasional establishment of SAR in *dir1* mutant lines in Ws accession was also previously reported by Carella et al., 2017 which this positive SAR was attributed to a functional DIR1-like protein that may only contribute to SAR in the absence of functional DIR1. This is perhaps a possible explanation for positive SAR-induction in *dir1* in the morning infiltration as shown in our study. In addition, *dir1* is able to establish SAR to the similar extent as wild-type Ws in the evening, suggesting that DIR1 is not associated with long-distance signaling during the evening.

AZI1 is another lipid-transfer like protein which is induced by AzA. *azi1* mutants were reported to be impaired in long-distance signaling but not in local bacterial infections (Jung et al., 2009; Cecchini et al., 2015). Since AZI1 and its close paralog EARLY1 are locally required for SAR, thus a possible role of AZI1 in production and/or translocation of SAR signal(s) rather than signal perception was estimated (Jung et al., 2009; Cecchini et al., 2015). In this study, we demonstrated that AZI1 is not necessarily required for SAR establishment during morning and evening infiltration of virulent and avirulent strains of *Psm*. Since both *azi1-2* and *dir1-1* are SAR-competent, we assume that there are multiple LTPs contributing to translocation of the putative SAR signal(s) in the absence of DIR1 and AZI1.

Our results indicate that *azi1-2* mutant lines are able to accumulate local and systemic SA and Pip when *Psm* is infiltrated in the morning. Upon evening infiltration of plants with *Psm*, *gli1-1* and *azi1-2* accumulate a higher level of systemic SA and Pip compared to Col-0, showing a regulatory role of GLI1 and AZI1 in systemic production of pathogen-induced Pip and SA only during evening-infiltration. According to (Yu et al., 2013) a feedback regulatory loop exists between LTPs (AZI1 and DIR1) and G3P and upstream of this loop is AzA to mediate SAR (Figure 1.8 on page 36). Although Wang et al., 2018 reported that Pip acts upstream of AzA and G3P in the feedback loop, our Pip feeding experiment showed that like *dir1-1*, *azi1-2* plants positively responded to exogenous Pip and SA, implying that AZI1 acts upstream of Pip and SA which is in contrast to Wang et al., 2018. Further studies should be conducted to find out the possible interaction of AZI1, DIR1, and G3P with Pip/FMO1-signaling. Whether and to which extend these signals interact with NHP, as a critical SAR regulator, is the question to be addressed in future. Important questions to resolve for future studies are whether the LDS mutant lines accumulate pathogen-induced NHP and how they interact with NHP-induced resistance.

6.3 Contribution of methylsalicylate (MeSA) to SAR

Methylsalicylate (MeSA) is a biological inactive methylated form of SA which is highly induced upon pathogen attack in Arabidopsis and tobacco and it accumulates in infected leaves and phloem exudate to be transmitted to systemic leaves (Park et al., 2007b; Attaran et al., 2009; Seskar et al., 1998). MeSA is also proposed to be an airborne signal which is emitted from infected plants to activate defense mechanisms in upper tissues of the same plant or in neighboring plants (Shulaev et al., 1997). MeSA is synthesized from SA by SA methyltransferases (SAMT/BSMT) which are also required for the phloem accumulation of MeSA. In distal tissue, MeSA is converted back to SA via MeSA esterase activity of the SA binding protein 2 (SABP2) (Chen et al., 2003; Forouhar et al., 2005).

There are several controversies on the role of MeSA in long-distance signaling. Park and colleagues in 2007 were among the first who considered MeSA as a critical SAR signal. It was reported that SABP2-silenced scions grafted onto wild-type or SABP2-silenced rootstocks of tobacco, failed to establish SAR against TMV and it was hypothesized that methylesterase activity of SABP2 is required in systemic leaves to convert systemic MeSA into its active form SA and SA-mediated inhibition of this esterase activity is required in primary infected leaves to induce SAR (Park et al., 2007b; Park et al., 2009). Subsequent studies reported on impaired-SAR in Arabidopsis *bsmt1* mutants which failed to accumulate systemic MeSA (Liu et al., 2010). Therefore, MeSA was considered as a mobile SAR signal and a model was proposed in which SA accumulating after TMV infection in tobacco, is converted to MeSA by methyltransferase activity of SAMT in local leaves and feedback inhibition of SABP2 in primary leaves ensures the sufficient amount of MeSA to be produced in infected leaves and to be transmitted to systemic leaves where it will be subsequently reconverted to active SA by methylesterase activity of SABP2 to further activate downstream defense mechanisms and SAR (Liu et al., 2010).

In contrast to these studies, the requirement of MeSA for SAR establishment was not confirmed when Attaran et al., 2009 reported on a wild-type-like SAR in Arabidopsis *bsmt1-1* and *bsmt1-2* mutant lines which failed to produce MeSA but not systemic SA. According to this publication, the role of MeSA as

a possible airborne signal for SAR was also excluded since *bsmt1* mutant plants failed to produced and emit MeSA, albeit their wild-type-like SAR phenotypes (Attaran et al., 2009).

Later on, these inconsistent conclusions in different research groups were attributed to differences in experimental setup such as developmental age of the plants, the light intensity, and/or the strain of bacterial pathogen used by these groups and a possible conditional role of MeSA in long-distance signaling was considered by Liu et al., 2011a. According to Liu et al., 2011a the requirement of MeSA for SAR, was defined by the length of light exposure after primary pathogen inoculation and MeSA was likely to be essential for SAR when primary infection of Arabidopsis with virulent and avirulent strains of pathogen took place in the late afternoon followed by little or no light prior to the night. MeSA could potentiate SAR after morning infiltration but it was not necessarily required for SAR establishment in the morning (Liu et al., 2011a).

In our study, we have manipulated several factors (such as time of infiltration (morning/evening) and strains of the pathogen (virulent/avirulent)) to assess whether these factors influence the ability of *bsmt1-1* Arabidopsis to develop SAR. Based on our data, *bsmt1-1* mutants are not defective to accumulate local and systemic SAR regulators (SA and Pip) in both morning and evening infiltrations (Table 6.1). Compared to Col-0, Arabidopsis *bsmt1-1* mutants induce a weaker but still significant SAR when virulent and avirulent strains of *Psm* attack in the morning, suggesting that MeSA is not required for SAR, however, it is essential for full SAR establishment under morning-infiltration which is in contrast with results from Liu et al., 2011a. When pathogen attacks in the absence of light (evening), *bsmt1-1* plants induces a slight reduced basal resistance compared to Col-0, suggesting that MeSA might associate to mechanisms underlying basal resistance only when pathogen attacks in the absence of light (Figure 3.5 d). Surprisingly, ANOVA statistical analysis indicated that under our experimental setup, SAR-induced in *bsmt1-1* was even higher when compared to wild-type upon evening-infiltration of *Psm* and this contradicts the finding by Liu et al., 2011a. In addition, *bsmt1-1* plants respond positively to exogenous Pip and SA, indicating that MeSA acts upstream of Pip and SA (Table 6.1).

Furthermore, irrespective to the type of pathogen (*Psm*, *Psm AvrRpm1*, and *Psm AvrRpt2*), *bsmt1-1* plants are still able to induce a weaker but still significant SAR in the morning (Figure 3.8 a_2). Although *bsmt1-1* are compromised to induce SAR against *Psm AvrRpt2*, induction of SAR upon *Psm* and *Psm AvrRpm1* in the evening-infiltration is obviously emphasizing that MeSA is not contributed to SAR (Figure 3.9 b_1). In addition, quantification of volatile MeSA in *bsmt1-1* shows that this mutant fails to emit as much as gaseous MeSA comparing to Col-0 (data not shown). Therefore, failure in emitting gaseous MeSA in *bsmt1-1* does not prevent the induction of SAR, suggesting that not only endogenous MeSA but also MeSA as an airborne signal does not efficiently contribute to SAR establishment.

Although MeSA may function to deliver SA to systemic leaves, it is obvious that it is not the only way and SA transportation to systemic leaves might be performed in a MeSA-independent pathway as well. Notably, one of the possible reasons that reject MeSA as a mobile SAR signal, is that the time frame in which the putative SAR signal is translocated in systemic leaves precedes the time frame of MeSA translocation to the systemic leaves which are 48 and 72 h post-primary infection (Park et al., 2009).

Contradiction to earlier findings by Liu et al., 2011a; Park et al., 2007b, we found out that Arabidopsis *bsmt1-1* mutants are capable to accumulate main defense regulators (SA and Pip) and they are able

to establish SAR irrespective to the time of infiltration and to the type of the pathogen. This led us to conclude that MeSA is not a crucial SAR signal, nonetheless it is contributed to basal resistance in the absence of light and to long-distance signaling mechanisms leading to establishing a full SAR in the morning. Future work will investigate whether there is a connection between MeSA and Pip-derived NHP, as a potent SAR inducer.

6.4 Summary table of Long Distance Signaling (LDS) results

The entire results of chapter 3 are all summarized in Table 6.1.

		Morning-infiltration							Evening-infiltration						
		Col-0	*gli1-1*	*gly1-1*	*azi1-2*	*bsmt1-1*	Ws	*dir1-1*	Col-0	*gli1-1*	*gly1-1*	*azi1-2*	*bsmt1-1*	Ws	*dir1-1*
SAR		+++	++	+++	+++	++	+++	++++	+++	++	+++	+++	++++	+++	+++
SA acc.	1°	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++++
	2°	+++	+++	+++	+++	+++	+++	+++	+++	++++	+++	++++	+++	+++	+++
Pip acc.	1°	+++	+++	++	+++	+++	+++	+++(+)[a]	+++	+++	++	++	+++	+++	+++
	2°	+++	++++	++	+++	+++	+++	+++	+++	++++	++	++++	+++	+++	+++

	Col-0	*gli1-1*	*gly1-1*	*azi1-2*	*bsmt1-1*	Ws	*dir1-1*
SA-IR	+++	++(+)[b]	+++	+++	+++	+++	+++
Pip-IR	+++	+++	+++	+++	+++	+++	+++

Table 6.1: Overview of SAR establishment, metabolite accumulation, and SA-/Pip-induced resistance in Arabidopsis LDS mutant lines upon morning and evening *Psm*-infiltration. In this table, we compared SAR establishment, metabolite accumulation, and SA-/Pip-induced resistance in LDS mutant lines to their respective wild-type lines (*gli1-1*, *gly1-1*, *azi1* and, *bsmt1-1* mutant compared to Col-0 and *dir1-1* lines compared to Ws). We use a (+++) parameter in each wild-type and then we compare each line with the respective wild-type by adding or distracting a plus +. Therefore, ++++ is used to show stronger resistance (SAR and SA-/Pip IR) or higher metabolite accumulation (SA and Pip accumulation in 1° inoculated or 2° systemic leaves) and (++) is used to show a weaker resistance or less accumulation of metabolites in comparison with wild-type. Red colors show all the points in the table that a different results in comparison to respected wild-type is observed (either ++++ or ++). a: This experiment was done only one time with higher Pip accumulation in comparison with Ws (thus ++++). To confirm the data the experiment must be repeated. b: This experiment was performed two times. In one experiment *gli1-1* showed induced a weaker SA-IR (++) and in the second experiment SA induced a wild-type-like resistance (+++). To confirm the data the experiment must be repeated. SAR: systemic acquired resistance, acc.: accumulation, SA-IR: SA-induced resistance, Pip-IR: Pip-induced resistance

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6.5 Effect of Light on SAR and plant defense responses at metabolite and transcriptional levels

Complex defense signaling network in plants are not only regulated by the physiological and developmental status of plants, but they are shown to be regulated by external factors such as light. In the present work, we aimed to clarify the influence of light on SAR, and biosynthesis and regulatory function of main SAR regulators SA and Pip. We furthermore intended to understand whether SA- and Pip-induced priming of PR1 expression is regulated by a light factor. In addition, we tested the direct effect of light on bacterial growth and biosynthesis of putative SAR signal Pip by applying different light period after *Psm* infiltration. In addition to investigate the influence of different light length with static intensity on plant defense responses, we checked whether fluctuation light, which is similar to light in nature, can affect the production of defense regulators (SA and Pip).

Consistent with the previous publication from our group (Zeier et al., 2004), our results in this study confirmed that execution of SAR and pathogen-induced production of SA require light. Development of SAR in Arabidopsis Col-0 plants is completely lost when the primary infection with *Psm* occurred in the absence of light (Figure 4.1). Light is proved to regulate several defense responses in SA-dependent signaling pathway including accumulation of SA, SA-induced PR1 expression, activation of phenylalanine ammonia-lyase (PAL), and execution of HR (Griebel and Zeier, 2008). Our time-course evaluation of *Psm*-induced SA accumulation confirmed that de novo biosynthesis of SA in both local and systemic leaves is highly dependent on the appearance of light after pathogen infection, although the plants are still capable to accumulate a very low level of SA induced by pathogen under dark condition (Figure 4.3). In a similar way, transcription of PAL as an involved enzyme in SA biosynthesis was also previously shown to be light dependent (Zeier et al., 2004). It is noteworthy to mention that accumulation of SA under normal light/dark cycle is proved to be rhythmic and SA has its highest peak at night which is a few hours before pathogen infection (Goodspeed et al., 2012). This could be one explanation that we observe a higher level of *Psm*-induced SA in the morning. Reduced level of SA in darkness, is not the only reason that causes impaired SAR in Arabidopsis Col-0 plants under dark condition, since plants under high light (PDF = 500 µmol photons m^{-2} s^{-1}) are shown to be SAR competent independent of systemic SA accumulation (Zeier et al., 2004) and *sid2* mutant plants establish a moderate but still significant SAR (Bernsdorff et al., 2016).

Pipecolic acid, as a crucial SAR signal, orchestrates SAR in SA-dependent and -independent pathways (Bernsdorff et al., 2016). We thus investigated whether biosynthesis and functional role of Pip in SAR development depend on a light-dependent factor. In comparison with mock-treated plants, *Psm*-induced Pip increases after 24 hpi, in infected leaves (Návarová, 2012). When plants translocate in constant darkness after *Psm* inoculation, they produce lower but still significant level of Pip in infiltrated (local) leaves after 24 and 48 hpi when compared to that of light (Figure 4.4), however, accumulation of systemic Pip is completely abolished in darkness. Moreover, no accumulation of Pip is observed in petiole exudate of inoculated leaves collected from dark-adjusted plants when compared to that of light-grown plants. Together these data highlight a critical role of light in the biosynthesis of local and systemic Pip in response to pathogen attack. Pip is not enriched in petiole exudate of local leaves collected in darkness, suggests that light or an unknown light-dependent factor is required for flow and mobility of Pip from local

to systemic leaves. Since local but not systemic accumulation of Pip is independent of FMO1, we need to further test whether FMO1 transcripts and function in Pip-signaling are regulated by light. A recent publication from our group demonstrates that FMO1 generates N-hydroxypipecolic acid (NHP) from Pip, which is a critical element for induced immunity against bacterial and oomycete infection (Hartmann et al., 2018). Whether light affects the functional role of FMO1 and consequently the production of NHP, is a question to be addressed.

As expected our experiments demonstrate that SA-induced PR1 expression is strongly light-dependent. Under normal light/dark cycle, exogenous application of SA induces the transcription of PR1, and co-application of SA and Pip markedly strengthens the expression of PR1 compared to solo-application. According to our results, SA strongly induces PR1 expression under light condition (Figure 4.9). Priming function of SA and Pip is not totally blocked when plants translocate in constant darkness after exogenous application, however, a highly reduced transcriptional level of PR1 is observed under dark condition, suggesting that light is essential to activate mechanisms of SA- and Pip-induced priming. [Due to high variations in Pip-priming results, more repetition of the experiment is required to confirm the light dependency of priming function of Pip on amplification of SA-inducible PR1 in future.]

6.6 Direct effect of light on induced resistance and defense metabolites

According to Griebel and Zeier, 2008, availability of long light period after pathogen attack, resulted in increased sets of defense responses in plants. Therefore, in this study, we investigated the direct effect of light by applying a light regime of 5, 10 (control condition), 15 h, and constant light after *Psm* attack. Surprisingly, determination of basal bacterial growth shows that length of light period applied to plants after *Psm* attack does not correlate with basal resistance towards *Psm*. The highest basal resistance is observed in plants adjusted to 5 h light after inoculation, comparing to plants situated in normal 10 h light or even more light period (15 h and constant light) (Figure 4.10). Inconsistent with this observation, the level of SA and camalexin, as two major defense metabolites required for basal resistance, positively correlates with increasing the length of the light period after *Psm* inoculation (Figures 4.10, 4.12, and 4.13). Under our experimental setup, a duration of 15 hours light application after pathogen inoculation, is sufficient to reach the maximal SA production, and further prolongation of the light period does not lead to higher SA production anymore. Perhaps, constant light application leads to saturation of SA-induction.

According to our ANOVA analysis, exposure of plants to only 5 hours light after *Psm* infiltration, is not sufficient to induce the accumulation of local and systemic Pip significantly. In infiltrated local leaves, the highest level of Pip (14.65 µg/g FW) was observed when plants remained in their growth condition (which is 10 h light application after *Psm*-infiltration) and this level of local Pip was lower when plants were exposed to 15 h and constant light after pathogen attack. In addition, the length of light period positively correlates with the systemic level of Pip (Figure 4.14 b), suggesting a direct effect of light on either mobility of Pip from local to systemic leaves or on de novo ALD1-regulated biosynthesis pathway of Pip in distal leaves. Future work will investigate whether systemic Pip accumulation correlates with the SAR efficiency. Therefore, SAR assay under the different light settings needs to be done in future.

Our result has further strengthened our confidence that the pathogen-induced production of several defense metabolites SA, camalexin, and systemic Pip are directly regulated by light and increasing the length of the light period in early hours after pathogen attack, results in higher accumulation of defense metabolites to a certain extent. Despite the increased level of local SA and camalexin in higher light period applied after pathogen attack, plants are incapable to reduce the bacterial growth, which suggests that Arabidopsis may employ different signaling networks rather than SA-dependent signaling pathways to limit the growth of bacteria under various light regimes. Application of more light period might deactivate other mechanisms plants employ to reduce the growth of bacteria. We hypothesize that the application of longer light period than standard 10 h light, may negatively regulate activation of SA-independent basal defense mechanisms.

6.7 Dynamic light and plant defense responses

Light intensity can change rapidly in nature and it affects several regulatory mechanisms leading to acclimation or adaptation of plants to a dynamic environment (Matsubara et al., 2016). Following the effect of light length on plant defense responses, we wanted to know whether short changes in light intensity (or fluctuation light) affects defense responses of Arabidopsis towards *Psm* in metabolic level.

In constant (control) light (CL) (PDF= 75 µmol photons m^{-2} s^{-1}), acclimation responses (such as growth, photosynthesis, metabolites, and gene transcription) were shown to be differently expressed in young and mature leaves (Caliandro et al., 2013). According to our data, compared to CL, fluctuation light (FL) significantly reduces the *Psm*-induced level of total SA in young-infiltrated leaves (local effect) and a significant reduction of SA was observed in young leaves distal to mature-infiltrated leaves (systemic effect) (Figures 4.15 and 4.16). In contrast to SA, there is no significant difference between the amount of *Psm*-induced Pip in both mature-infiltrated and systemic young leaves in CL and FL, suggesting that production of Pip is not affected by FL (Figure 4.17). Hence, FL impacts only the production of SA particularly in the systemic leaves and it might consequently affects the strength of SAR. Further work needs to be carried out to establish whether SAR performance differs under FL and CL.

In the absence of the pathogen, FL can affect the performance of the photosynthetic apparatus and high light intensity induces long-term acclimation responses involving protection against photo-oxidative stress and reduced carbon gain in plants (Alter et al., 2012; Matsubara et al., 2016). In comparison to mature leaves, young leaves can quickly redesign their leaf anatomy and photosynthetic apparatus, to optimize it to the new light conditions (Bielczynski et al., 2017). In addition, SA as a hormone involves in photoprotective mechanisms elicited by FL (Karpiński et al., 2013). In Arabidopsis, 90% of pathogen-induced SA production is via the isochorismate pathway in chloroplasts (Garcion et al., 2008; Wildermuth et al., 2001). Therefore, it is likely that a regulatory network among FL, acclimation responses of photosynthesis apparatus under FL in productive young leaves, and functional SA as a photo-protective component exists which could be perturbed upon pathogen attack via affecting the photosynthesis apparatus. We showed here that acclimation to FL negatively affects systemic immune responses, however, an important question to resolve for future studies is whether the acclimation to FL affects SAR performance and other plant immune inducers such as NHP (as a central SAR regulator).

6.8 Nitrogen as an external defense regulator

Nitrogen is a limiting factor for plant growth and development and it modulates different aspects of plant physiological and metabolism. In this study, the contribution of nitrogen to the regulation of SAR and SAR-related metabolites SA and Pip in an interaction between Arabidopsis and *Pseudomonas syringae* in a hydroponic system have been investigated. Our data shows that establishment of SAR and production of Pip are highly dependent on plant's nitrogen supply (Figures 5.1 and 5.3). Arabidopsis plants grown under nitrogen-limiting conditions (0 mM N in the hydroponics solution) for five days before *Psm* inoculation, fail to establish SAR. However, increasing the concentration of nitrogen (N) in hydroponic solution to 1.18, 2.35, and 4.7 mM resulted in a positive SAR establishment in these plants. A full SAR was observed when the concentration of nitrogen in solution was 2.34 mM, and a weaker but still a positive SAR was observed in plants grown in 4.7 mM N-concentrated solution (Figure 5.1). We conclude that the execution of SAR is thus not only dependent on the availability of nitrogen but also on the concentration of nitrogen in plants growth solution (Figure 5.1).

At metabolite level, lack of nitrogen supply in plant's growth solution does not block the production of local and systemic *Psm*-induced Pip, however, it highly reduces the level of Pip when compared to that of plants grown in solutions with available nitrogen. In local leaves, the level of Pip induced by *Psm* at 24 hpi, positively correlates with the concentration of nitrogen in the growth solution (Figure 5.3 a). A positive correlation between nitrogen concentration and induced-Pip level in local and systemic leaves at 48 hpi indicates that nitrogen availability directly affects the biosynthesis of Pip. Notably, a higher level of Pip compared to control buffer (2.8 mM N) is observed at the concentration of 1.4 mM N in both local and systemic leaves at 48 hpi, suggesting that nitrate availability affects the production of Pip in a concentration-dependent manner. During SAR in Arabidopsis, the amino acid profile is modified upon *Pseudomonas syringae* infection (Návarová et al., 2012). We assume that the change in the level of amino acids could be as a consequence of protein degradation or autophagy, since autophagy is induced in response to nitrogen deficiency due to recycle the nutrients in plants (Havé et al., 2018). To investigate whether the nitrogen deficiency impacts the amino acids content and subsequently the concentration of growth- and defense-related metabolites, future work will evaluate the level of all *Psm*-induced amino acids in Arabidopsis plants grown under different N concentrations. Moreover, future studies need to focus on Pip-derived NHP, as the main SAR regulator, in the context of nitrogen supply.

Interestingly, the local level of *Psm*-induced SA negatively correlates with nitrogen concentrations. One possible explanation for this result is based on the Carbon-Nitrogen Balance (CNB) hypothesis. According to CNB predictions, when plant grow under limited nitrogen, carbohydrates will accumulate in plant tissues and consequently, this increase will lead to synthesis of carbon-based secondary metabolites such as phenolics as we observed higher accumulation of SA in local leaves which are closer to the roots and to the nitrogen source (Hamilton et al., 2001). In contrast, in the systemic leaves, a positive correlation exists between nitrogen concentration and systemic SA showing that pathogen-induced accumulation of phenolic compounds (SA) is dependent on the availability of nitrogen source in systemic young leaves (Figure 5.2 d).

In our experiments, we infiltrated Arabidopsis mature leaves as local leaves and we collected both mature and young systemic leaves for quantification of secondary metabolites. However, it should be

considered that chlorophyll content and management of nitrogen assimilation could be differentially expressed in young and mature leaves and this could affect the allocation of nitrogen from photosynthesis to biosynthesis of secondary metabolites for defense (Hirel et al., 2005; Zeier, 2005).

In addition, irrespective of the concentration of nitrate in the area, uptake of nitrogen is an essential step in nitrate assimilation and nitrate-depended metabolism (Remans et al., 2006). Plants may undergo several physiological changes to uptake the nitrate when they are translocated from a full buffer into low-concentrated N solutions. A more direct approach to study the role of nitrogen on plant defense mechanisms would be to include Arabidopsis nitrate reductase mutant lines in future work.

7 Materials and methods

7.1 Plant material and growth conditions

7.1.1 Soil-grown plants

Arabidopsis (*Arabidopsis thaliana*) plants were grown in individual pots containing a mixture of soil (Klasmann-Deilmann; Substrat BP3), vermiculite, and sand (8:1:1), under controlled condition inside plant chambers with 10-h-day/14-h-night (9 AM-7 PM), photon flux density (PDF) of 100 µmol photons m^{-2} s^{-1}, humidity of 60%, and temperature of 21℃ and 18 ℃ during day and night respectively. Based on the experiments, to apply different light periods (e.g. 5, 10, 15 hpi) or even constant light/darkness, plants were grown in percivals in which the conditions such as day cycle, light, temperature, and humidity could be adjusted accordingly. Experiments in section 4.6 were performed at Jülich research institute, Germany. Arabidopsis Col-0 plants were kept in growth light condition of 75 µmol photons m^{-2} s^{-1} for four weeks and then half of the plant population was transferred to a fluctuating light where 20 s pulses of high light (1000 µmol photons m^{-2} s^{-1}) were applied with three halogen lamps (Haloline; Osram) every 6 min under the background light intensity of 75 µmol photons m^{-2} s^{-1} during the day. For infiltration, 5-6 week old unstressed and uniform Arabidopsis plants were used. The table shows a list of mutant lines used in this study. Homozygous T-DNA insertion lines were identified by PCR according to Alonso et al., 2003 and alignment analysis of G3P mutant lines is shown in section 7.2.

Mutant	Background	Line	Mutant line confirmation
gli1-1	Col-0	Kang et al., 2003	This study (page 136)
gly1-1	Col-0	Kachroo et al., 2004	This study (page 136)
azi1-2	Col-0	SALK_085727	Done by Katrin Gruner
bsmt1-1	Col-0	SALK_140496	-
fmo1-1	Col-0	Mishina et al., 2007	-
ald1	Col-0	SALK_007673	-
npr1	Col-0	NASCID: N3801	-
dir1-1	Ws	Maldonado et al., 2002	Done by Katrin Gruner

Table 7.1: Mutant lines used in this study

7.1.2 Hydroponic system

Due to change the concentration of nitrate supply, a hydroponic system (Araponics system) was used in which plants were grown in a full and adjusted nitrate medium containing 1.50 mM $Ca(NO_3)_2 \cdot 4\,H_2O$, 1.25 mM KNO_3, 0.75 mM $Mg(SO_4)_2 \cdot 7\,H_2O$, 0.50 mM KH_2PO_4, 0.1 mM $Na_2O_3Si \cdot 5\,H_2O$, 72 µM Fe-EDTA, 50 µM KCl, 50 µM H_3BO_3, 10 µM $MnSO_4$, 2 µM $ZnSO_4$, 1.5 µM $CuSO_4$, 0.075 µM $(NH_4)_6Mo_7O_{24}$.(Tocquin et al., 2003; Gibeaut et al., 1997). The final PH was adjusted to 6.0. The seed holders were filled with 0.6 % agar and seeds were set in agar after sterilization with VE-water and one drop of tween. Plants were grown in full medium and adjusted to experimental conditions almost 4 days before infiltration.

7.2 Alignment-based analysis of Arabidopsis G3P mutant lines:

7.2.1 GLY1: NAD-dependent glycerol-3-phosphate dehydrogenase 1

GLY1 (also known as SFD1 (SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1) is a protein-coding gene which is located in Arabidopsis thaliana (ecotype Columbia) chromosome 2 (Locus tag: AT2G40690). Arabidopsis knockout lines were generated by a G to A point mutation at the end of exon 1 (base pair 258) (Kachroo et al., 2004; Chanda et al., 2011). In order to find individual plants carrying a point mutation in GLY1, genomic DNA was extracted from leaves of predicted mutant lines and Col-0 as the control. Primers were designed to amplify a selected fragment (145 base pair length) within the coding region as shown below. The amplified segment was sequenced at Eurofins overnight sequencing services. Alignment of original sequence (selected segment from the coding region in wild-type) and expected mutant sequence (with point mutation) proves substitution of a single nucleotide and consequently inactivation of the coding gene GLY1. Sequence data shown here were taken from NCBI under accession numbers NM_129631.2 (GLY1).

The sequence of GLY1 coding region (Primer binding sites are highlighted in red).

```
   1 ATGGCGGCTT CGGTGCAACC TGCATGCTTA GACCTCCACT TCTCCGGAAA GCATCCACCG CTTCTTAAAC
  71 ACAACGCTAT TATCGTCCGC TGCGTTTCTT CTCCAAATGT AATTCCCGAA GCTGACTCCA TCTCTGGTCC
 141 GCCTGATATC ATCAATACGA ACCGTGACCA CCGCAAAGTG GTTCGTATCG CTTGGGAGAA GTTGGTTCGA
 211 TGGTCTCGTT CTTTGCGCGC TAAAGCTAAA ACCGATGTTC TTGAGCGTAC TCGCAAGGTT GTTGTTCTTG
 281 GTGGAGGTTC GTTTGGTACT GCAATGGCTG CTCATGTAGC TAGAAGGAAA GAGGGATTAG AGGTTAATAT
 351 GCTTGTTCGT GACTCTTTTG TTTGTCAATC TATCAACGAG AACCACCATA ATTGTAAGTA TTTTCCTGAG
 421 CACAAGTTAC CTGAGAATGT GATTGCTACA ACTGATGCGA AAGCTGCATT GCTTGATGCT GATTACTGCC
 491 TTCATGCCGT GCCTGTGCAG TTTAGCTCTT CGTTTCTAGA GGGAATTGCC GATTATGTCG ATCCAGGATT
 561 GCCTTTTATA TCTCTTAGCA AAGGTCTGGA GCTTAATACT CTTAGGATGA TGTCTCAGAT CATTCCCATT
 631 GCGCTTAAGA ATCCCCGGCA ACCTTTTGTT GCTCTTTCTG GCCCGTCATT TGCTCTGGAG CTGATGAACA
 701 ATTTACCAAC TGCAATGGTG GTTGCCTCAA AAGATAAGAA ATTGGCCAAT GCTGTTCAGC AGCTTCTTGC
 771 TTCTAGTTAC TTGAGAATAA ATACTTCCAG TGATGTTACA GGCGTGGAAA TCGCCGGTGC CCTGAAGAAT
 841 GTTCTAGCAA TAGCTGCAGG AATTGTTGAT GGAATGAATC TCGGTAACAA CTCTATGGCA GCTCTTGTGT
 911 CCCAAGGTTG TTCAGAGATA AGATGGTTAG CCACAAAGAT GGGTGCAAAG CCAACAACCA TTACTGGTTT
 981 ATCAGGAACT GGGGACATAA TGCTTACGTG TTTTGTAAAT CTTTCAAGAA ACCGAACAGT TGGAGTCAGG
1051 TTAGGGTCAG GGGAGACACT AGATGACATA CTAACCTCTA TGAATCAGGT TGCAGAAGGT GTAGCAACAG
1121 CCGGGGCAGT GATAGCATTA GCACAGAAAT ACAATGTGAA ACTGCCGGTT TTGACAGCCG TAGCTAAGAT
1191 AATAGATAAT GAACTGACCC CGACTAAGGC TGTTCTTGAG CTCATGAACC TTCCTCAGAT TGAAGAAGTA
1261 TGA
```

Oligos which used to amplify the selected segment

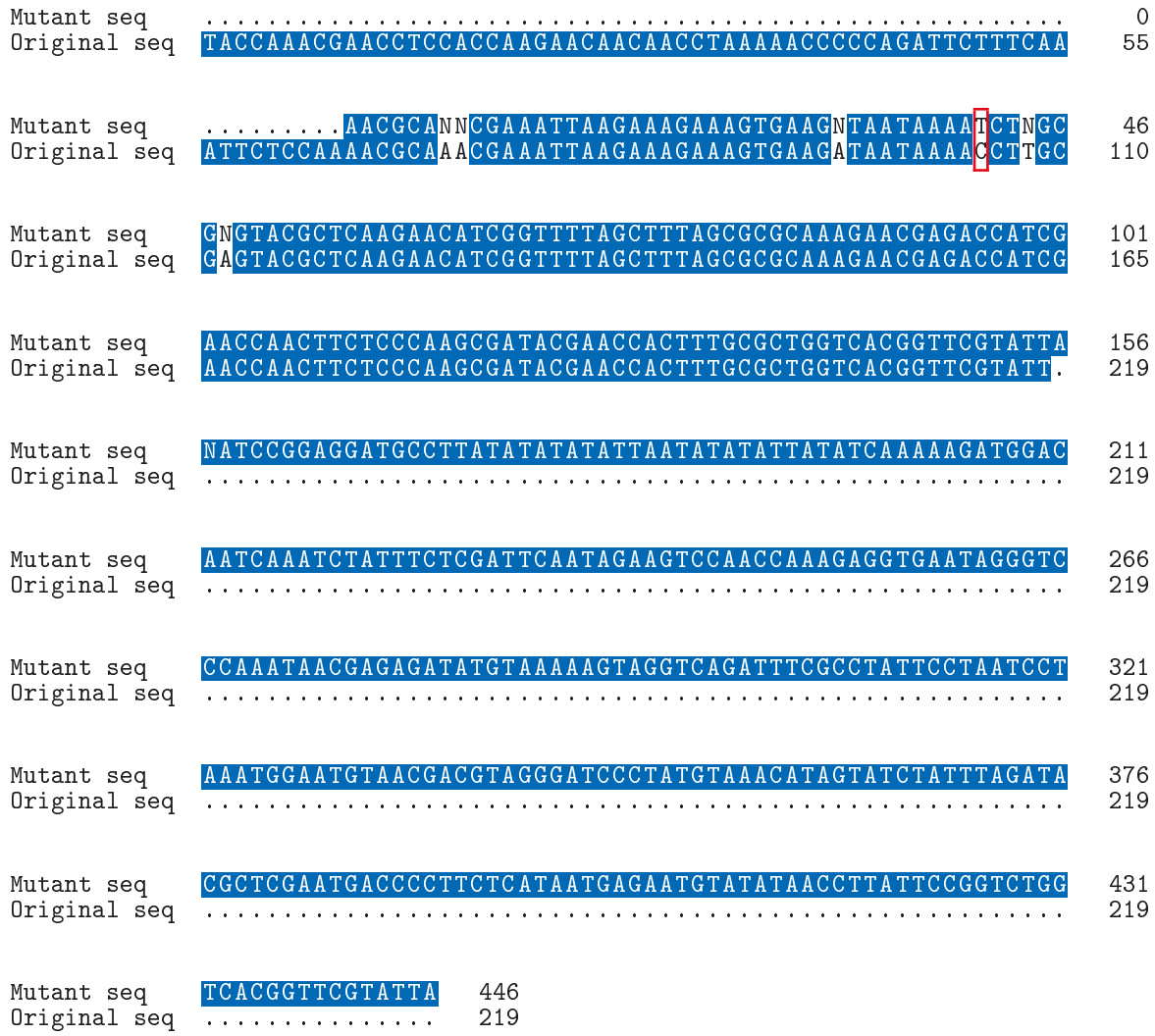
forward primer AATACGAACCGTGACCAG complementary reverse primer TACCAAACGAACCTCCAC

Amplified segment cDNA: 145bp (Primer binding sites are highlighted in red and point mutation nucleotide is in green)

```
   1 AATACGAACC GTGACCAGCG CAAAGTGGTT CGTATCGCTT GGGAGAAGTT GGTTCGATGG TCTCGTTCTT
  71 TGCGCGCTAA AGCTAAAACC GATGTTCTTG AGCGTACTCG CAAGGTTTTA TTATCTTCAC TTTCTTTCTT
 141 AATTTCGTTT GCGTTTTGGA GAATTTGAAA GAATCTGGGG GTTTTTAGGT TGTTGTTCTT GGTGGAGGTT
 211 CGTTTGGTA
```

location of point mutation: AACCGATGTTCTTGAGCGTACTCGCAAG—-G—TT End of Exon 1

Alignment of original amplified sequence and mutant segment (red box shows the location of point mutation)



7.2.2 GLI1: glycerol kinase

GLI1 also known as NHO1 (nonhost resistance to *P. s. phaseolicola 1*) is a protein-coding gene with glycerol kinase activity which converts glycerol to glycerol-3-phosphate. GLI1 is located on Arabidopsis thaliana chromosome 1 (Locus tag: AT1G80460). Arabidopsis Knockout lines were generated by a single nucleotide substitution of G-to-A which causes a stop codon in coding sequence as shown below (Kang et al., 2003) To detect individual defective Arabidopsis in GLI1, genomic DNA was extracted from leaves of predicted mutant lines and Col-0 as the control. Primers were designed to amplify a selected fragment (407 base pair length) within the coding region, in which the point mutation is expected as shown below. Arabidopsis *gli1-1* lines were verified by alignment-based comparison of two sequences (the original segment and the expected point mutation segment). Sequence data shown here were taken

from NCBI under accession number NM_001036230.1 (GLI1). amplified fragments were sequenced at Eurofins overnight sequencing services.

The sequence of GLI1 coding region (Primer binding sites are highlighted in red).

```
   1 ATGGCAAAAG AAAATGGATT TATAGGATCA ATCGATCAAG GAACCACCAG CACCAGATTC ATCATTTACG
  71 ACCACGATGC TCGTCCTGTT GCTTCTCATC AAGTCGAGTT CACTCAGTTC TATCCCGAAG CTGGATGGGT
 141 GGAACACGAT CCAATGGAGA TACTGGAAAG TGTGAAAGTG TGCATTGCAA AGGCTCTCGA CAAAGCCACT
 211 GCCGATGGAC ACAACGTCGA CGGTGGCTTG AAGGCCATTG GGCTTACAGA TCAGAGAGAG ACTACTGTTG
 281 TTTGGAGCAA ATCCACTGGC CTTCCTCTCC ACAAGGCTAT TGTCTGGATG GATGCTCGTA CCAGCTCCAT
 351 CTGCAGGAGA CTAGAGAAAG AACTCTCGGG AGGAAGATCC CATTTTGTGG AGTCTTGCGG CTTGCCAATA
 421 AGCACATACT TCTCTGCCAT GAAGCTGCTT TGGCTCATGG AGAATGTGGA TGATGTCAAA GACGCTATCA
 491 AGAAAGGGGA TGCCATCTTT GGCACTATCG ACACATGGTT GATCTGGAAC ATGACTGGCG GTATCAATGG
 561 CGGCCTTCAT GTCACTGATG TCACCAATGC TTCACGGACA ATGCTCATGA ACCTCAAAAC CTTGAGCTGG
 631 GACCAGGACA CTTTGAAGAC ACTTGGCATA CCGGCTGAAA TCTTGCCCAA GATTGTCAGC AATTCAGAAG
 701 TGATTGGAGA GATCTGCAAA GGCTGGCCTA TTCCCGGTAT CAAGATTGCT GGATGTCTTG GTGATCAGCA
 771 TGCTGCGATG TTGGGGCAAG CTTGCAGAAA AGGCGAGGCG AAGAGTACTT ATGGCACAGG TGCTTTCATT
 841 CTTCTCAACA CCGGAGAAGT GCCAATCAAA TCAGGTCATG GTCTTCTGAC CACGTTGGCC TACAAGCTCG
 911 GTCCTCAAGC ACAGACAAAC TATGCATTGG AGGGTTCGAT TGCCATAGCA GGAGCTGCTG TTCAGTGGCT
 981 TAGAGACAGC CTTGGGATAA TCAAAAGCGC CTCTGAGATC GAAGATTTGG CAGCAATGGT AGATTCTACA
1051 GGAGGAGTGT ACTTTGTGCC AGCGTTCAAC GGCTTGTTTG CTCCTTGGTG GAGAGAAGAC GCACGTGGTG
1121 TGTGCATTGG AATCACGAGG TTCACCAACA AGTCTCACAT TGCTCGGGCT GTGCTGGAGA GCATGTGTTT
1191 CCAGGTGAAA GACGTCCTTG ACTCCATGAA CAAAGATGCA GGTGAAAAGG GTTCCCTTAA TAACGGGAAA
1261 GGGGAGTTCT TGCTCAGAGT TGATGGTGGT GCCACAGCTA ACAACCTTCT GATGCAGATT CAGGCTGATC
1331 TGATGGGAAG TCCGGTGGTG AGGCCAGTGG ACATAGAGAC AACAGCATTA GGAGCAGCCT ATGCAGCTGG
1401 ATTAGCTGTG GGATTCTGGA AGGAAGCAGA CATATTCGAG TCGGGAGAGA AGGCGAAGAA CTCCAAAGTT
1471 TTCAGACCCG CTATGGAAGA AGGAATCAGG AAGAAGAAAG TGGCGTCTTG GTGCAAAGCG GTGGAAAGAA
1541 CATTTGATCT CGCTGACCTC TCTATCTAA
```

Oligos which used to amplify the selected segment Forward primer: ATTGCAAAGGCTCTCGAC Complementary reverse primer: TCCACATTCTCCATGAGC

Amplified segment cDNA: 407bp (Primer binding sites are highlighted in red and point mutation nucleotide is in green)

```
   1 ATTGCAAAGG CTCTCGACAA AGCCACTGCC GATGGACACA ACGTCGACGG TGGCTTGAAG GCCATTGGGC
  71 TTACAGATCA GAGAGAGACT ACTGTTGTTT GGAGCAAATC CACTGGCCTT CCTCTCCACA AGGCTATTGT
 141 CTGGATGGAT GCTCGTACCA GCTCCATCTG CAGGTACATA ACTTTCATTG ATCTCTTTCT TTTCTTCTTG
 211 TATGGTTTGT CTACTATCAT CTTCAGGATT GCTGAGGGAT TTGGTTTCTG ATGAAAGCTT TCTTTGGTGA
 281 TCCTTAATTA CAGGAGACTA GAGAAAGAAC TCTCGGGAGG AAGATCCCAT TTTGTGGAGT CTTGCGGCTT
 351 GCCAATAAGC ACATACTTCT CTGCCATGAA GCTGCTTTGG CTCATGGAGA ATGTGGA
```

location of point mutation: CCTTCCTCTCCACAAGGCTATTGTCTG—-G—-ATG (G to A point mutation which causes an stop codon)

Alignment of original amplified sequence and mutant segment (red box shows the location of point mutation)

```
Mutant seq    .A.....................................TANGTGCTTATTG    14
Original seq  TCCACATTCTCCATGAGCCAAAGCAGCTTCATGGCAGAGAAGTATGTGCTTATTG    55

Mutant seq    GC.AGCCGC.AGACTCNANAAAATGGGATCTTCCTCCCGAGAGTTCTTTCTCTAG    67
Original seq  GCAAGCCGCAAGACTCCACAAAATGGGATCTTCCTCCCGAGAGTTCTTTCTCTAG   110

Mutant seq    TCTCCTGTAATTAAGGATCACCAAAGAAAGCTTTCATCAGAAACCAAATCCCTCA   122
Original seq  TCTCCTGTAATTAAGGATCACCAAAGAAAGCTTTCATCAGAAACCAAATCCCTCA   165

Mutant seq    GCAATCCTGAAGATGATAGTAGACAAACCATACAAGAAGAAAAGAAAGAGATCAA   177
Original seq  GCAATCCTGAAGATGATAGTAGACAAACCATACAAGAAGAAAAGAAAGAGATCAA   220

Mutant seq    TGAAAGTTATGTACCTGCAGATGGAGCTGGTACGAGCATCCATTCAGACAATAGC   232
Original seq  TGAAAGTTATGTACCTGCAGATGGAGCTGGTACGAGCATCCATCCAGACAATAGC   275

Mutant seq    CTTGTGGAGAGGAAGGCCAGTGGATTTGCTCCAAACAACAGTAGTCTCTCTCTGA   287
Original seq  CTTGTGGAGAGGAAGGCCAGTGGATTTGCTCCAAACAACAGTAGTCTCTCTCTGA   330

Mutant seq    TCTGTAAGCCCAATGGCCTTCAAGCCACCGTCGACGTTGTGTCCATCGGCAGTGG   342
Original seq  TCTGTAAGCCCAATGGCCTTCAAGCCACCGTCGACGTTGTGTCCATCGGCAGTGG   385

Mutant seq    CTTTGTCGA.ANCCTTTTGCAAT   364
Original seq  CTTTGTCGAGAGCC.TTTGCAAT   407
```

7.3 Bacteria cultivation and plant inoculation

Pseudomonas syringae pv. maculicola strain ES4326 (*Psm*), *Psm* carrying the *avrRpm1* avirulence gene (*Psm AvrRpm1*), *Psm* carrying the *avrRpt2* avirulence gene (*Psm AvrRpt2*), and *Psm* carrying the *luxCDABE* operon from *Photorhabdus luminescens* under the control of a constitutive promoter (*Psm lux*) were grown in King's B medium supplemented with the appropriate antibiotics (50 µg ml^{-1} rifampicin for the virulent *Psm* strain; 50 µg ml^{-1} rifampicin and 15 µg ml^{-1} tetracycline for the avirulent strains *Psm AvrRpm1* and *Psm AvrRpt2*; and 50 µg ml^{-1} and 25 µg ml^{-1} kanamycin for the *Psm lux* strain) at 28 ℃ under overnight permanent shaking (240 rpm) (Fan et al., 2008; Zeier et al., 2004). Log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to different optical densities at 600 nm (OD_{600}) for leaf inoculation. The bacterial solution was infiltrated from the abaxial side into the leaves using 1 ml syringe without a needle. The time of infiltration varied among the experiments (10 AM for morning-inoculation and at 6 PM for evening-inoculation). For SAR and metabolite analysis three leaves per plant were infiltrated with either suspension of bacteria or with 10 mM $MgCl_2$ as control buffer. Depending on the experiment, inoculated (local) leaves and leaves distal from inoculation site (systemic) were harvested at different time-points (based on experiments), fresh weight was measured and leave materials were stored in liquid nitrogen and stored at -80 ℃ freezer for later analysis.

7.4 Assessment of basal resistance and SAR

7.4.1 Basal resistance

Due to measure basal resistance, three leaves per plant were infiltrated with *Psm lux* (OD_{600}=0.001) either at 10 AM (for morning measurement) or at 6 PM (for evening measurement). At least six to seven replicate plants were used for each treatment and for each genotype. Amount of bacteria in inoculated leaves were measured by luminescence of the *Psm lux* strain in a leaf disc with defined size (one disc per leaf, three discs per plant) and an exposure time of 10 s using a "Sirius FB12" luminometer (Berthold Detection Systems GmbH). Bacterial growth rates were expressed as relative light units per cm^2 of leaf area (RLU/cm^2) (Fan et al., 2008).

7.4.2 Systemic acquired resistance (SAR)

For SAR assessment, plants were infiltrated in three leaves defined as local leaves with a suspension of *Psm* (OD_{600}=0.005) or one of the two avirulent strains of *Psm AvrRpm1* (OD_{600}=0.005) or *Psm AvrRpt2* (OD_{600}=0.01). Then three upper leaves of pretreated leaves (defined as systemic leaves) were infiltrated with *Psm lux* (OD_{600}=0.001), 48 h after primary inoculation. Bacterial growth in systemic leaves was measured by luminometer as described above.

7.5 Sample extraction for metabolite analysis using gas chromatography/mass spectrometry GC/MS

7.5.1 Petiole exudate collection and analysis

Six leaves (per plant) of 5-week-old Arabidopsis (grown under 10 h light/14 h dark) were infiltrated either with *Psm* (OD_{600}=0.005) or 10 mM $MgCl_2$ as control. After infiltration half of the plants were transferred to constant dark percival. After 6 hours, leaves were cut at the base of their petioles and sterilized by successive dipping in 50 % ethanol for 10 sec and rinsed with 1 mM EDTA (pH=8.0). The petioles were submerged in eppendorfs filled with 2 ml of fresh EDTA (pH=8.0). Eppis were located in a bigger box on top of drenched papers and they were located back to their respective light conditions. Exudate was continuously collected in the period from 6 to 48 hpi. For the determination of amino acid (defense metabolite) content, 100 µl of collected exudate was analyzed as described in subsection 7.5.2.

7.5.2 Extraction of amino acids

To extract free amino acids, the EZ:faast free amino acid analysis kit for GC/MS (Phenomenex) was used. 50-100 mg of homogenized leaf material was treated with 500 µl of extraction buffer (25 % acetonitrile in 0.01 N HCl). Samples were vortexed for 15 min at room temperature and centrifuged at 14,000 rpm, for 4 min. An aliquot (100 µl) of the supernatant was extracted following the EZ:faast user's manual (Phenomenex). According to this method, free physiological amino acids were adsorbed to an SPE sorbent tip, eluted after a washing step, and treated with propyl chloroformate (Kugler et al., 2006). This method leads to highly stable derivatization of both the amine and carboxyl groups of amino acids. The dry residue was dissolved in 70 µl of dichloromethane and subjected to GC/MS analysis. The sample mixture (4 µl) was separated on a silica capillary column (ZB-AAA 10m x 0.25mm, Zebron, Phenomenex). The initiation injection was at 250 °C and then the metabolites were separated by a temperature program: 3 minutes at 70 °C followed by an increase to 240 °C (in 8 °C per minute steps) and a further increase in temperature to 320 °C (in 20 °C per two minutes steps); the final temperature of 320 °C was maintained for 2 minutes. To quantify amino acid levels, following peaks originating from selected ion chromatograms were integrated: Gly (m/z 116), Ala (m/z 130), Val (m/z 158), β-Ala (m/z 116), Leu (m/z 172), Ile (m/z 172), GABA (m/z 130), Ser (m/z 146), Thr (m/z 101), Pro (m/z 156), Pip (m/z 170), Aad (m/z 244), Asp (m/z 216), Glu (m/z 84), Asn (m/z 69), Gln (m/z 84), Cys (m/z 248), Orn (m/z 156), Lys (m/z 170), His (m/z 282), Phe (m/z 148), Tyr (m/z 107), and Trp (m/z 130). The peak area of a substance was related to the peak area of the internal standard norvalin (m/z 158; included in reagent 1) and experimentally determined correction factors for each substance were used in the calculation of the substance amount. In cases with no available correction factor, for example for unknown substances, a correction factor of 1 was used and the relative amount of substance per gram fresh weight calculated.

Calculation:

$$\frac{\text{Area}_{m/z}(\text{substance}) * 11.7 * \text{correction factor}}{\text{Area}_{m/z}(\text{internal standard}) * FW}$$

7.5.3 Vapor Phase Extraction (VPE)

A modified Vapor-phase-extraction method was used to extract and analyze plants metabolites (Schmelz et al., 2004). Briefly, 100 mg of homogenized plant material was mixed with 600 µl of extraction buffer (H2O: 1-propanol: HCl = 1: 2: 0.005). 30 µl of a standard solution (D4-salicylic acid, dihydro-jasmonic acid, indolepropionic acid [IPA]; 3.33 ng / µl each) and 1 ml of dichloromethane (GC ultra-grade, Roth) added to each sample, mixed strongly and centrifuged for 1 minute at 14,000 rpm. The lower organic phase was dried via water-free Na_2SO_4 and 2 µl of 2 M trimethylsilyldiazomethane (Sigma-Aldrich) in hexane was added to the samples to convert carboxylic acid groups into their corresponding methyl esters. the samples to convert carboxylic acid groups into their corresponding methyl esters. The reaction was stopped after 10 minutes by adding 2 µl of 2 M acetic acid in hexane to each sample and incubating the samples another 10 minutes. The metabolites were evaporated at 70 °C and collected in a volatile collector trap packed with a Porapak-Q absorbent (VCT- 1/4X3-POR-Q, Analytical Research Systems),

with a final evaporation step at 200 °C for 2 minutes. Samples were eluted by 1 ml of dichloromethane and reduced to 30 µl under the stream of gaseous nitrogen and subjected to GC/MS-analysis.

For extraction of glycosidic bound forms of SA (SAG), 30 µl of standard solution (D4-salicylic acid, dihydro-jasmonic acid, indolepropionic acid; 3.33 ng / µl each) and 1 ml of 0.1 N HCl were added to the upper hydrous phase and the pellet and samples were incubated at 100 °C for 30 minutes to convert the bounded SA to free SA. After cooling down, the aqueous phase was extracted three times by 2ml of dichloromethane and organic extracts were dried by water-free Na_2SO_4. The organic solvent was completely removed under gaseous nitrogen and dissolved in 300 µl of dichloromethane and 60 µl of methanol. Then it was methylated and subjected to VPE as described above. A volume of 4 µl of the sample mixture was separated on a gas chromatograph (GC 7890 A; Agilent Technologies) equipped with a fused silica capillary column (ZB-5MS 30m x 0.25mm, Zebron, Phenomenex) and combined with a 5975C (EI) mass spectrometric detector (Agilent Technologies). The initiation injection was at 250 °C and then the metabolites were separated by a temperature program: 50 °C/3min with 8 °C/min to 240 °C, with 20 °C/min to 320 °C/3 min, under constant flow of helium, 1.2 ml/min. For the quantitative determination of metabolites, peaks originating from selected ion chromatograms were integrated. The area of a substance peak was related to the peak area of the corresponding internal standard [SA (m/z 120) – D4-salicylic acid (m/z 124), camalexin (m/z 200) – indolepropionic acid (m/z 130)], and jasmonic acid (m/z 224) – dihydrojasmonic acid (m/z 156). Experimentally determined correction factors for each substance/standard pair were considered.

Calculation:

$$\text{Area}_{m/z}\ (\text{substance}) * 100 * \text{correction factor} / \text{Area}_{m/z}\ (\text{internal standard}) * \text{FW}$$

7.5.4 Determination of VOC emission including MeSA

To assess *Psm*-induced VOC emission including MeSA, five-week-old Arabidopsis plants were infiltrated with *Psm* OD_{600}=0.001 from the abaxial side using 1 mL syringe without a needle. Control treatments were performed by infiltrating a 10 mM $MgCl_2$ solution. Volatiles emitted by individual plants were collected in a push-pull apparatus essentially as described by (Attaran et al., 2008). The system consisted of six independent circular glass chambers (13 cm in diameter, 12 cm in height) that allowed for simultaneous collection. Plants were placed in chambers about 30 min after leaf infiltrations, and trapping filters consisting of glass tubes packed with a Super-Q absorbent (VCT- 1/4X3-POR-Q, Analytical Research Systems) were attached in a way so that the tip of each filter was a distance of 1 cm from each plant rosette. Charcoal- filtered and humidified air was pushed into each sampling chamber at a rate of 1.2 liters per min. The air flow containing plant volatiles was pulled through the trapping filter with a vacuum pump (ME2; Vacuubrand, Wertheim, Germany). After collecting volatiles for 48 h, trapping filters were removed, extracted, and analyzed. Fresh weight of six infiltrated leaves was also measured. Trapping filters were eluted with 1 ml dichloromethane after each collection, and D3-MeSA was added as an internal standard. The mixture was concentrated to a volume of 25 µl under a gentle stream of nitrogen, strictly avoiding evaporation to dryness, and was analyzed by GC/MS.

7.6 Sample extraction for metabolite analysis using Liquid chromatography/mass spectrometry LC-MS/MS

Water-soluble metabolites were extracted using a modified protocol, which was previously published in Arrivault et al., 2009. 30 mg of leave material mortared and 350 µl of chloroform/methanol (105 µl chloroform + 245 µl methanol) was added to samples while samples were on liquid nitrogen. After vortex, samples were stored at -20 ℃ for one hour. Then, 560 µl of ice-cold MilliQ water was added to samples. The aqueous fraction was collected after centrifugation (2 min, 13,500 rpm, 4 ℃) and the chloroform fraction was washed two additional times with 560 µl ice-cold water. The total aqueous phase was divided into two parts and an additional 280 µl of MilliQ water was added to each part. Samples were freeze-dried overnight (Alpha 2-4; Christ) and resuspended in 250 µl water and stored at -80 ℃. Metabolites such as G3P and DHAP were analyzed using LC-MS (Agilent 1200 HPLC and 6490 QQQ with jet stream source) and glycerol was analyzed using GC-MS (Gerstel Autosampler MPS2, Agilent 7890B GC, Agilent 7200 QTof) at metabolic profiling center (Plant biochemistry institute, HHU Düsseldorf).

7.7 Exogenous application of Pip and SA

7.7.1 Exogenous Pip application

To test Pip-induced priming, 5 week old plants were either watered with 10 ml water or with 10 ml of 1 mM (10 µmol) D,L-Pip (S47167; Sigma-Aldrich) one day prior to infiltration of three leaves (in each plant) with *Psm lux* (OD_{600}=0.001). The bacterial measurement was assessed 2.5 days later as explained in section 7.4. To determine Pip-induced priming effect on metabolites, one day after treatment of plants with water or Pip (explained above), three leaves of each plant were infiltrated with *Psm* (OD_{600}=0.005) or with 10 mM $MgCl_2$ as a control. Infiltrated (local) and systemic leaves were harvested at 10, 24, and 48 hpi.

7.7.2 Exogenous SA application

SA was infiltrated into three leaves (per plant) in a concentration of 0.5 mM SA (S5922; Sigma-Aldrich) with a pH of 7.0. Control infiltrations were performed with ddH2O. The same leaves of plants were infiltrated with *Psm lux* (OD_{600}=0.001) 4 hours after water/SA infiltration and bacterial measurement was assessed 2.5 days later as explained in section 7.4.

7.7.3 Co-application of SA and Pip for analysis of gene expression

5-week-old plants were either watered with 10 ml water or with 10 ml of 1 mM (10 µmol) D,L-Pip (S47167; Sigma-Aldrich) one day prior to leaf-infiltration. Three leaves of each plant were infiltrated either with water as control or 0.5 mM SA (S5922; Sigma-Aldrich). After application of water/Pip and also after infiltration with water/SA, plants were adjusted to different light conditions according to the respective experiment. Samples were collected 4 hours after water/SA infiltration for q-PCR analysis as explained in section 7.8.

7.8 Analysis of gene expression by quantitative real-time PCR analysis (qPCR)

7.8.1 RNA extraction

Total plant RNA was isolated from frozen tissue using the peqGOLD TriFast (PEQLAB) extraction reagent following the manufacturer´s instructions. 80-100 mg of plant material homogenized in a tissue homogenizer (TissueLyser II, Qiagen), treated with 1 ml PeqGold TriFast reagent and mixed thoroughly till thawed. Samples were treated with 200 µl chloroform, vortexed strongly to form a homogenous suspension and centrifuged at 12,000 x g for 5 minutes. The upper organic phase was pipetted into a reaction tube containing 500 µl of cold isopropanol. RNA was precipitated by incubating the samples at -20 °C for 15-20 minutes and centrifuged again 12,000 x g for 10 minutes. Pellet was washed twice with 75 % cold ethanol and dried in the SpeedVac (Concentrater plus, eppendorf). RNA was dissolved in 44 µl of RNase-free water at 65 °C. RNA purity was checked in 1 % agarose gel and RNA concentration determined by measuring absorption at 260 nm (BioPhotometer plus, Eppendorf). RNA stored at -80 °C or was directly used for cDNA synthesis.

7.8.2 cDNA synthesis

1 µg of RNA was treated with DNase and samples were incubated for 30 min at 37 °C to remove genomic DNA. This reaction was inactivated by adding 25 mM EDTA and incubating the samples at 70 °C for 15 min. RNA was transcribed to cDNA by adding 10 µM Oligo dTs, 10 mM dNTPs, 10 x reaction buffer, and 3 units reverse transcriptase. Samples were incubated at 42 °C for 60 min. To stop the reaction, samples were incubated at 70 °C for 15 min. the samples were diluted 1:10 with RNase-free water prior to use in quantitative real-time PCR.

7.8.3 Quantitative real-time PCR analysis

cDNA was amplified with 0.75 µM gene-specific primers and 5 µl of SYBR-Green reaction mix in a 10 µl reaction volume. The qPCR was performed in triplicates with a cycling program of a two minutes activation step at 95 ℃ and 35 to 40 cycles of 5 s at 95 ℃ to denature the DNA strands and 30 s at 60 ℃ to amplify the new DNA strands. POLYPYRIMIDINE TRACT-BINDING PROTEIN 1 (PTB) (At3g01150), which is nonresponsive to *Pseudomonas syringae* gene infection, was used as a reference gene (Czechowski, 2005). The qPCR data were analyzed using the Rotor-gene Q 2.0.2 software, with a threshold for normalized fluorescence set to 0.05. The Ct and amplification values were used to calculate the relative mRNA abundance; expression of the genes of interest was normalized to the expression of PTB and expressed as fold-change expression relative to the $MgCl_2$-treated wild-type control sample. list of primers used in qPCR is shown in supplemental table S.1.

7.9 Statistical analysis

In this thesis, we used two statistical techniques (either t-test or ANOVA) to analyze our data in different experiments. In the legend of each figure, we explained the technique that we used for that specific experiment. Most of the data shown in section 3 resulted from three to four independent experiments with the same results. At least three samples were analyzed for metabolic experiment and data represent the mean ±SD (SD=standard deviation). Statistical significance was assessed by ANOVA with type II sum of squares using the R statistical package (https://www.r-project.org/), and subsequent posthoc Tukey's HSD test (Bernsdorff et al., 2016; Brady et al., 2015). Bacterial numbers values represent mean value ±SD of colony forming units (cfu) per square centimeter from at least 6 replicate samples, each consisting of three leaf disks. In several experiments, we also used two-tailed Student's t-test in which we showed the differences of compared groups by showing asterisks above each group. Asterisks denote statistically significant differences between *Psm* and $MgCl_2$ samples (*P < 0.05, **P < 0.01, ***P < 0.001, ns. not significant).

7.10 Accession numbers

Sequence data from genes described in this study can be found in the Arabidopsis Genome Initiative or GenBank/EMPL databases under the following accession numbers: ALD1 (At2g13810), FMO1 (At1g19250), NPR1 (At1g64280), PTB (At3g01150), PR1 (At2g14610), AZI1 (AT4G12470), GLY1 (AT2G40690), GLI1 (At1g80460), BSMT1 (At3g11480), DIR1 (At5g48485).

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Supplementary data

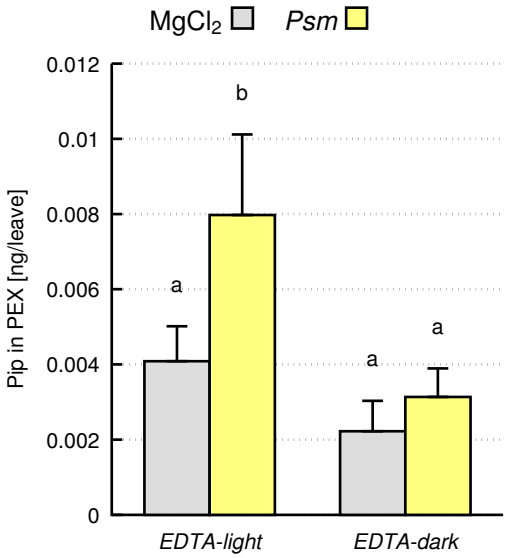

Figure S.1: Comparison analysis of Pip levels in petiole exudate (PEX) of *Psm*-infiltrated leaves of Arabidopsis Col-0 under normal light (10 h light/ 14 h dark cycle) and constant dark conditions (As depicted in Figure 4.2). Six leaves of 5-week-old plants (grown under 10 h light/ 14 h dark cycle) were infiltrated with either 10 mM $MgCl_2$ or with *Psm* OD_{600}=0.005 at 9 AM and plants were either kept grown light condition or translocated to constant darkness (Figure 4.2). PEX of inoculated leaves was collected between 6 to 48 h after inoculations using EDTA. Data represent means ± SD of at least three biological replicates from different plants, each replicate consisting of twelve leaves from two plants. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Usage
ALD1	GTGCAAGATCCTACCTTCCCGGC	CGGTCCTTGGGGTCATAGCCAGA	qRT-PCR
FMO1	TCTTCTGCGTGCCGTAGTTTC	CGCCATTTGACAAGAAGCATAG	qRT-PCR
PR1	GTGCTCTTGTTCTTCCCTCG	GCCTGGTTGTGAACCCTTAG	qRT-PCR
PTB1	GATCTGAATGTTAAGGCTTTTAGCG	GGCTTAGATCAGGAAGTGTATAGTCTCTG	qRT-PCR
GLY1	AATACGAACCGTGACCAG	TACCAAACGAACCTCCAC	PCR
GLI1	ATTGCAAAGGCTCTCGAC	TCCACATTCTCCATGAGC	PCR

Table S.1: Primers used in this study for qRT-PCR and PCR

<0.5 0.5-0.8 0.8-1.6 1.6-3.0 3.0-5.0 5.0-20.0 >20

	light					dark				
Amino acids	**Mean mock**	**±SD**	**Mean *Psm***	**±SD**	**Ratio P/M**	**Mean mock**	**±SD**	**Mean *Psm***	**±SD**	**Ratio P/M**
Alanin	108.58	12.24	176.13	51.92	1.6	77.40	7.89	6.20	11.09	0.1
Glycin	21.37	2.79	54.22	22.70	2.5	26.18	6.11	30.25	7.94	1.2
valin	8.71	0.95	45.02	16.89	5.2	50.02	4.60	79.93	21.17	1.6
leucin	2.86	0.28	30.83	10.83	10.8	32.02	1.42	40.18	8.94	1.3
isoleucin	2.28	0.40	17.44	7.72	7.6	23.05	4.00	40.67	13.41	1.8
GABA	1.59	0.50	7.47	4.03	4.7	3.69	1.06	3.96	0.92	1.1
Threonin	54.05	6.70	115.34	49.57	2.1	60.45	2.64	114.56	32.26	1.9
Serin	89.27	8.92	193.57	83.31	2.2	63.90	11.01	66.49	21.91	1.0
Prolin	15.51	3.32	22.48	5.34	1.4	43.16	5.70	24.70	5.42	0.6
Pip	1.35	0.64	41.11	11.04	30.5	0.27	0.06	10.53	4.95	39.3
Asparagin	37.42	5.33	56.22	26.28	1.5	170.26	42.75	413.56	176.86	2.4
Aspartic acid	552.75	72.55	388.22	127.06	0.7	245.34	53.67	404.77	150.98	1.6
Phenylalanin	3.14	0.45	25.44	9.85	8.1	22.88	1.90	59.73	18.97	2.6
Glutamic acid	274.38	46.47	398.59	153.26	1.5	241.33	24.63	180.09	64.05	0.7
Alpha amino adipic acid	0.20	0.04	1.47	0.47	7.2	2.22	0.31	2.94	0.99	1.3
Glutamin	262.00	42.53	291.31	104.42	1.1	204.93	66.05	391.54	138.90	1.9
ornithin	0.70	0.09	0.49	0.18	0.7	12.34	3.78	3.34	2.26	0.3
Lysin	2.04	0.47	4.93	3.19	2.4	22.40	4.93	21.41	6.05	1.0
Tyrosin	0.68	0.15	10.36	3.30	15.3	15.34	0.62	12.49	2.11	0.8
Tryptophan	0.15	0.03	1.25	1.25	8.3	2.20	1.36	4.33	2.14	2.0

Table S.2: Changes in the levels of Free Amino Acids at 24 hpi in local leaves of Col-0 plants upon *Psm*-infiltration in light and dark conditions. Mean values for leaf samples are given in µg/g fresh weight (FW) ± standard deviation (SD) from at least three replicate samples. Mock treatments were performed by infiltration of leaves with a 10 mM $MgCl_2$ solution.

<0.5 0.5-0.8 0.8-1.6 1.6-3.0 3.0-5.0 5.0-20.0 >20

	light					dark				
Amino acids	**Mean mock**	**±SD**	**Mean *Psm***	**±SD**	**Ratio P/M**	**Mean mock**	**±SD**	**Mean *Psm***	**±SD**	**Ratio P/M**
Alanin	74.76	18.48	112.32	10.29	1.5	55.39	6.22	42.33	8.90	0.8
Glycin	47.28	5.55	40.08	9.69	0.8	16.30	10.07	56.04	16.30	3.4
valin	6.63	0.88	13.71	2.44	2.1	87.86	10.49	88.84	8.90	1.0
leucin	1.40	0.37	4.95	0.94	3.5	56.84	5.54	57.30	9.56	1.0
isoleucin	2.07	0.84	4.33	0.60	2.1	43.28	12.01	41.12	3.29	1.0
GABA	1.71	0.85	2.11	1.09	1.2	3.49	0.83	4.83	3.10	1.4
Threonin	38.48	15.99	56.20	16.48	1.5	63.07	2.55	48.23	10.60	0.8
Serin	40.52	22.10	33.71	15.24	0.8	18.91	10.01	5.06	1.49	0.3
Prolin	38.28	5.62	14.86	1.56	0.4	29.14	6.16	22.49	3.49	0.8
Pip	0.58	0.48	18.56	3.15	31.9	0.55	0.50	0.24	0.03	0.4
Asparagin	67.22	1.92	36.51	6.34	0.5	334.89	36.97	379.82	52.13	1.1
Aspartic acid	444.60	120.77	462.13	11.06	1.0	107.31	30.76	47.27	3.73	0.4
Phenylalanin	6.35	0.90	8.54	1.45	1.3	49.68	3.51	46.37	4.93	0.9
Glutamic acid	293.94	10.90	301.99	28.56	1.0	271.07	21.82	268.28	29.84	1.0
Alpha amino adipic acid	0.13	0.05	0.85	0.21	6.4	4.70	0.42	3.74	0.70	0.8
Glutamin	406.75	104.22	168.18	52.10	0.4	63.54	14.70	112.03	39.28	1.8
ornithin	0.81	0.19	0.37	0.12	0.5	40.76	5.22	63.16	15.00	1.5
Lysin	1.59	0.21	1.58	0.16	1.0	39.17	3.09	45.13	9.83	1.2
Tyrosin	0.40	0.08	1.75	0.28	4.4	29.25	1.07	26.25	3.67	0.9
Tryptophan	0.14	0.14	0.25	0.16	1.8	3.56	0.80	1.70	1.08	0.5

Table S.3: Changes in the levels of Free Amino Acids at 48 hpi in systemic leaves of Col-0 plants upon *Psm*-infiltration in light and dark conditions. Mean values for leaf samples are given in µg/g fresh weight (FW) ± standard deviation (SD) from at least three replicate samples. Mock treatments were performed by infiltration of leaves with a 10 mM $MgCl_2$ solution.

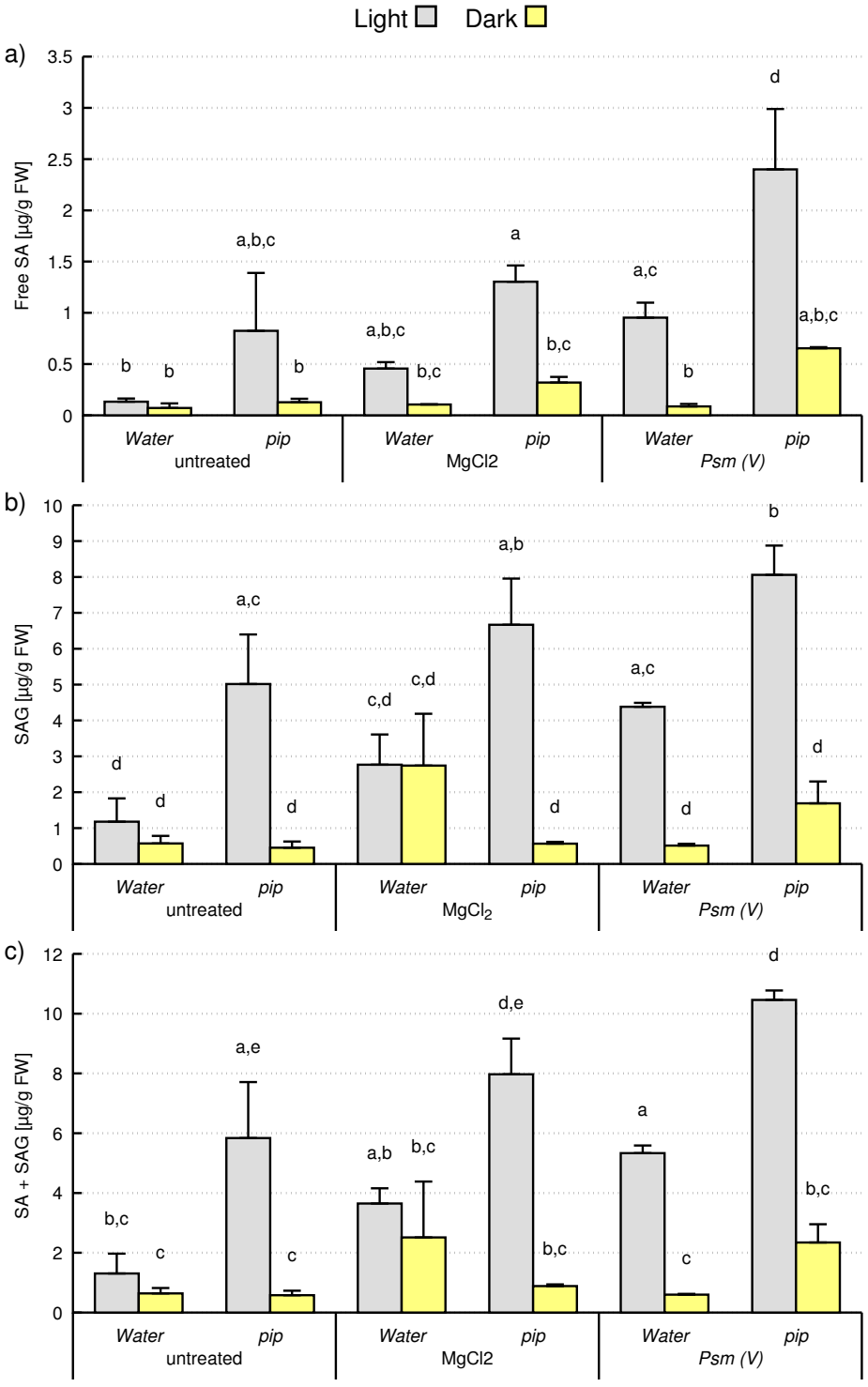

Figure S.2: Priming effect of exogenous Pip on defense metabolites SA and camalexin under light and constant darkness. See Figure S.3 for details.

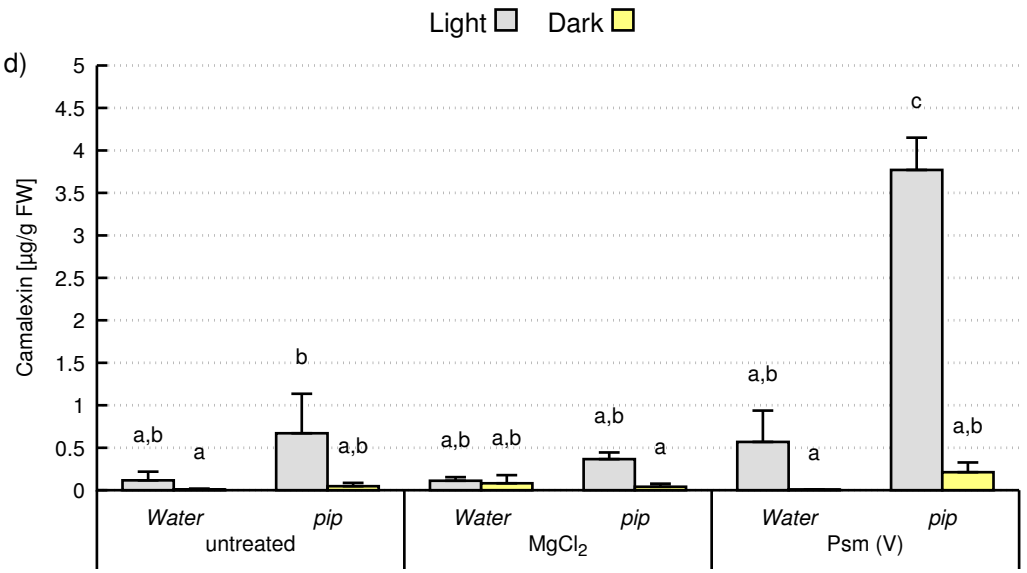

Figure S.3: Priming effect of exogenous Pip on defense metabolites SA and camalexin under light and constant darkness. 5-week-old Col-0 plants (grown under 10 h light/ 14 h dark cycle) were supplied with 10 ml of 1 mM D,L Pip (= dose of 10 µmol) or with 10 ml of H_2O (control treatment) via the root system one day before pathogen inoculation. Three leaves of water/Pip treated plants were inoculated with either 10 mM $MgCl_2$ or *Psm* OD_{600}=0.005 and plants were either kept in grown light condition (control group) or translocated to constant darkness (as shown in Figure 4.2). [Note: To show the absorption of Pip and its priming effect on basal level of SA and camalexin, we included several untreated samples which were not infiltrated with $MgCl_2$ or *Psm*]. Infiltrated leaves were collected at 10 hpi for quantification of free SA (a), glucosidic SA (b), total SA (c), and camalexin (d). Data represent means ± SD of at least three biological replicates from different plants, each replicate consisting of six leaves from two plants. Different letters above the bars denote statistically significant differences ($P < 0.05$, ANOVA and posthoc Tukey's HSD test.

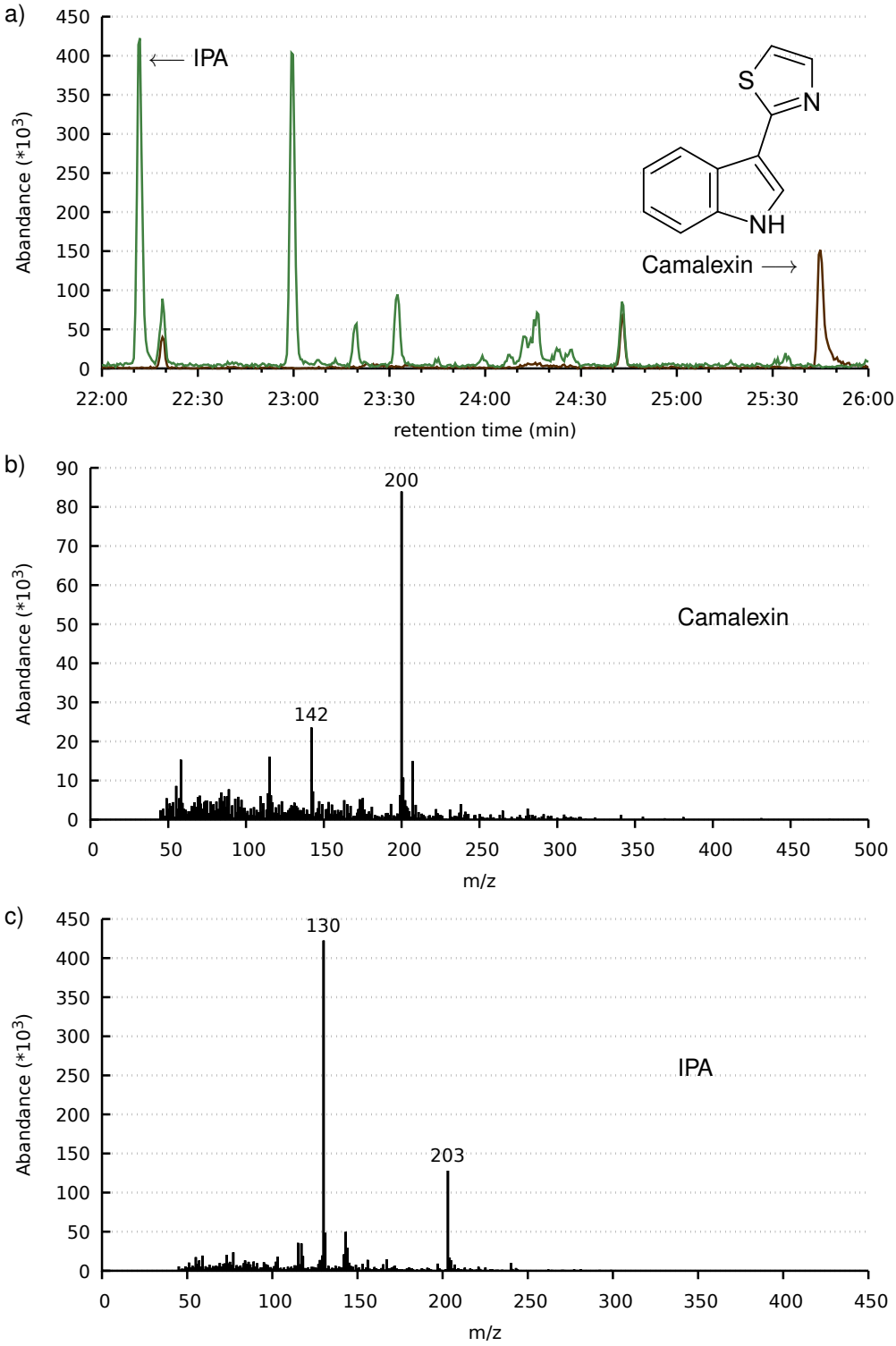

Figure S.4: a: Chromatograms, mass-spectra of camalexin and its internal standard Iso propionic acid (IPA), and structure of camalexin b: Single-ion-chromatograms for camalexin [m/z 200], c: IPA [m/z 130]. Mass-spectra were recorded in the in the electron ionization mode.

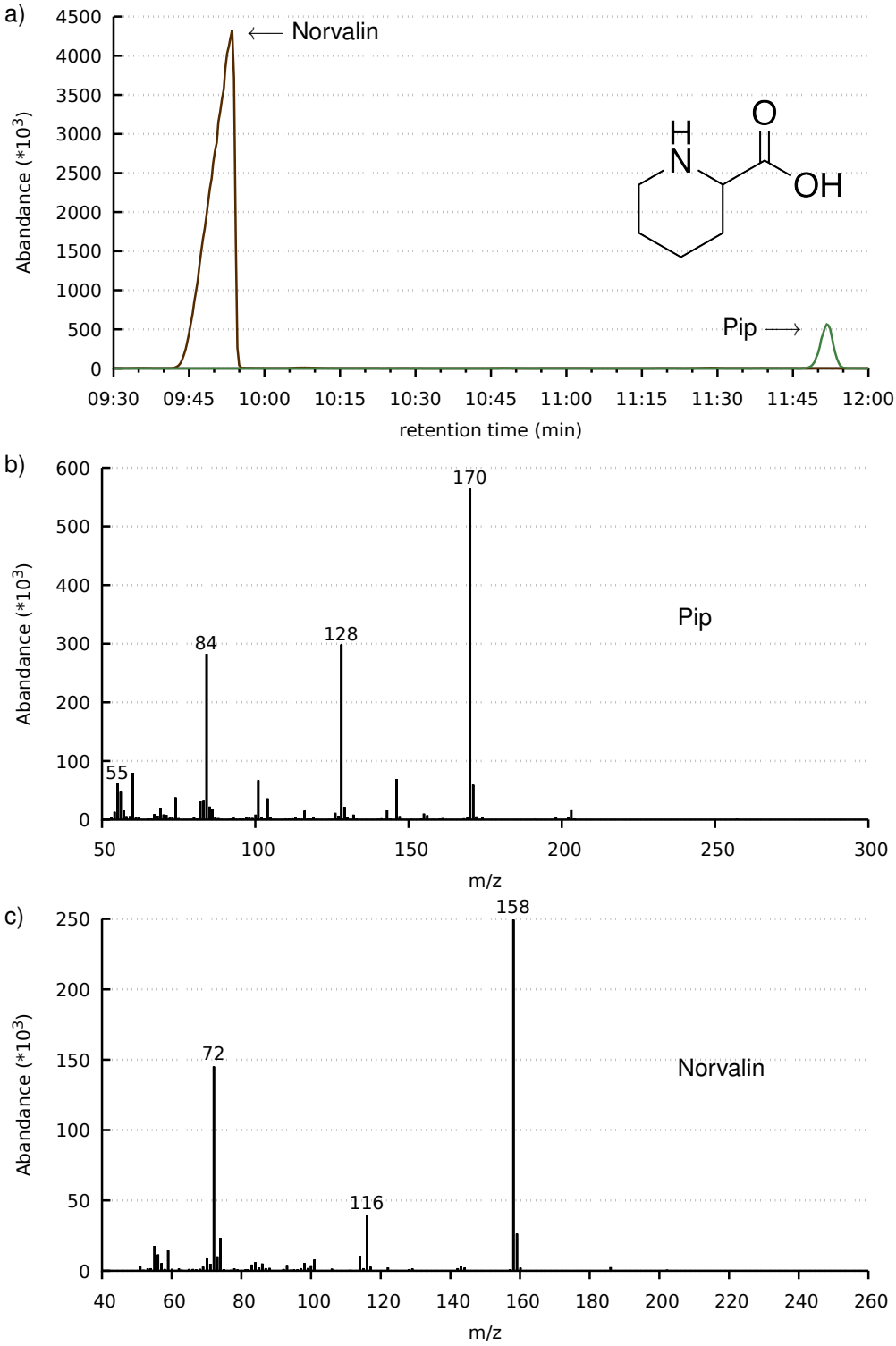

Figure S.5: a: Chromatograms, mass-spectra of Pip and its internal standard norvalin, and structure of Pip b: Single-ion-chromatograms for Pip [m/z 170], c: for norvalin [m/z 158]. Mass-spectra were recorded in the in the electron ionization mode.

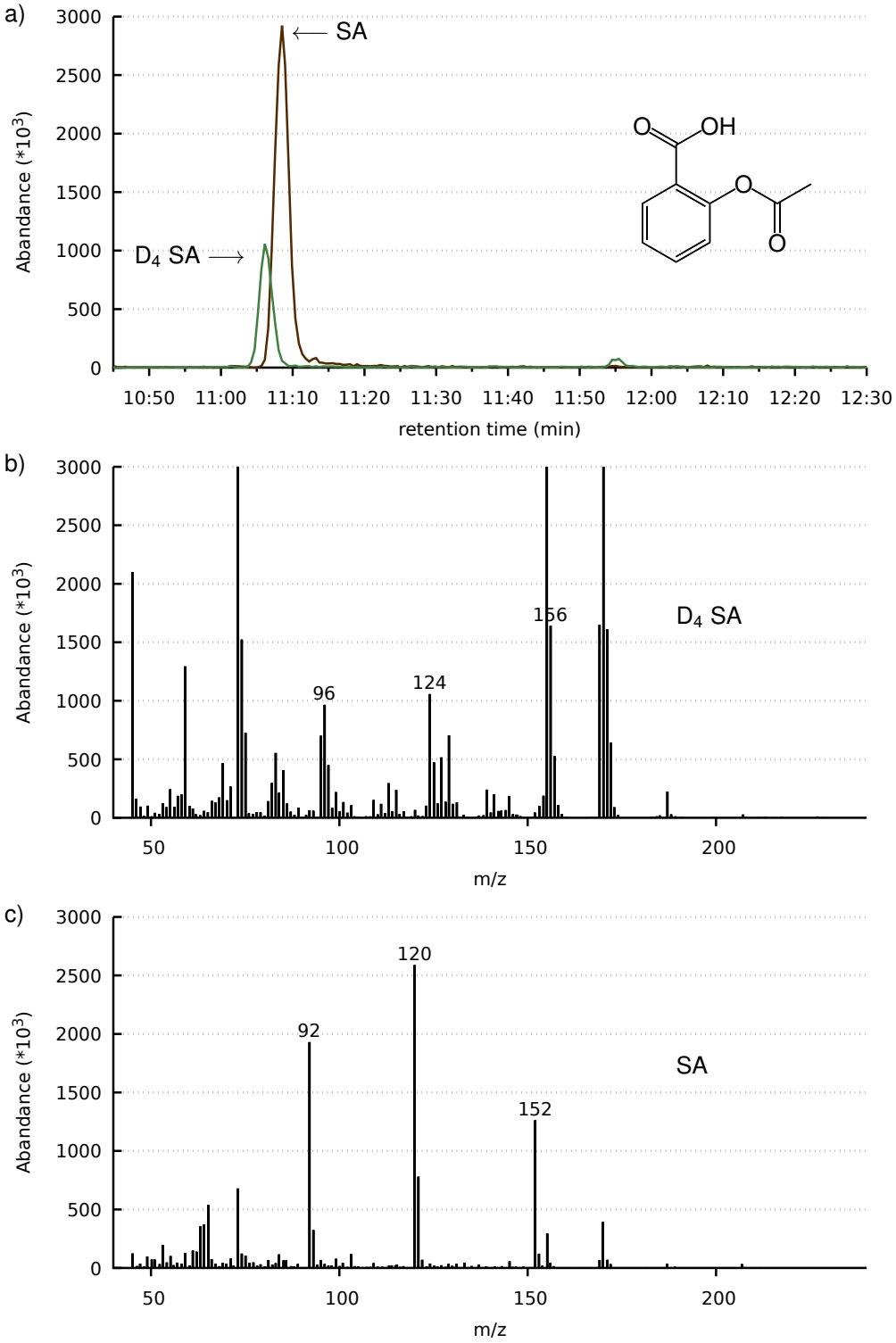

Figure S.6: a: Chromatograms, mass-spectra of SA and its internal standard D_4 SA, and structure of SA b: Single-ion-chromatograms for SA [m/z 120], c: for D_4 SA [m/z 124]. Mass-spectra were recorded in the in the electron ionization mode.

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Thanks for all your encouragement!

Abbreviations

Abberivation	Description
℃	Celcius
ABA	Abscisic acid
ACP	Acyl carrier protein
ACT	Acyltransferase
AGD	ABERRANT GROWTH AND CELL DEATH
ALD	AGD2-LIKE DEFENSE RESPONSE PROTEIN
AM	Ante Meridiem
Avr	avirulence
AzA	Azelaic acid
AZI	AZELAIC ACID INDUCED
BABA	β-aminobutyric acid
BAK	Brassinosteroid insensitive1-associated kinase
bp	Base pair
BSMT	Salicylic acid/benzoic acid carboxyl methyltransferase
BTH	Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
CC	coiled-coil
CCA1	CIRCADIAN CLOCK ASSOCIATED
cDNA	complementary DNA
cfu	Colony forming unit
CoA	coenzym A
COI1	Coronatine insensitive
Col	Columbia
DA	Dehydroabietinal
DAMPs	damage-associated molecular patterns
DHAP	dihydroxyacetone phosphate
DIR	DEFECTIVE IN INDUCED RESISTANCE
DP	dehydropipecolic acid
EDS	ENHANCED DISEASE SUSCEPTIBILITY
EDTA	Ethylen-diamine tetra-acetic acid
EF-Tu	Elongation factor Tu
ER	endoplasmic reticulum
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FMO	FLAVIN-DEPENDENT MONOOXYGENASE
FW	fresh weight
G3P	glycerol-3-phosphate
GABA	γ-amino butyric acid
h	hour(s)
HMW	High molecular weight
hpi	hours post inoculation
HR	hypersensitive response
ICS	ISOCHORISMATE SYNTHASE

Abberivation	Description
INA	2,6-dichloro-isonicotinic acid
IR	Induced resistance
ISR	Induced systemic resistance
JA	jasmonic acid
JA-Ile	jasmonoyl-isoleucine
LMW	Low molecular weight
LPS	lipopolysaccharides
LRR	leucine-rich repeat
LTP	lipid transfer protein
MAMP	microorganism-associated molecular patterns
MAP	mitogen-activated protein
MES	MeSA esterase
MeSA	methyl salicylate
MGD1	MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE
ml	millilitre
mM	Millimolar
NahG	salicylate hydroxylase
NB-LRR	nucleotide-binding – leucine rich repeats
NDR1	NON RACE SPECIFIC DISEASE RESISTANCE
NHP	N-hydroxypipecolic acid
NO	Nitric oxide
NPR	NON-EXPRESSOR OF PR GENES
OD	Optical density
ONA	9-oxononanoic acid
PAD	PHYTOALEXIN DEFICIENT
PAL	phenylalanine ammonia-lyase
PAMP	pathogen-associated molecular patterns
PBS3	AvrPphB SUSEPTIBLE 3
PCD	programmed-cell-death
PEX	Petiole exudate
PFD	Photon flux density
Pip	Pipecolic acid
PM	Post meridiem
PR	pathogenesis-related
PRR	pattern recognition receptors
Psm	Pseudomonas syringae pv. maculicola ES4326
PTI	PAMP-triggered immunity
qPCR	quantitative RT-PCR
ROS	Reactive oxygen species
s	second
SA	salicylic acid
SABP	SA BINDING PROTEIN
SAG	SA 2-O-β-glucoside
SAR	systemic acquired resistance
SD	standard deviation
SGE	Salicyloyl glucose ester
SID	Salicylic acid induction deficient
TCV	Turnip Crinkle Virus
T-DNA	transposable DNA
TIR	toll, interleukin 1R and resistance
TMV	tobacco mosaic virus
TTSS	Type-III secretion system
VOC	Volatile organic compound
Ws	Wassilewskija

Affidavit

I declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the *Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf*.

I never submitted this dissertation or a similar dissertation to another faculty or university and I never tried to obtain a Ph.D. degree or doctor degree before.

Ziba Ajami-Rashidi