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der Christian-Albrechts-Universität zu Kiel

**Investigation on the role of plant defensin proteins in  
regulating plant-*Verticillium longisporum* interactions  
in *Arabidopsis thaliana***

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vorgelegt von  
M.Sc. Shailja Singh  
aus Lucknow, India

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Dekan/in: Professor Dr. Dr. Christian Henning

1. Berichterstatter: Prof. Dr. Daguang Cai

2. Berichterstatter: Prof. Dr. Professor Joseph-Alexander Verreet

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## List of Abbreviations

<b>ABA</b>	Abscisic acid
<b>AOC3</b>	ALLENE OXIDE CYCLASE 3
<b>ATP</b>	Adenosine triphosphate
<b>BLAST</b>	Basic local alignment search tool
<b>bp</b>	Base pair
<b>CaMV</b>	Cauliflower mosaic virus
<b>CDB</b>	Czapek Dox Broth
<b>cDNA</b>	Complementary DNA
<b>CF</b>	Compatibility factor
<b>Ct</b>	Threshold cycle
<b>DAG</b>	Days after germination
<b>DBP</b>	DNA binding protein
<b>DNA</b>	Deoxyribonucleic acid
<b>dpi</b>	Days post infection
<b>ds</b>	Double strand
<b>EDS1</b>	ENHANCED DISEASE SUSCEPTIBILITY 1
<b>ET</b>	Ethylene
<b>ETI</b>	Effector-triggered immunity
<b>ETS</b>	Effector-triggered susceptibility
<b>FAO</b>	Food and Agriculture Organization of the United Nations
<b>flg22</b>	Flagellin22
<b>FW</b>	Fresh weight
<b>GA</b>	Gibberellin
<b>GSL</b>	Glucosinolates
<b>GUS</b>	$\beta$ -glucuronidase
<b>HR</b>	Hypersensitive response
<b>JA</b>	Jasmonic acid
<b>KO</b>	Knockout
<b>KD</b>	Knockdown
<b>LB</b>	Luria-Bertani medium
<b>LR</b>	Lateral root
<b>LRR</b>	Leucine-rich repeat
<b>mRNA</b>	Messenger RNA
<b>MS</b>	Murashige and Skoog medium
<b>MYB</b>	MYELOBLASTOSIS
<b>NASC</b>	Nottingham <i>Arabidopsis</i> Stock Centre

<b>NBS</b>	Nucleotide-binding-site
<b>NBS-LRR</b>	Nucleotide binding domain-leucine-rich repeat
<b>nt</b>	Nucleotide
<b>OD600</b>	Optical density at 600 nm wavelength
<b>OE</b>	Overexpression
<b>ORF</b>	Open reading frame
<b>PDA</b>	Potato dextrose agar medium
<b>PDF</b>	Plant defensin proteins
<b><i>PR</i> genes</b>	<i>PATHOGENESIS RELATED</i> genes
<b><i>PDF1.2</i></b>	<i>PLANT DEFENSIN 1.2</i>
<b>PRR</b>	Pattern recognition receptor
<b>PTI</b>	PAMP-triggered immunity
<b>qPCR</b>	Quantitative PCR
<b>R genes</b>	Resistance genes
<b>RNA</b>	Ribonucleic acid
<b>rpm</b>	Rounds per minute
<b>RT</b>	Room temperature
<b>SA</b>	Salicylic acid
<b>TAIR</b>	The Arabidopsis Information Resource
<b>T-DNA</b>	Transfer DNA
<b>TF</b>	Transcription factor
<b>TIR</b>	Toll-Interleukin receptor domain
<b><i>VI43</i></b>	<i>Verticillium longisporum</i> strain 43R
<b>WRKY70</b>	WRKY DNA-BINDING PROTEIN 70

## Chapter I: General Introduction

### Introduction

Plants are a rich source of nutrients for many microorganisms. Due to the lack of an adaptive immune system, plants have developed effective strategies to defend themselves against pathogen attacks, which are either dependent on constitutive barriers or on the activation of multi-component defense responses. Constitutive defenses include preformed mechanisms such as cell walls, waxy epidermal cuticles as well as chemical substances with antimicrobial effects (phytoanticipins) (Zhang et al. 2018). The primary mechanical and structural barriers prevent the physical penetration of pathogens. For chemical defense, plants produce non-specific secondary antimicrobial chemicals to counteract pathogen invasion. Phytoalexins, acting as antimicrobial toxins, can be synthesized de novo very rapidly in response to pathogen infection (Ahuja et al. 2012). The synthesis of camalexin, as a major phytoalexin in *Arabidopsis*, can be regulated by two major enzymes: *Arabidopsis* cytochrome P450 monooxygenase 71A13 (CYP71A13), and *Arabidopsis* cytochrome P450 monooxygenase 71B15/ phytoalexin-deficient 3 (PAD3). The increased susceptibility of the mutant *pad3* toward *Alternaria brassicicola* was caused by camalexin biosynthesis impairment (Thomma et al. 1999). Phytoanticipins are constitutively produced metabolites against pathogens in plants (Pedras et al. 2015). Glucosinolates, as well-characterized phytoanticipins, play a pivotal role in defense against herbivores, bacteria, and fungi (Field et al. 2006; Burow and Halkier 2017). Glucosinolates are produced in the trichomes of *Arabidopsis* and their biosynthesis is regulated by the basic helix-loop-helix (bHLH) transcription factors (TFs): MYC2, MYC3, and MYC4 (Schweizer et al. 2013). Plant defensins are small cysteine-rich peptides that are active against bacteria and fungi (Vriens et al. 2014; Finkina and Ovchinnikova 2018; Lacerda et al. 2014). The importance of defensins in killing pathogens are well proven in many plant species, including *Arabidopsis* (Vriens et al. 2016), *Solanum lycopersicum* (tomato) (Cui et al. 2018), and *Nicotiana tabacum* (Lee et al. 2018). In addition, plants can detect pathogens and activate defense mechanisms including the production of toxic chemicals, enzymes, and programmed cell death. If pathogens overcome these defense mechanisms, they often cause devastating diseases in plants (Zhang et al. 2018).



Different types of plant microbe-interactions have been described. In necrotrophic interactions, pathogens kill infected plant cells, e.g. *Botrytis cinerea* (Colmenares et al. 2002) while in biotrophic interactions resources from living host cells are exploited by pathogens, e.g. *Cladosporium fulvum* (Joosten and De Wit 1999) and in symbiotic interactions, both partners benefit from each other e.g. *Laccaria bicolor* (Lammers et al. 2004). On the other hand, many pathogens first colonize their host plant as biotrophs and then switch to a necrotrophic phase by killing the host plant in the later stages of infection e.g. *Verticillium* species (Klosterman et al. 2009). Such pathogens are called hemibiotrophs. The co-evolution of plants and their associated microbes have given rise to a diverse array of exchanged signals and responses (Bent 1996; HammondKosack and Jones 1997).

## **1 Induced plant defense responses**

Plants can activate defense responses after detecting pathogens or damage-associated molecular patterns (PAMPs/DAMPs). A well-studied PAMP is a flagellin (Zipfel and Felix 2005) which is recognized by the membrane-bound pathogen recognition receptor (PRR) FLS2 (FLAGELLIN INSENSITIVE2, Gómez- Gómez and Boller 2002). Flg22 is a 22-amino-acid peptide from a conserved flagellin domain, which is sufficient to induce many cellular responses (Felix et al. 1999), trigger an immune response (Zipfel 2008) and initiate diverse downstream signaling events that ultimately result in the activation of a basal resistance. This is called PAMP-triggered immunity (PTI) (Jones and Dangl 2006). The downstream signaling events include the activation of MAPK (mitogen-activated protein kinase) cascades via MAPK3 or MAPK6; altered RNA metabolism via GRP7 (glycine-rich RNA-binding protein) (Fu et al. 2007) and the induction of vesicle trafficking. The plant PRRs identified so far are mostly located in the plasma membrane (Zipfel 2008) and are represented mostly by receptor protein kinases (RPKs) (Tena et al. 2011) or receptor-like kinases (RLKs) (Shiu and Bleecker 2001; Greeff et al. 2012). Hence, the first line of plant defense comprises PTI that can limit further colonization of pathogens in plants (Jones and Dangl 2006). During evolution, pathogens acquired effector molecules (Gohre and Robatzek 2008) that are transported into the host cell to suppress PTI and promote virulence of the pathogen and which results in so-called effector-triggered susceptibility (ETS) (Jones and Dangl 2006). Effector molecules, in contrast to PAMPs are species-,

race- or strain-specific and contribute to pathogen virulence (Nurnberger et al. 2011). These effector molecules are secreted into the host cell via a pathogen-derived secretion system, among which the so-called type-III-protein-secretion-system (T3SS) is mostly used. The T3SS is generally used by gram-negative bacterial pathogens to deliver effectors across the plant cell wall and plasma membrane (Alfano and Collmer 2004); but also by oomycete effectors (Fabro et al. 2011; Bozkurt et al. 2012). T3SS are substantially conserved among different bacteria, but the effector molecules they deliver are unique for each bacterial species (Galan and Collmer 1999). Not only the delivery of the right effector molecules is an important function of the T3SS, but also its ability to recognize and secrete substrates in a defined order is of exceptional importance. Protein secretion must be precisely coordinated to successfully deliver effector proteins through the eukaryotic host cell membrane and successively circumvent plant defense (Lara-Tejero; Kato et al. 2011). To combat ETS plant resistance (*R*) genes come into action (Belkhadir et al. 2004; Martin et al. 2003; Van der Biezen and Jones 1998). *R* proteins recognize specific effectors, resulting in a secondary immune response called effector-triggered immunity (ETI). Most *R* genes encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Collier and Moffett 2009). These proteins are named NB-LRR proteins because of the presence of a conserved central nucleotide-binding (NB) domain and a variable C-terminal leucine-rich repeat (LRR) domain. Considering their N-termini, two major groups can be distinguished within the NB-LRR proteins (Meyers et al. 1999). Members of the first class possess an N-terminal TIR (Toll and Interleukin-1 Receptor homology) domain, whereas the members of the second class possess few variable N-terminal domains, some of which are predicted to form a coiled-coil (CC) structure. Studies indicate that the C-terminal LRR domain plays a role in the specificity of pathogen recognition (Shen et al. 2003; Ellis et al. 2007). However, also the N-terminus of the NB-LRR proteins are involved in pathogen recognition, leading to a two-step recognition model. This model involves interactions of an effector with both cellular co-factors and the LRR domain of the associated *R* protein, which in turn activates the molecular switch leading to disease resistance (Collier and Moffett 2009). ETI culminates the programmed cell death around and inside the infection site. This is called the hypersensitive response (HR) that effectively arrests pathogen growth (Mur et al. 2008). Development of an HR triggers the downstream mechanisms, e.g. autophagy (Hofius et al. 2009), reactive oxygen species (ROS) production (Torres et

al. 2006), MAPK cascade activation (Asai et al. 2002) and hormone signaling (Jones and Dangl 2006). The plant immunity can be explained by a so-called “zigzag” model (Jones and Dangl 2006; Chisholm et al. 2006; Nishimura and Dangl 2010).

### **1.1 Phytohormones - general role in plants and special role in plant defense**

Infection of plants with diverse pathogens initiates changes in the levels of plant hormones. Plant hormones are small endogenous signaling molecules, including gibberellin (GA), auxin indole-3-acetic acid (IAA), cytokinin (CK), brassinosteroids (BRs), abscisic acid (ABA), ethylene (ET), jasmonic acid (JA), salicylic acid (SA), and strigolactone (SL).

Based on the interactions that have been studied, a general rule of hormonal action has been proposed, in which resistance response to biotrophs require salicylic acid (SA), whereas responses to necrotrophs require jasmonic acid (JA) and ethylene (ET) (Feys and Parker 2000). Roles of SA, JA and ET have also been proposed in regulation to susceptible responses (Bent et al. 1992; Greenberg et al. 2000; Lund et al. 1998; Pilloff et al. 2002).

The investigation of Arabidopsis mutants with defects in salicylic acid (SA) biosynthesis and signaling pathways for altered pathogen susceptibility have demonstrated that SA is a crucial defense signal molecule against biotrophs (Gaffney et al. 1993; Wildermuth et al. 2001; Ádám et al. 2018). SA is required for the activation of both PTI and ETI. Parallel approaches have demonstrated that phytohormones, ethylene, and jasmonate, play a major role in defense responses against necrotrophs. The Arabidopsis jasmonate- or ethylene-insensitive mutants display enhanced susceptibility to the necrotrophic *Botrytis cinerea*. Those mutants have no effect on resistance to biotrophs (Thomma et al. 1998; Thomma et al. 1999). The infection of Arabidopsis plants with biotrophic *P. syringae*, triggers SA-mediated defense response, resulting in significantly compromised resistance against necrotrophs by suppression of the jasmonate/ethylene (JA/ET) signaling pathway (Spoel et al. 2007). This experiment demonstrated the existence of crosstalk between SA and ET/JA signaling pathways. The classical phytohormones, such as abscisic acid (ABA), auxin, brassinosteroid (BR) and cytokinin (CK) were adopted to fine-tune the plant defense response (Robert-Seilantantz et al. 2007; 2011; Yu et al. 2018). The roles and

models of those compounds have been comprehensively discussed in many studies and are beyond the scope of this thesis. Here, I briefly compile recent progress in the biosynthesis regulation and signaling of the JA/ET pathway.

## **1.2 JA/ET-mediated defense against pathogens and signaling pathways**

Both the jasmonic acid (JA) and ethylene (ET) signaling pathways are required for the activation of plant defense against necrotrophic pathogens. In either JA- or ET-insensitive mutants, the induction of pathogen defense genes (i.e., *PDF1.2*) is drastically reduced (Penninckx et al. 1998). JA signaling has also been shown to mediate defense against some biotrophic and hemibiotrophic pathogens that obtain nutrients primarily from a living plant cells and the examples include rice resistance to *Meloidogyne graminicola* and *Xanthomonas oryzae* (Nahar et al. 2011; De Vleeschauwer et al. 2013). Jasmonate (JA) and its derivatives are oxygenated-lipids (oxylipins)-based hormones that play important roles in the regulation of plant defense and development (Wasternack and Hause 2013).

The biosynthesis of JA starts with the oxygenation of the lipid substrate, linolenic acid (18:3), in chloroplasts (Fig. 1A). The end product of a series of reactions catalyzed by 13-lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) is 12-oxophytodienoic (OPDA). In Arabidopsis, *ghd* mutation of the AOS gene results in a complete loss of JA production. OPDA produced in chloroplasts is transported into peroxisomes, where it is subsequently reduced by OPDA Reductase 3 (OPR3) and oxidized by acyl-CoA-oxidase 1 (ACX1) resulting in JA formation. The genes participating in JA synthesis are inducible by JA, thus providing a positive feedback loop. JA produced in peroxisomes is transported to the cytosol. To control the activity of JA in plants, it undergoes differential modifications, for instance, JA hydroxylation, decarboxylation, glycosylation, methylation catalyzed by a JA methyltransferase (JMT) and amino acid conjugation by a JA conjugate synthase (JAR1, jasmonate resistant 1). JA-Ile, produced by JAR1, is the final biological active compound in plants (Staswick et al. 2004; Suza et al. 2008). JA-Ile was identified as the ligand of the jasmonate receptor complex, consisting of Coronatine Insensitive 1 (COI1), JA-Ile and member of the Jasmonate ZIM Domain (JAZ) proteins (Fonseca et al. 2009; Katsir et al. 2008). A major breakthrough in understanding the JA signaling pathway was the isolation of JAZ proteins, which were

later found to be components of the JA co-receptors (Pauwels and Goossens 2008). JAZs are suppressors of JA-induced transcriptional response. In the absence of JA, JAZ proteins recruit the transcriptional co-repressor TPL via interaction with the bridging protein Novel Interactor of JAZ (NINJA) (Pauwels et al. 2010). Upon stress, accumulated JA-Ile binds to the F-box protein COI1 to facilitate the formation of COI1-JAZs complex, resulting in ubiquitination and the ultimate degradation of JAZ repressors via the 26S proteasome (Chini et al. 2007; Thines et al. 2007). Downstream of COI1-JAZ perception, the JA signaling pathway can be divided into two distinct branches: the MYC-branch and the ERF-branch (Pieterse et al. 2009).

The MYC-branch is mainly responsible for wounding- and insect-induced JA signaling pathway. This branch is controlled by the basic helix-loop-helix leucine zipper transcription factors MYC2, MYC3 and MYC4. In the absence of JA, JAZ repressors interact with MYC proteins and recruit the co-repressor TPL (Fig. 1B). A recent study by Zhang et al. (2015) showed that the JAZ interaction with MYC protein competitively block their interaction with the MED25 subunit of the transcriptional Mediator complex (Zhang et al. 2015). Activation of MYC-branch upon removal of JAZs leads to expression of a large set of JA-responsive genes, including JA marker gene *VSP2*, JA synthesis gene *LOX2* and JA signaling repressor *JAZ* genes.

The ERF-branch is induced upon necrotrophic pathogen infection. This branch is synergistically regulated by the ET-signaling pathway and controlled by the AP2/ERF-Domain transcription factors, OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59) and ETHYLENE RESPONSE FACTOR 1 (ERF1), which directly activate the expression of ERF-branch marker genes, like *PDF1.2*. ORA59 and ERF1 specifically bind to the GCC-box motif via the ERF domain. The GCC-boxes are essential for the activation of *PDF1.2* expression (Pré et al. 2008). However, whether JAZ repressors interact directly or indirectly with ERFs are unknown. Zhu et al. (2011) reported that JAZ proteins directly interact with EIN3 to repress EIN3 induced *ORA59* and *ERF1* expression. Within the JA responsive pathway, the MYC- and ERF-branches are mutually antagonistic.

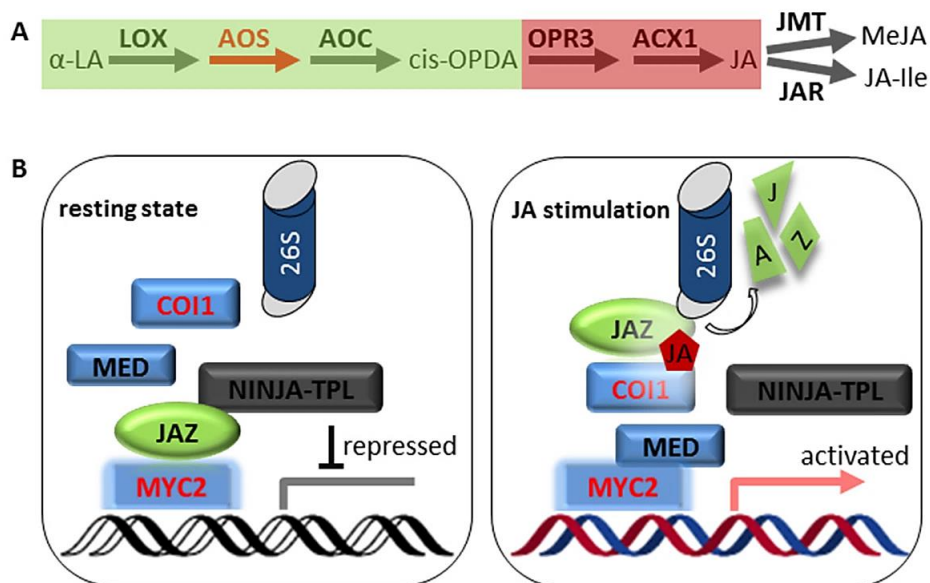


Figure 1 | Jasmonate (JA) biosynthesis and signaling transduction pathway.

(A) Model for the JA biosynthesis pathway. The intermediate OPDA is synthesized in the chloroplasts. JA is synthesized in the peroxisomes and exported to the cytosol, where it is converted to other bioactive derivatives (i.e., JA-Ile). The key enzyme AOS is highlighted in red. (B) Model for the JA signaling transduction pathway of the MYC-branch in Arabidopsis. In the non-induced cells (left, low JA level), MYC2 activity is repressed by JAZ proteins that interact with NINJA to recruit transcriptional repressor TPL. In the JA-stimulated cell (right, high JA level), JAZ proteins are degraded by the SCFCOI1-mediated 26S-proteasome. MYC2 is released to interact with the transcriptional mediator to activate JA-responsive gene expression. Abbreviations:  $\alpha$ -LA,  $\alpha$ -linolenic acid; ACX1, acyl-CoA-oxidase 1; AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1; JA, jasmonic acid; JA-Ile, Jasmonic acid-isoleucine conjugate; JAR, jasmonate resistant; JAZ, jasmonate ZIM domain; JMT, JA methyltransferase; LOX, 13-lipoxygenase; MeJA, methyl jasmonate; MED, mediator; NINJA, novel interactor of JAZ; OPDA, 12-oxophytodienoic; OPR3, OPDA Reductase 3; TPL, TOPLESS (Li et al. 2019).

ET is a gaseous hormone that has been recognized as a plant growth regulator for more than a century. The ET biosynthetic pathway, also known as the Yang cycle, begins with the amino acid methionine (Bradford and Yang 2008) (Fig. 2A). 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) is a rate-limiting enzyme of ET biosynthesis, which converts S-adenosyl methionine (SAM) to ACC (Yoon 2015). As a diffusible, gaseous, and non-degradable hormone, ET biosynthesis has to be tightly controlled. Therefore, the regulation of ACS activity confers strict control of ET production. After accumulation, ET is perceived by endoplasmic reticulum (ER)-localized receptors, which act as negative regulators of the ET signaling pathway (Ju and Chang 2015). Upon ET binding, the ER-localized EIN2 becomes dephosphorylated due to the inactivation of the Raf like kinase CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) associated with the receptors. Dephosphorylated EIN2 releases its C-terminal domain (CEND), which enters into the

nucleus and conveys signals to the EIN3 transcription factor (Alonso et al. 1999; Ju et al. 2012; Qiao et al. 2012).

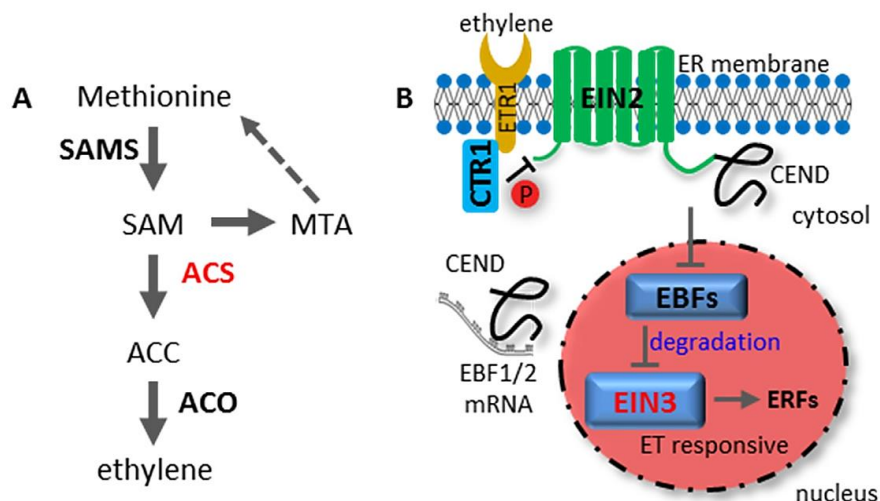


Figure 2 | Ethylene (ET) biosynthesis and the signaling cascade pathway.

(A) Model for the ET biosynthesis pathway. The precursor SAM is produced by SAMS with methionine as substrate. SAM is converted to the intermediate chemical ACC by ACS with the release of MTA as byproduct. MTA is recycled to methionine through the so-called Yang cycle. The rate-limiting enzyme ACS is highlighted in red. (B) Model for the ET signaling cascade. In the absence of ET, CTR1 phosphorylates EIN2 and the ET pathway is therefore blocked. In the presence of ET and when it is perceived by ET receptor (i.e., ETR1, ETHYLENE RESISTANT 1), the kinase activity of CTR1 is inactivated, the EIN2 CEND becomes dephosphorylated and cleaved. CEND subsequently translocates into the nucleus to attenuate EBFs E3 ligase function. In addition, CEND may bind to the UTR of EBF1/2 mRNA to perturb EBF1/2 translation in cytosol. Stabilized EIN3 protein then activates ERF transcription factors (i.e., ERF1 and ORA59) to elicit the ET response. Abbreviations: ACC, 1-Aminocyclopropane-1-carboxylic acid; ACO, ACC-oxidase; ACS, ACC synthase; CEND, C-terminal end of EIN2; CTR1, constitutive triple response 1; EBF1/2, EIN3-binding F-Box 1/2; EIN, ethylene insensitive; ER, endoplasmic reticulum; ERF, ethylene-response factor; ET, ethylene; ETR1: ethylene-resistant 1; MTA, methylthioadenosine; SAM, S-adenosyl methionine; SAMS, SAM synthase. (Li et al. 2019).

EIN3 directly activates the expression of an array of ET-responsive transcription factors such as *ERF1* and *ORA59*, which magnify and elicit the ET response (Pré M et al. 2008; Solano et al. 1998). ET also stabilizes EIN3 protein by eliminating two F-box proteins, EIN3 BINDING F-BOX PROTEIN 1 (EBF1) and EBF2, which target EIN3 for proteasomal degradation in the absence of ET (Potuschak et al. 2003). The expression of EBF1 and EBF2 are induced by EIN3, providing a negative feedback loop for the ET signaling pathway (Konishi et al. 2008). Both Li et al. and Merchante et al. (2015) discovered that ET-released C-terminal portion of EIN2 directly bound to 3'UTR of EBF1/2 mRNA for translational repression (Fig. 2B).

### 1.3 Crosstalk between the phytohormones ET, JA, and SA

Plant defense responses against environmental pathogens are energy consuming. Ideally, plants employ a specific pathway upon recognition of distinct pathogens. Extensive crosstalk between different signaling pathways provides the potential for efficient energy allocation. Due to their different roles in plant development as well as in plant defense, SA, JA and ET may also affect each other. It is hypothesized that plant defense pathways interact synergistically or antagonistically to fine-tune responses according to the challenging organisms (Pieterse et al. 2009). An example of synergism between JA and ET is the pathogen-induced expression of the plant defensin gene *PDF1.2* in *Arabidopsis*, which requires activation of the JA and ET signaling pathway for full expression (Penninckx et al. 1998). Cross-communication between hormone signaling pathways provides the plant with a large regulatory capacity that may tailor its defense response to different types of attackers (Pieterse et al. 2009). This section will focus on the crosstalk between JA/ET and SA signaling, and some key hormones involved in plant defense response. The primary mode of interaction is mutually antagonistic (Gupta et al. 2000; Doares et al. 1995). For instance, JA insensitive *coi* mutants are impaired in JA signaling and insensitive to the bacterial effector and JA analog coronatine. Simultaneously, SA-dependent gene expression is enhanced in these mutants. At the same time, SA synthesis and SA-mediated defense responses upon infection are hyper-activated (Kloek et al. 2001). While mutations that disrupt SA signaling (e.g. *npr1*) lead to the concomitant increases in the basal or induced levels of the JA marker gene *PDF1.2* (Kazan and Manners 2008). However, synergism between SA and JA signaling has also been reported (Koornneef et al. 2008; Koornneef and Pieterse 2008; Niki et al. 1998; Vidal et al. 1997). Whether crosstalk between SA and JA will be synergistic or antagonistic, it depends at least in part on the concentrations of the hormones (Mur et al. 2006; Schenke et al. 2005). This means that the outcomes of JA-SA interactions could be tailored to the pathogen/pest attack by the relative concentration of each hormone. Moreover, ET influences crosstalk between SA and JA. It potentiates SA-responsive *PR1* expression (De Vos et al. 2006), but also affects the outcome of the JA response; after infection by necrotrophic pathogens ET synergistically interacts with JA to trigger defense (Lorenzo et al. 2003; Anderson et al. 2004; Pré et al. 2008). Accordingly,



Leon-Reyes et al. (2010) showed that ET counteracts the antagonistic effect of SA on JA-responsive gene expression. Fig. 3 illustrates various players involved in the SA, JA, and ET hormone signaling pathways. This figure includes proteins/genes that are required to initiate different response pathways that effectively target pathogens. Moreover, the negative and positive effects among these different players are indicated, highlighting the complexity of hormonal crosstalk in plant defense. Identity of the different regulators shown in Fig. 3 has not been discussed thoroughly.

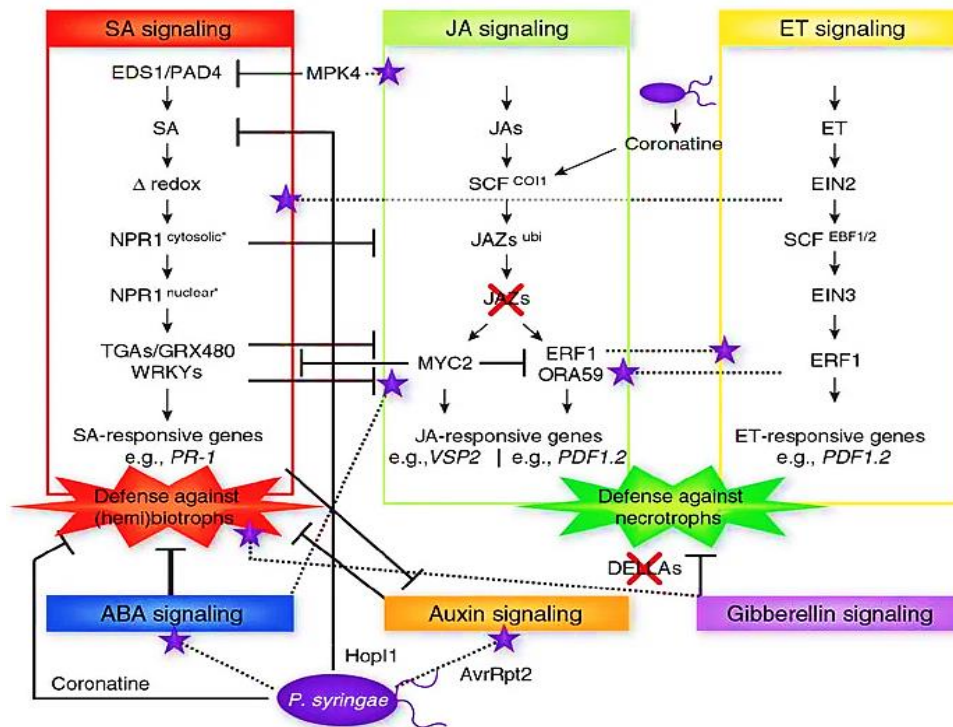


Figure 3 | Networking by phytohormones in the plant immune response.

JA and ET signaling is involved in the defense response against necro-trophs, SA signaling in response to (hemi) biotrophs. The different hormones are influenced by each other either positively or negatively. Blocked arrows: negative effect, purple stars: positive effect. (Pieterse et al. 2009).

Pathogens can take advantage of the plant's ability of hormonal crosstalk by mimicking hormones of one pathway to interfere with another host immune response pathway (Spoel and Dong 2008). One example of "hormonal-mimicry" is the phytotoxin coronatine (COR) (Bender et al. 1999) produced by some *Pseudomonas syringae* strains. COR structurally resembles JA derivatives, including JA-isoleucine (Staswick, 2008), and is subsequently able to interfere with SA signaling, thus promoting virulence of the COR-producing pathogen (Spoel and Dong 2008).

## **2 Role of plant defensins in immunity**

Although plants are continuously exposed to high numbers of microorganisms, the manifestation of infectious diseases remains a rare event. This may in part be attributed to the occurrence of a solid cell wall around plant cells (Iiyama et al. 1994). Besides this preformed barrier, plants can activate a whole array of defense responses against microorganisms that succeed in passing the cell wall or that use natural openings to enter plant tissues. A whole set of genes encoding pathogenesis-related (PR) proteins can be activated not only at the site of infection but also systemically in healthy leaves of infected plants (Ward et al. 1991; Uknes et al. 1992). PR proteins are categorized into structurally homologous families. Some of these PR-protein families have direct antimicrobial activities. To date, seventeen PR protein groups have been categorized across many plant species (Tab. 1) (Buonaurio et al. 2009). The total number of PR genes count up to 479 in Arabidopsis.

### **2.1 Plant defensins (PDFs)**

Among antimicrobial peptides, PR12, or plant defensins are known to be the most important antifungal peptides in plants. The in-vitro studies revealed that plant defensins show antifungal activity against many fungal pathogens (Terras et al. 1995; Jha and Chattoo, 2009). Plant defensins are small cysteine-rich proteins of 45-54 amino acids that are closely related to insect and mammalian defensins (Thomma, et al. 2002; Zasloff 2002; Lay and Anderson, 2005). They are expressed in most, if not all, plants. Although abundant in seeds, they are expressed in almost all organs of a plant. A majority of plant defensins are synthesized as precursor proteins and post-translational processing cleaves out the C-terminal mature defensin peptide from the secretory signal peptide. Although a majority of defensins are secreted to the extracellular space, a few floral defensins are targeted to the vacuole. They are either constitutively expressed in storage and reproductive organs or produced upon pathogenic attack or injury as part of a systemic defense response (Broekaert et al. 1997). Antifungal activity is the most common function and is a well-characterized function of plant defensins. Interestingly, Lacerda et al. (2016) reported the first time that transgenic *Pichia pastoris* plants expressing defensin gene lead to enhanced resistance against obligate biotrophic fungal pathogens, *Fusarium tucumaniae* and *Colletotrichum gossypii* var. In addition,

overexpression of plant defensin peptides both in model and crop plants have shown enhanced and long-lasting disease resistance (Kanzaki et al. 2002; Chen et al. 2004; Anuradha et al. 2008; Ntui et al. 2010; Ghag et al. 2012; Kaur et al. 2016). Besides their role in biotic stress, they are also induced by diverse abiotic stresses including cold (Koike et al. 2002), drought (Do et al. 2004; Maitra et al. 1998; Maitra et al. 1994), heavy metals (Mirouze et al. 2006) and wounding (Rawat et al. 2017). In addition, the production of plant defensins is also induced in response to signalling molecules, including methyl jasmonate, ethylene, and salicylic acid (Hanks et al. 2005; Manners et al. 1998).

Table 1 | Families of pathogenesis related proteins (PRPs).

Sources: Niderman et al. (1995); Van Loon et al. (1998); Sels et al. (2008); Okushima et al. (2000); Irigoyen et al. (2020).

PRP family	Protein activity	Targeted pathogen sites	Copies in <i>At</i>
PR-1	Unknown	Active against oomycetes	23
PR-2	1,3 $\beta$ -glucanase	Cell wall glucan of fungi	70
PR-3	Chitinases	Cell wall chitin of fungi	21
PR-4	Chitinase type I, II	Active against oomycetes	6
PR-5	Thaumatococin	Unknown?	42
PR-6	Proteinase inhibitor	Active on nematodes and insects	7
PR-7	Aspartic endoproteases	Microbial cell wall dissolution	78
PR-8	Endochitinase with lysozyme activity	Cell wall chitin of fungi and mucopolysaccharide cell wall of bacteria	1
PR-9	Peroxidase	Strengthening of plant cell wall	97
PR-10	Ribonuclease-like		3
PR-11	Endochitinase	Cell wall chitin of fungi	9
PR-12	Defensins	Antifungal and antibacterial activity	15
PR-13	Thionin	Antifungal and antibacterial activity	4
PR-14	Lipid transfer proteins	Antifungal and antibacterial activity	23
PR-15	Oxalate-oxidase	Produce H <sub>2</sub> O <sub>2</sub> that inhibits microbes and also stimulates host defence	74
PR-16	Oxalate-oxidase-like with SOD activity	Production of H <sub>2</sub> O <sub>2</sub>	
PR-17	Uncharacterized	Unknown	8
			$\Sigma$ 479

## 2.2 Structure of plant defensins

Structure of plant defensins typically comprises a cysteine-stabilized  $\alpha\beta$ -motif (CS $\alpha\beta$ ) with a prominent  $\alpha$ -helix and a triple-stranded antiparallel  $\beta$ -sheet that is stabilized by four disulphide bridges (Bruix et al. 1995; Fant et al. 1998; Fant et al. 1999). This structure resembles defense peptides in insects and mammals, revealing a common evolutionary origin and conservation between invertebrates, plants, and vertebrates (Thomma et al. 2002). A subclass of the plant defensin family comprises defensins with ten cysteine residues, resulting in a total of five disulphide bonds. The fifth disulphide bond appears to

reinforce a conserved hydrogen bond and is likely to confer additional thermodynamic stability of the defensin, as compared to other defensins, by replacing non-covalent hydrophobic interactions or hydrogen bonds with a covalent bond (Janssen et al. 2003). This extra pair of cysteines has only been reported for *PhD1* and *PhD2*, both floral defensins isolated from *Petunia hybrida* (Lay et al. 2003; Janssen et al. 2003).

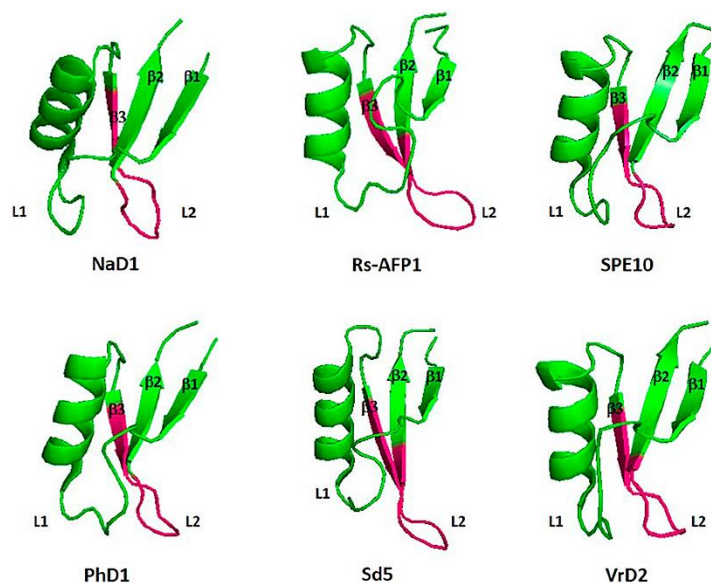


Figure 4 I Three-dimensional structure of six antifungal defensins from plants. Pink region highlight the  $\gamma$ -core motif of each peptide.  $\beta$ 1:  $\beta$ -sheet 1;  $\beta$ 2:  $\beta$ -sheet 2;  $\beta$ 3:  $\beta$ -sheet3; L1: Loop1; L2: Loop 2; NaD1: *Nicotiana alata* defensin 1 (Accession: 4ABO\_A); Rs-AFP1: *Raphanus sativus* antifungal peptide 1 (Accession: 1AYJ\_A); SPE10: *Pachyrhizus erosu* peptide (Accession: 3PSM\_A); PhD1: *Petunia hybrida* defensin 1 (Accession: 1N4N\_A); Sd5: *Saccharum officinarum* defensin 5 (Accession: 2KSK\_A); VrD2: *Vigna radiata* defensin 2 (Accession: 2GL1\_A) (Lacerda et al. 2014).

According to the structure of their precursor protein, plant defensins can be subdivided into two groups. A first group comprises defensins in which the precursor is composed of a signal sequence and a mature defensin domain. The signal sequence targets the protein to the endoplasmic reticulum, where it is folded and subsequently enters the secretory pathway. In a second and less common group, the precursor protein contains an additional C-terminal prodomain that is proteolytically removed during or after transit through the secretory pathway (Lay et al. 2003, Lay et al. 2005). These types of defensins have been identified in solanaceous plants, such as *Nicotiana alata* and *Petunia hybrida* (Lay et al. 2003). Fig. 4 shows the three-dimensional structural conformation of six antifungal defensins from plants (Lacerda et al. 2014).

### 2.3 Modes of action of plant defensins

The mode of antifungal action of selected antifungal defensins and defensin-like proteins from plants are shown in Fig. 5. The mechanism of antifungal defensins are most likely subject to electrostatic interactions in the middle of peptides and hyphal films, prompting a disturbance by a fast instigation of  $K^+$  efflux and  $Ca^{2+}$  uptake and consequently preventing parasitic growth. Notably, two major scientific hypotheses - the carpet model and the pore model, have been postulated to elucidate the model of action antimicrobial defensin peptides. According to both models, defensins preferentially interrelate with negatively charged structures of pathogens' cell membrane, resulting in increased membrane permeability and cell leakage followed by necrotic cell death. The carpet model explicates the pore formation of several peptides into the cell membrane, whereas the pore model demonstrates the formation of oligomers of those peptides, which then produce numerous pores into the membrane (Lacerda et al. 2014). On the contrary, several reports have discussed an alternative mechanism without damaging the cell membrane of pathogens.

Thereby, the defense peptides are internalized into the intracellular environment, leading to elevated ion permeability of the membrane and to the formation of transient pores and/or results in (active) protein transport into the host cell where these antifungals interact with intracellular targets (Hegedus and Marx 2013; Wilmes et al. 2011). This antifungal activity increases the intracellular level of reactive oxygen species (ROS) and triggers programmed cell death (PCD) (Hegedus and Marx 2013; Wilmes et al. 2011). Thus, plasma membrane leakage could occur at a later time point after protein contact with the fungal cell as a secondary effect of extensive intracellular ROS formation, as proposed for plant defensins (Thevissen et al. 2003). The location of positively charged amino acids at loops or  $\beta$ -sheet regions have been reported to be useful for antifungal potentiality, suggesting that the interaction of positively charged Rs-AFP1 peptide with fungal pathogens might occur via electrostatic interfaces (Fant et al. 1998).

Some other reports focusing on the structural assessments of plant defensins also recognized the significance of positively charged amino acid residues (located at the loop region) for antifungal activities, as well as working as a specificity factor against a range of pathogenic fungi (Lay et al. 2003). Sagaram et al. (2011) reported the presence of





activity (Sagaram et al. 2011). An illustration of the proposed modes of action of the plant defensins is shown in Tab. 2 (Vriens et al. 2014).

Table 2 | Summary of the current knowledge regarding the mechanisms of antifungal action and additional biological functions of selected antifungal defensins and defensin-like proteins from plants  
n.d. -not determined; cellular uptake: – no uptake; + intracellular localization.

Defensin producing Plants	Interaction molecules of the fungal envelope	Cellular uptake	Mechanism of action and signalling pathways involved in fungi-AMP interaction	Biological functions beyond antimicrobial activity	References
DmAMP1 <i>Dahlia merckii</i>	Sphingolipid M(IP) <sub>2</sub> C	n.d.	K <sup>+</sup> efflux, Ca <sup>2+</sup> uptake, alkalinization of the medium, membrane potential changes, membrane permeabilization	n.d.	(Thevissen et al. 1996; 2000;2005 ; 2003b; 1999)
RsAFP2 <i>Raphanus sativus</i>	Sphingolipid GlcCer	n.d.	K <sup>+</sup> efflux, Ca <sup>2+</sup> uptake, alkalinization of the medium, membrane potential changes, plasma membrane permeabilization, induction of ROS accumulation, MAPK signalling	Plant root growth inhibition	(Allen et al. 2008; Thevissen et al.1996; 2004; 1999; Aerts et al. 2007; Ramamooorthy et al. 2007b)
HsAFP1 <i>Heuchera sanguinea</i>	Interaction with plasma and microsomal membranes	n.d.	Plasma membrane permeabilization, ROS accumulation, apoptosis, MAPK signalling	n.d.	(Aerts et al. 2011; Thevissen et al. 1997; 1999)
MsDef1 <i>Medicago sativa</i>	Sphingolipid GlcCer	n.d.	MAPK signalling	Mammalian Ca <sup>2+</sup> channel blocker, plant root growth inhibition	(Allen et al. 2008; Spelbrink et al. 2004; Ramamoorthy et al. 2007a,b)
MtDef2 <i>Medicago truncatula</i>	n.d.	n.d.	MAPK signalling	Plant root growth inhibition	(Allen et al. 2008; Ramamooorthy et al. 2007b)
Psd1 <i>Pisum sativum</i>	n.d.	+	Interaction with cyclin F and interferes with cell division	n.d.	(Lobo et al. 2007)
AhPDF1.1 <i>Arabidopsis halleri</i>	n.d.	n.d.	n.d.	Tolerance against zinc and selenite, inhibition of plant root growth	(Oomen et al. 2011; Mirouze et al. 2006; Tamaoki et al. 2008)
DEF2 <i>Solanum lycopersicon</i>	n.d.	n.d.	n.d.	Influence of pollen viability, seed production, and the growth of various organs of the producing plant	(Stotz et al. 2009a)
ZmES4 <i>Zea mays</i>	n.d.	n.d.	n.d.	Inter-gametophyte signalling, induction of pollen tube burst	(Amien et al. 2010)

Ha-DEF1 <i>Helianthus inbred line</i> (LR1)	n.d.	n.d.	Plasma membrane permeabilization	Parasitic plant defence	(de Zelicourt et al. 2007)
VrD1 <i>Vigna radiata</i>	n.d.	n.d.	n.d.	Insect inhibitory activity	(Liu et al.2006; Chen et al. 2004; 2002)
Sesquin <i>Vigna sesquipedalis</i>	n.d.	n.d.	n.d.	Inhibitory activity against cancer cells, inhibitory effect towards HIV reverse transcriptase	(Wong and Ng 2005)
Limyin <i>Phaseolus limensis</i>	n.d.	n.d.	n.d.	Inhibitory activity against cancer cells	(Wang et al. 2009)

## 2.4 Role of defensins in plant defense

Many reports have revealed that defensins are an essential component of the plant's inherent immunity (Selitrennikoff et al. 2001). De Beer and Vivier (2011) isolated four defensin genes (Hc-AFP1-4) with homology and clustering closest to defensins isolated from other *Brassicaceae* species. The same study also used propidium iodide assays to reveal the anti-fungal potential of all newly isolated defensin genes against *Botrytis cinerea*. A light microscopy analysis confirmed that the anti-fungal activity was related to an increase in membrane permeabilization (De Beer and Vivier 2011). Most of the plant defensins exhibited a constitutive expression pattern with upregulation following pathogen attack, injuries, and abiotic stresses. Defensins are widely distributed and identified in flowers, tubers, leaves, pods, and seeds, where these peptides play a significant

protective role during seed germination and seedling development (Garcia-Olmedo et al. 1998). Besides, plant defensins are also found in different tissues such as stomata, xylem, stomata, and parenchyma cells, and other peripheral regions (Chen et al. 2002). Interestingly, plant defensins presented broad-spectrum antimicrobial activities, and some reports described the production of transgenic plants with the constitutive expression of foreign defensins. Therefore, these transgenic plants possess multiple biological potentialities, such as antibacterial, antifungal, and insecticidal activities, protein synthesis inhibition, inhibitors of digestive enzymes, and abiotic stress and heavy metal resistance (de Carvalho and Gomes 2009; 2011). Due to their potential biological activities, these defensins are categorized as promiscuous proteins. For instance, different homologous



forms of a family of defensins isolated from *V. unguiculata* may present antibacterial and antifungal activities, as well as enzyme inhibition (Franco 2011). Though they display numerous biological activities, the antimicrobial role of plant defensins is predominantly observed against a range of pathogenic fungi.

## **2.5 Biotechnological applications**

The biological activities presented by plant defensins make them attractive for biotechnology. Among these characteristics are antimicrobial, insecticidal, and even anti-parasitic activities (De Carvalho and Gomes 2009). These qualities make the defensins a good candidate for use in protein engineering and transgenic production of agronomically important plants that may combat against pathogens and pests, as exemplified by the defensin from *V. radiata* (De Carvalho and Gomes 2009). Furthermore, the antifungal activity provides another advantage since one peptide is active against several phytopathogens, as demonstrated by the defensin from *R. sativus* Rs-AFP2 which inhibits many fungal pathogens such as *C. lindemuthianum*, *F. culmorum*, *F. oxysporum*, *N. haematococca*, *B. cinerea*, *V. dahliae*, and *M. fijiensis* at concentrations ranging from 1 to 3 mgmL<sup>-1</sup> (Terras et al. 1992). Plant defensins have also displayed indirect responses towards phytopathogenic fungi in transgenic plants, when other foreigner genes are being overexpressed (Murad et al. 2007). Earlier reports showed that a peptide from Arabidopsis, named AtPep1 stimulated the transcription activation of the defensin gene *pdf1.2* (Huffaker et al. 2006). When *AtPep1* precursor gene PROPEP1 was expressed into transgenic Arabidopsis plants, the transcription of PDF1.2 was also observed. Moreover, the expressed defensin stimulated root development, which, consequently, improved plant resistance against the filamentous fungus *Pythium irregular* (Huffaker et al. 2006). Thus, these are obvious advantages over plant transformation with race-specific R genes. Additionally, defensins can work together with another antimicrobial compounds, conferring, or enhancing resistance (Oh B-J et al. 1999). In an attempt at transforming two different genes at the same time in tomato plants was performed using the genetic material of a defensin and a glucanase from alfalfa, to analyze their efficiency towards phytopathogenic fungi. Therefore, T1-generation transgenic plants revealed enhanced tolerance to *R. solanacearum*, when compared to non-transformed plants, indicating the existence of a synergic effect of both proteins as

antifungal molecules in tomato cultivars (Chen et al. 2006). Plant defensins have also displayed indirect responses when an ionotropic glutamate receptor (RsGluR) was transformed into Arabidopsis plants. The expression of RsGluR led to an up-regulation of defensins, causing an increase of the plant resistance towards *Botrytis cinerea* (Kang et al. 2006). Microarray analyses later confirmed that up-regulated defensins and jasmonic acid-responsive genes were produced after overexpression of RsGluR in Arabidopsis. Furthermore, the same plant species was transformed with a cotton non-symbiotic hemoglobin for tolerance against fungal pathogens. However, the foreigner gene could also induce a constitutive expression of the PR protein K (*PR1*) as well as the defensin *PDF1.2*, providing enhanced resistance to *Verticillium dahliae* (Qu et al. 2006)

### **3 The plant pathogen *Verticillium longisporum***

*Verticillium longisporum* is a hemibiotrophic, soil born endophytic pathogen which causes symptoms on the stem of oilseed rape therefore, the disease is termed as 'Verticillium stem striping' instead of Verticillium wilt (Depotter et al. 2016). Its occurrence as a pathogen mainly comprises the northern parts of Europe (Zeise and von Tiedemann 2002; Steventon et al. 2002). However, this disease recently emerged in the United Kingdom (Depotter et al. 2017; Gladders et al. 2011) as well as in Canada (CFIA 2015). It is assumed that *V. longisporum* diseases in total are responsible for an economic loss of about €3 billion in the 20 most-affected hosts worldwide (Depotter et al. 2016). Until today, this pathogen cannot be controlled by fungicide application and no effective genetic resistance could be identified in the present gene pool of oilseed rape so far (Depotter et al. 2016). The host-range of the near-diploid fungus *V. longisporum*, named after its characteristic spores, is limited to *Brassicaceae*, where it causes severe crop loss on oilseed rape (Zeise and von Tiedemann 2002, Klosterman et al. 2011).

#### **3.1 Taxonomy and morphology of *Verticillium longisporum***

*Verticillium longisporum* is a member of the Flavnonexudans (a subclass of Sordariomycetes) lineage and thus lacks the ability to produce yellow hyphal pigmentation (Inderbitzin et al. 2011a). *V. longisporum* was first described as a variety of *V. dahliae*, as *V. dahliae* var. *longisporum* (Stark, 1961), and was then elevated to species rank 37 years later (Karapapa et al. 1997). Although first contested, the name *V. longisporum* is

now widely adopted (Inderbitzin and Subbarao 2014). The evolutionary history of *V. longisporum* is unique among *Verticillium* species, as *V. longisporum* is an amphidiploid hybrid that evolved repeatedly by hybridization among four different ancestors (Inderbitzin et al. 2011b; Ingram, 1968). The differentiation of *V. longisporum* from related species may be based on morphological and cultural features (Tab. 3). In general, *V. longisporum* conidia are longer than those of its close relative *V. dahliae* (Karapapa et al. 1997; Stark, 1961) and, with respect to *V. dahliae*, *V. longisporum* has been reported to have elongated microsclerotia and a tendency towards the presence of three phialides in each whorl (Karapapa et al. 1997). In addition, no morphological characteristics allow for the differentiation of the different hybrid lineages in *V. longisporum*. *Verticillium longisporum* has also been referred to as a ‘near-diploid’ as its nuclear DNA content is  $\pm 1.7$ -1.8 times that of short-spored isolates (Collins et al. 2003; Karapapa et al. 1997; Steventon et al. 2002). The *V. longisporum* lineage A1/D3 rDNA region was derived from *V. dahliae*, whereas the *V. longisporum* lineage A1/D1 and A1/D2 rDNA regions were derived from species A1. Parasexual recombination has been proposed as the underlying mechanism (Karapapa et al. 1997), although parasexual processes generally end with chromosome loss to regain a haploid state after the fusion of hyphae and nuclei (Caten 1981). Thus, the stability of *V. longisporum* as a hybrid makes the hypothesis of interspecific hyphal fusion followed by nuclear fusion more plausible than parasexual processes (Inderbitzin et al. 2011b).

Table 3 | Non-molecular criteria for the taxonomic discrimination of *Verticillium longisporum*.

Source: (Karapapa et al. 1997, Bhat and Subbarao 1999, Zeise and Tiedemann 2001, Zeise and Tiedemann 2002, Steventon et al. 2002, Inderbitzin et al. 2011b).

Parameter	<i>V. longisporum</i>
Microsclerotial shape	Mostly elongate
Conidial size	Mostly long (7.1-8.8 $\mu\text{m}$ )
Extracellular polyphenol oxidase activity	Mostly none
Culture filtrate fluorescence	Yes
Host range	Mainly restricted to Brassicaceae

### 3.2 Symptom development and life cycle of *Verticillium longisporum*

Infected oilseed rape develops dark, unilateral striping on the stem late in the growing season, indicating the necrosis of cortical tissue (Heale and Karapapa 1999). Symptom

development coincides with increased pathogen colonization of root and shoot tissues (Dunker et al. 2008). In the final stages of the disease, the fungus forms black microsclerotia in the stem cortex. In contrast with the disease caused by this pathogen on other crops, conventional wilting symptoms are typically not observed on oilseed rape. Rather, the crop ripens prematurely, making disease symptoms difficult to distinguish from natural senescence.

Interestingly, disease development in oilseed rape on artificial inoculation differs from disease development under field conditions. Whereas symptoms in the field involve dark unilateral striping on the stem late in the growing season, on root dip inoculation in the seedling stage, oilseed rape plants exhibit chlorosis, vascular discoloration and stunting at an early stage (Eynck et al. 2007; 2009b; Floerl et al. 2008; Zeise and Tiedemann, 2002). Moreover, clear biomass reduction is observed, whereby roots are significantly more affected than shoots (Keunecke 2009). It is currently not understood why these differences in disease development occur. The typical disease symptoms of *V. longisporum* infection that are, leaf chlorosis, stunted growth and reduced biomass of its host oilseed rape (*Brassica napus*) and model host plant *Arabidopsis* (*Arabidopsis thaliana*) (Johansson et al. 2006b).

Stem striping is a monocyclic disease (Klosterman et al. 2011). *V. longisporum* produces melanized microsclerotia (Stark 1961) for survival to bridge the gap between hosts. Microsclerotia are clusters of melanized, thick-walled fungal cells, which are derived from hyphal cells through lateral budding of the hyaline mycelium (Klebahn 1913). In the absence of a host, *V. longisporum* microsclerotia can remain dormant and viable in the soil for many years (Karapapa et al. 1997; Depotter et al. 2016). Root exudates stimulate the germination of *V. longisporum* microsclerotia, after which hyphae grow towards the root of the plant (Berlanger and Powelson 2000; Leino, 2006). Subsequently, hyphae colonize the surface of the root hairs and grow towards the root surface (Eynck et al. 2007; Zhou et al. 2006). On oilseed rape (*Brassica napus*), the fungus enters the root by direct penetration of rhizodermal cells of lateral roots or root hairs. Once inside the root, hyphae initially grow both intercellularly and intracellularly in the root cortex towards the central cylinder, where the pathogen enters the xylem (Eynck et al. 2007). Next, conidia may be produced that are carried upwards with the transpiration stream. Conidia that become

trapped in pit membranes or at vessel end walls may germinate and penetrate into adjacent vessels (Garber and Houston 1966). The colonization induces occlusions of the vessels, which may disturb the sap stream in the xylem (Kamble et al. 2013). Only during senescence does the pathogen grow out of the xylem vessels, invades the stem parenchyma, and form microsclerotia beneath the stem epidermis and in the stem pith. The microsclerotia are released into the soil during tissue decomposition (Heale and Karapapa 1999).

### **3.3 Plant response to *V. longisporum* infection**

The influence of plant secondary metabolites on the interaction between *V. longisporum* and its host plants have been explored just for few. Glucosinolates are constitutively expressed sulfur-containing phytochemicals that are predominantly found in brassicaceous plants (Wittstock and Halkier 2002). Upon tissue damage, glucosinolates are hydrolyzed with the formation of biologically active and sometimes toxic compounds. Levels of glucosinolates in the roots of *V. longisporum*-infected *Arabidopsis* plants are higher than in non-inoculated plants. However, the increase in glucosinolates is not accompanied by an increase in glucosinolate breakdown products in the roots (Witzel et al. 2015). *Verticillium longisporum* infection induces the transcriptional activation of genes involved in tryptophan biosynthesis and tryptophan-derived secondary metabolism. Furthermore, genetic disruption of tryptophan-derived secondary metabolism leads to enhanced susceptibility. In contrast with the protective role of the tryptophan-derived secondary metabolites, monoterpenes produced by the monoterpene synthase TPS23/27 stimulate *in-vitro* conidial germination and subsequent invasion of *V. longisporum* in *Arabidopsis* roots (Roos et al. 2015). The ability of *V. longisporum* to synthesize aromatic amino acids and the cross-pathway control of amino acid biosynthesis are required for pathogenicity. Silencing mutants impaired in chorismate synthase or CPC1, the conserved transcription factor of cross-pathway control, caused less disease and showed reduced growth in the hypocotyl of *B. napus* and *Arabidopsis*. Chorismate is essential for the biosynthesis of tryptophan, phenylalanine and tyrosine, whereas cross-pathway control allows fungi to increase amino acid biosynthesis on amino acid starvation (Singh et al. 2010; Timpner et al. 2013). *B. napus* xylem sap contains only low concentrations of amino acids, and aromatic amino acids are especially scarce

(Singh et al. 2010). Increased production of plant secondary metabolites in response to *V. longisporum* infection probably further depletes amino acid concentrations in the xylem. Hence, the fungus requires a functional cross-pathway control to overcome the imbalance in amino acid supply in the xylem.

To date, the involvement of typical plant hormone signaling pathways in the interaction with *V. longisporum* remains unclear, and the role of the various plant hormones in the defense of *A. thaliana* and *B. napus* against *V. longisporum* appears to be different (Ratzinger et al. 2009). *V. longisporum* infection increases the level of jasmonic acid (JA) in Arabidopsis and activates the corresponding marker genes *VSP2* and *PDF1.2*, but biosynthesis and signaling mutants do not show major differences in disease susceptibility when compared with wild-type plants (Johansson et al. 2006b; Ralhan et al. 2012). However, the treatment of Arabidopsis plants with methyl jasmonate (MeJA) results in enhanced resistance towards *V. longisporum* (Johansson et al. 2006b). Moreover, *V. longisporum* requires JA-independent CORONATINE INSENSITIVE1 (COI1) function in the roots to elicit disease symptoms in Arabidopsis shoots (Ralhan et al. 2012). In oilseed rape, JA concentrations increase over time in both healthy and infected plants (He et al. 2002; Ratzinger et al. 2009). In Arabidopsis, metabolites of the salicylic acid (SA) pathway, salicylic acid glucoside (SAG), and dihydroxybenzoic acid increase after *V. longisporum* infection, and the SA marker genes *PR1* and *PR2* are activated. However, mutants in the SA pathway (*eds1-1*, *NahG*, *npr1-3*, *pad4-1*, and *sid2-1*) do not exhibit enhanced susceptibility, indicating that SA signaling may not contribute to *V. longisporum* resistance in Arabidopsis (Johansson et al. 2006b; Ralhan et al. 2012). In contrast, SA appears to play a role in *B. napus* susceptibility to *V. longisporum* infection (Ratzinger et al. 2009). However, the exact role of the enhanced levels of SA and SAG in xylem sap after infection with *V. longisporum* is not clear (Ratzinger et al. 2009). Ethylene (ET) production and the expression of ET-dependent plant defenses are induced by *V. longisporum* in Arabidopsis. Moreover, pretreatment with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) enhances host resistance to *V. longisporum* (Johansson et al. 2006b). The Arabidopsis mutants impaired in ET signaling, *ein4-1*, *ein2-1* and *ein6-1*, were more susceptible than the wild-type to *V. longisporum*. In contrast, the Arabidopsis mutant *etr1-1* showed enhanced resistance and

a higher chlorophyll content compared with the wild-type, indicating that prolonged ET perception via ETR1 enhances susceptibility via the induction of senescence (Johansson et al. 2006b; Veronese et al. 2003). ABA levels increase after infection with *V. longisporum* in Arabidopsis (Ralhan et al. 2012; Roos et al. 2014; Behrens et al. 2019). The ABA-deficient mutant *aba2-1* is susceptible to *V. longisporum* and accumulates less anthocyanin than wild-type plants, whereas ABA-insensitive mutants do not show enhanced susceptibility (Johansson et al. 2006b; Veronese et al. 2003). Interestingly, *B. napus*, ABA concentrations in roots was altered by *V. longisporum* in the initial phase of infection (Behrens et al. 2019).

### **3.4 Disease Management**

The management of Stem striping is challenging; as current disease control strategies do not provide appropriate protection. Protective or curative control by conventional fungicides is not an option for *V. longisporum*. Although heat treatment of the soil can similarly reduce the viability of microsclerotia, steam-mediated heat treatment and most other heat treatment methods are energy consuming and not cost-effective in most commercial field production systems (Pullman et al. 1981). Nevertheless, there is no evidence of effective heat treatment in the greenhouse conditions.

The more confined host range of *V. longisporum*, in comparison with *V. dahliae*, theoretically facilitates the use of crop rotation as a disease management strategy. However, the persistence of microsclerotia, potential non-brassicaceous reservoir plants (Johansson et al. 2006a) and inadequate weed management may jeopardize the effects of crop rotation.

Few crop rotation studies have been conducted with *V. longisporum*, and more long-term research is needed to determine whether crop rotation could be an effective management strategy. Hitherto, only studies on the impact of fallow treatment in cauliflower fields have been conducted. These suggest that fallow treatment does not reduce microsclerotia accumulation in the soil (França et al. 2013; Subbarao and Hubbard 1999). Moreover, even a 4-year fallow period after a long history of cauliflower cropping did not reduce the microsclerotia density in the soil (França et al. 2013).

Several microorganisms, including bacteria and fungi, can reduce the colonization by, and deleterious effects of, *V. longisporum*, and can thus potentially serve as biological control agents (BCAs), provided that an ecologically fit and effective agent is developed. Specific, non-pathogenic *Verticillium* isolates, such as the *V. isaacii* isolate Vt305, can suppress disease symptoms caused by pathogenic isolates. The strain Vt305 was isolated from a *Verticillium* wilt-suppressive cauliflower field in Belgium (França et al. 2013). Vt305 appears to be an endophyte of cauliflower and shows effective biological control capacities under controlled conditions (Tyvaert et al. 2014).

Resistance breeding is the most favored means of *Verticillium* disease management, and several crops with polygenic *V. longisporum* resistance have been reported (Fradin and Thomma 2006; Kemmochi et al. 2000; Rygulla et al. 2008). Unfortunately, a genuine resistance (*R*) gene against *V. longisporum* has not yet been found, and *Ve1* presently remains the only *R* gene that has been described against *Verticillium* wilts (Fradin et al. 2009; 2011). Although *Ve1* was initially identified in tomato (Kawchuk et al. 2001), functional *Ve1* homologues have also been identified in other plant species, such as *Nicotiana glutinosa* (Zhang et al. 2013), lettuce (Hayes et al. 2011) and cotton (Zhang et al. 2011; 2012). Tomato *Ve1* confers resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum* (presently *V. alfalfae*) which contain the *Ave1* gene (de Jonge et al. 2012). *Ave1* encodes an effector protein that activates *Ve1*-mediated resistance, but *Ave1* contributes to fungal virulence in susceptible plants that lack *Ve1*. Thus far, the functionality of *Ve1*-mediated resistance has not been demonstrated against *V. longisporum*, which has been attributed to the observation that the currently investigated isolates do not carry the *Ave1* gene (Fradin et al. 2011). However, there are genetic resources that may be used to reduce the susceptibility of brassicaceous plants to *V. longisporum* such as deletion of susceptibility genes (Pröbsting et al. 2020) or overexpression of plant defensin genes (Ntui et al. 2010; Ghag et al. 2012; Kaur et al. 2016)

In addition to breeding, resistance sources from outside the Brassicaceae may improve the resistance of current *V. longisporum* hosts. These include sugar beet; whose *BvGLP-1* gene reduces *V. longisporum* disease symptoms in *Arabidopsis*. *BvGLP-1* has high sequence homology to a set of plant germin-like proteins and is highly induced after



nematode (*Heterodera schachtii*) infection of resistant sugar beet plants containing the single dominant resistance gene *Hs1<sup>pro-1</sup>* (Knecht et al. 2010). Furthermore, recently Pröbsting et al. (2020) reported that Loss-of-function of *CRT1a* (Calreticulin) reduces plant susceptibility to *Verticillium longisporum* in both *Arabidopsis thaliana* and oilseed rape (*Brassica napus*).

#### **4 The model plant *Arabidopsis thaliana***

The availability of complete genome sequence and having a small genome size together with the extensive collection of new mutants and germplasm as well as the presence of specialized transformation techniques made *Arabidopsis*, an ideal model plant for plant biology, genetic and molecular researches in general. In addition to this, *Arabidopsis* is susceptible to a limited number of pathogens including viruses, bacteria, fungi, nematodes, insect, pests, and it responds to the pathogen attack in a similar fashion to those of other higher plant species, therefore *Arabidopsis* has been intensively used for plant-pathogen interaction studies. *Arabidopsis* has been established as a host model for *V. dahlia* (Tjamos et al. 2005; Pantelides et al. 2010) and *V. longisporum* (Johansson et al. 2006). The plant can be infected by up-rooting, root dip inoculation and repotting. Disease symptoms such as early senescence, stunting, chlorosis, reduced leaf area and early flowering proved to be associated with infection (Veronese et al. 2003; Johansson et al. 2006).

Thus, the *Arabidopsis-Verticillium* interaction is a suitable model pathosystem for molecular studies. To analyze genetic determinants associated with *V. longisporum*-induced disease, several studies applied pathogen-related mutants, such as mutants involved in hormone biosynthesis or signaling (Veronese et al. 2003; Johansson et al. 2006). Due to different infection systems, which are mainly based on *in-vitro* culture, these studies are often not comparable and differ considerably in their results. Nevertheless, the plant hormone ethylene (ET) appears to be important, as the *etr1-1* receptor mutant shows reduced *V. longisporum*-induced symptoms. In addition, several ET insensitive mutants (*ein2-1*, *ein4-1* and *ein6-1*) are more tolerant to *Verticillium* infection (Veronese et al. 2003; Johansson et al. 2006). Genetic studies identified the *VET1* locus (*VERTICILLIUM-TOLERANCE 1*), which controls both transition to flowering and

increased tolerance to *Verticillium* (Veronese et al. 2003). Disease symptoms could be linked to development (Behrens et al. 2019).

## 5 Aim of the thesis

Plant defensins (PDFs) are known to have antimicrobial/antifungal activities. Up till now, fifteen PDFs have been characterized in *A. thaliana*, yet the specific role and molecular mechanisms of most of them are largely unknown. In this thesis, *A. thaliana-Verticillium longisporum* pathosystem was employed to systematically identify *AtPDF* genes being involved in plant-fungus interaction, particularly in the roots. The primary objective of this study was to identify and distinguish the roles of plant defensin genes (*PDFs*) in the plant-*Verticillium longisporum* interaction. A deep understanding of the molecular mechanisms underlying might greatly help to develop novel strategies and approaches for improvement of plant resistance against this pathogen. This thesis includes the following four chapters:

**Chapter I:** A general overview of plant-pathogen interactions with the focus on plant hormone defense pathways (JA/ET/SA), the role of PDFs and the genetic and molecular aspects of *V. longisporum* pathosystems.

**Chapter II:** This chapter is a manuscript that has been submitted to the Journal “Plant Molecular Biology” for publication. In a previously conducted study, several genetic factors referred to as compatibility factors (CFs) were identified in *Brassica napus* and *Arabidopsis*. Knockout of these factors in oilseed rape as well as in *Arabidopsis* strongly reduced plant susceptibility to *V. longisporum* but along with the significantly elevated *AtPDF2.2* expression in plants. This prompted us to assume that *AtPDF2.2* might act as an antifungal protein. Following this, transcriptional responses of main plant defensin genes of different classes to the fungal infection were characterized and investigated, demonstrating that *AtPDF2.2*, one member of the Class-II defensin presents an antifungal protein and a positive regulator of plant hormone signalings in the plant-*Verticillium* interactions.

**Chapter III:** This chapter is a manuscript to be submitted to the journal “Frontiers in Plant Science” for publication. The study was conducted to comprehend the difference in early transcriptional responses between the two classes of *AtPDFs* to *V. longisporum* infection

in Arabidopsis and their possible impact on the plant-Verticillium interactions. To this end, the gene *AtPDF1.2a*, a well-known JA associated gene from Class-I defensins of Arabidopsis, was employed for this study. It is demonstrated that *AtPDF1.2a* is regulated by JA/ET pathway upon *V. longisporum* infection and negatively interacts with *AtPDF2.2*.

**Chapter IV:** A general discussion, in which the results presented in Chapter II and III are in-depth discussed and evaluated in the light of current literature and knowledge. Finally, a functional model illustrating the role of *AtPDF2.2* and *AtPDF1.2a* in regulating the Arabidopsis -*V. longisporum* interactions are presented.

**6 Reference (see page 146)**

**Chapter II: The fungus *Verticillium longisporum* suppresses plant-defensin *AtPDF2.2* to achieve its colonization on its host *Arabidopsis***

Submitted to *Plant Molecular Biology*

Shailja Singh, Wanzhi Ye, Daguang Cai

Institute of Phytopathology, Department of Molecular Phytopathology and Biotechnology, Christian-Albrechts-University of Kiel, Hermann Rodewald Str. 9, D-24118 Kiel, Germany

**Running head:** Plant defensin *AtPDF2.2* in plant-fungus interactions

**Keywords:** Plant defence response, plant defensin, *AtPDF2.2*, *Verticillium longisporum*, plant-fungus interactions

## 1 Abstract

Plant defensins proved to be functional in plant resistance against phytopathogenic fungi. In oilseed rape and Arabidopsis plants, we had observed that knockout of a candidate gene for susceptibility (CF-1) resulted in plant resistance to the *Verticillium longisporum* infection, accompanied by a strongly enhanced expression of *AtPDF2.2*. To identify the antifungal activity of this gene, we generated *AtPDF2.2*-overexpressing (OE-PDF2.2) and knockdown (*KD-pdf2.2*) Arabidopsis lines and challenged these with the fungal infection. As a result, the knockdown plants showed highly susceptible with much more pronounced fungal colonization and symptom development. However, the overexpression lines promoted resistance and showed impaired development of symptoms and fungal colonization as well. Transcript- and promoter-GUS histochemical staining assays revealed that the *AtPDF2.2* expression levels were drastically changed during the infection process primarily suppressed starting from hyphal penetration till 6 dpi and then gradually elevated in the infected roots and leaves. These results support the hypothesis that *AtPDF2.2* is an antifungal protein and involved in plant resistance defense response to the *V. longisporum* infection. Moreover, the suppression of *AtPDF2.2* expression at the early infection stage might belong to the virulence strategy of *V. longisporum* to initiate/establish a compatible plant-fungus interaction.

## 2 Introduction

*Verticillium longisporum* is a soil-borne fungal pathogen, which especially thrives in northern Europe where it particularly attacks Brassica oil crops (Fahleson et al. 2003; Johansson et al. 2006; Zeise and Tiedemann 2002). *V. longisporum* is a hemibiotrophic, soil born endophytic pathogen, which causes symptoms on the stem of oilseed rape (Depotter et al. 2016). To date, the economic importance and acreage of oilseed rape are increasing because of the growing demand for oil crops for nutrition and bio-fuels (Nath et al. 2016; Robson et al. 2002). This is accompanied by a spread of *Verticillium* diseases, which may cause yield losses as high as 10 to 50% (Dunker et al. 2008). Since conventional fungicides cannot effectively control diseases caused by *V. longisporum*, hence the use of resistant cultivars is the only alternative that has been recommended (Depotter et al. 2016). Breeding of resistant cultivars requires an in-depth understanding of the molecular biology of plant-pathogen interactions that can help in developing novel strategies to breed resistant cultivars.

*Verticillium* infect their hosts by root penetration and subsequently colonize the xylem, where they cause partial clogging of the vessels (Schnathorst 1981; Eynck et al. 2007). These obstructions are expected to affect water and nutrient transport. Therefore, typical disease symptoms such as wilting, stunting, chlorosis, and premature senescence have been observed as consequences of water limitations and insufficient nutrient supply (Johansson et al. 2006). In contrast to other target plants, wilting symptoms were not observed in oilseed rape or in *Arabidopsis* infected with *V. longisporum* (Floerl et al. 2008). Analyses of the plant's nutrient status during the *V. longisporum* infection cycle did not reveal nutrient limitations in these plant species (Floerl et al. 2008; Floerl et al. 2010). Instead, the *V. longisporum* infected plants exhibited severe stunting indicating that the plant-pathogen interaction resulted in extensive re-modeling of plant architecture (Floerl et al. 2008; Floerl et al. 2010; Ratzinger et al. 2009). Therefore, the disease is termed as 'Verticillium stem striping' instead of Verticillium wilt (Depotter et al. 2016).

Antimicrobial peptides (AMPs) are believed to serve as one of the first lines of defense against pathogen invasion and are one of the key contributors to plant innate immunity in plants (Goyal et al. 2014).

Plant defensins (PDFs) are a group of (AMPs) with a molecular weight in the range of 5 kDa that have been found ubiquitously in the plant kingdom (Osborn et al. 1995; Broekaert et al. 1997; Shewry and Lucas 1997; Osborn and Broekaert 1999; Thomma et al. 2002). So far, over 300 cysteine-rich defensin-like genes in *Arabidopsis* (Silverstein 2015) and *Medicago truncatula* (Graham 2004) were reported. Van der Weerden and Anderson (2013) have classified plant defensins into 18 groups. These are all small, basic, and cysteine-rich peptides that exhibit a three-dimensional folding pattern stabilized by eight cysteine residues linked by four disulfide bridges (Broekaert et al. 1995; Almeida et al. 2002). They have been isolated from seeds (Broekaert et al. 1995; Thomma et al. 2003), roots (Sharma and Lönneborg 1996), leaves (Segura et al. 1998; Do et al. 2004) and pods (Chiang and Hadwiger 1991).

Increasing data demonstrate that plant defensins are involved in the resistance of plants against pathogens, especially fungi. Generally, PDFs are abundantly present in the stomatal and peripheral cells, the entry point of plant pathogens, it shows their primary potential effect against on the invading pathogens (Broekaert et al. 1995). Some of the PDFs are found to be involved in signal transduction pathways (Lay and Anderson 2005). In growing seeds, they are expressed to prevent the newly formed radical tissues from fungal invasion (Stotz et al. 2009). Some PDFs, as reported, also inhibited protein formation (Mendez et al. 1990). In addition, overexpression of a tomato PDF, *DEF2* in transgenic tomato caused decreased pollen viability and low seed yield (Stotz et al. 2009). Expression of the radish plant defensin *AFP2* in tobacco resulted in a reduction of the average lesion area after inoculation with *Alternaria longipes* (Terras et al. 1995). In some cases, it has been shown that plant defensins are induced after pathogen attack (Terras et al. 1995; Chiang et al. 1991). Transgenic plants expressing PDFs have been reported to have an increased resistance to diverse fungal pathogens. For example, *WT1* from wasabi when overexpressed in rice; potato and orchid resulted in increased resistance against *Magnaporthe grisea*, *Erwinia carotovora* and *Botrytis cinerea* (Kanzaki et al. 2002; Lay et al. 2005; Stotz et al. 2009). Transgenic tomato plants containing PDF, *Rs-AFP2* decreased the activity of phytopathogenic fungi, including *Alternaria solani*, *F. oxysporum*, *Phytophthora infestans*, and *Rhizoctonia solani* (Lacerda et al. 2014). Overexpression of *Rs-AFP2* in transgenic rice (*Oryza sativa*) reduces *Magnaporthe*

*oryzae* and *Rhizoctonia solani* infection. These two fungi are the main causative agents for rice blast and sheath blight diseases, which leads to rice losses in agriculture (Jha et al. 2010). Pea PDFs enhanced resistance towards blackleg diseases in *B. napus*, which is caused by *Leptosphaeria maculans* (Wang et al. 1999). Similarly, transgenic *Triticum aestivum* genotypes expressing a chimeric gene encoding an apoplast-targeted antifungal plant PDF, *MtDef4.2* from *M. truncatula*, displayed resistance leaf rust pathogens without affecting the root colonization of a beneficial arbuscular mycorrhizal fungus, *Rhizophagus irregularis*. Nevertheless, the underlying mechanism remains largely unclear, so far.

It has been demonstrated that plant defensins can directly attack or inhibit invading microbes and parasitic plants (de Zélicourt et al. 2007). Initially, it was reported as barley (Mendez et al. 1990) and wheat (Colilla et al. 1990)  $\gamma$ -thionins, which possess structural similarity to animal defensins (Bruix et al. 1993; Terras et al. 1995). Analogous to animal defensins plant defensins (PDFs) interfere with pathogen protein synthesis and enzyme function (Mendez et al. 1990; Méndez et al. 1996) and defensins can move into pathogen cytoplasm (Van Der Weerden et al. 2008; van der Weerden et al. 2010). *Triticum aestivum* defensin 1 (*Tad1*) is expressed in the crown and possesses antipathogen properties (Koike et al. 2002). Defensins inhibit proteases (Wijaya et al. 2000), trigger pathogen ROS production (Aerts et al. 2007), and block ion signaling (Spelbrink et al. 2004). Unlike animal defensins that inhibit bacterial growth, many plant defensins are antifungal and are especially active in seeds. They make up 0.5% of the total seed protein and a substantial amount of proteins that are released from the seed coats (Stotz et al. 2009). Defensins have been found in many tissues (García-Olmedo et al. 1998) and induced during seasonal changes (Koike et al. 2002). It has been reported that C-terminal hydrophobic and  $\gamma$ -core regions are critical for membrane interaction and antifungal activity, respectively (De Paula et al. 2011; Lacerda et al. 2014). In addition, defensins contain scorpion toxin-like, knottin, and purothionin domains with conserved cysteine residues that form a cysteine-knot structure, composed of disulfide bridges. Scorpion toxins and some plant defensins block potassium channels using similar protein domains (Vriens et al. 2016; Almeida et al. 2004). Defensins can cause an increase in pathogen membrane permeability that initiates necrosis (Brogden 2005).



TBLASTN search on the fully sequenced genome of Arabidopsis revealed a total of 15 putative plant defensin genes (Thomma et al. 2002). The deduced putative protein sequences can be classified into three families, the PDF Class-I, II and III (Thomma et al. 2002; Penninckx et al. 1996; Epple et al. 1997; Thomma and Broekaert 1998).

Class-I contains seven genes encoding defensins (*AtPDF1.1* to *AtPDF1.5*), including the three to date purified plant defensins *AtPDF1.1*, *AtPDF1.2* and *AtPDF1.3* (Penninckx et al. 1996; Terras et al. 1993; Silverstein et al. 2007) respectively. Among Class-I, five genes show high sequence similarity (*AtPDF1.1* to *AtPDF1.3*); three genes (*AtPDF1.2a*, *b*, and *c*) encode the same mature peptide (*AtPDF1.2*), only showing a difference in their corresponding signal peptide sequences. The *AtPDF1.2a* and *AtPDF1.2c* gene sequences are located in tandem repeat on chromosome 5, while *AtPDF1.2b* and *AtPDF1.3* are clustered in tandem repeat on chromosome 2 (Jan et al. 2008). The first family members *AtPDF1.1*, *1.2a*, *1.2b*, *1.2c*, *1.3* and to some extent *1.4* were found induced in the non-host response of Arabidopsis to the barley powdery mildew fungus (Jan et al. 2008). Overexpression of *AtPDF1.1* resulted in enhanced resistance of Arabidopsis plants against *Cercospora beticola* and *P. carotovorum* (De Coninck et al. 2010; Pao-Yuan et al. 2017). *AtPDF1.2* gene is induced by pathogen challenge both locally at the site of inoculation by incompatible fungal pathogen and systemically in remote non-inoculated regions of the plant (Penninckx et al. 1996). However, whether the second family plant defensins mediate fungal tolerance remains unknown.

Class-II members are found in close proximity. The putative protein sequences of the *AtPDF2*-family show relatively more variation at the amino acid level (Thomma et al. 2002). The *AtPDF2.1*, *2.3* and *2.6* occur in a tandem array and *AtPDF2.2* is separated from this array by one gene on chromosomes 2, while *AtPDF2.4* and *AtPDF2.5* are located on chromosomes 1 and 5, respectively (Jan et al. 2008). Possibly this separation is caused by shuffling, a phenomenon that frequently happened in the Arabidopsis genome (The Arabidopsis Genome Initiative 2000). A six-member multigenic family that is well described in Arabidopsis (Thomma et al. 2002), is of particular importance. Among these defensins, *AtPDF2.3* possesses potassium channel-blocking activity (Kim et al.

2016), *AtPDF2.5* and *AtPDF2.6* significantly enhanced Cd tolerance (Jin-Song et al. 2019). But so far, no information about the function of *AtPDF2.2*.

Class-III contains two genes. They were identified to encode proteins containing only a plant defensin domain (Thomma et al. 2002). Gene *AtPDF3.1* (*At4g30070*) encodes a protein of 129 amino acids, of which the C-terminal domain displays the conserved cysteine pattern shared by all plant defensins in addition to a number of residues that are conserved among the Arabidopsis plant defensin gene family. The second gene, *AtPDF3.1* (*At5g38330*) encodes a similar protein consisting of 122 amino acids, sharing 56% identical residues with the protein encoded by the gene *At4g30070*. Both proteins have a putative 26-amino-acid signal sequence. The amino-terminal domains of both proteins are rich in cysteine residues but do not share significant homology to any previously characterized protein. Possibly, these proteins are fusion proteins. Alternatively, these proteins could be precursors, as reported for a tobacco defensin and thionins (Bohlmann and Apel 1991; Gu et al. 1992). Their exact functions are not known yet.

It was observed that knockout of a candidate compatibility factor gene (CF-1) resulted in plant resistance to the *V. longisporum* infection, which was however accompanied with a strongly enhanced expression of *PDF2.2* (Pröbsting et al. 2020). This observation prompted us to hypothesize an antifungal role/function of *PDF2.2*. Here, we demonstrated that *AtPDF2.2* is functionally involved in plant resistance response to the *V. longisporum* infection. However, the fungus has evolved a virulence strategy to suppress the expression of *AtPDF2.2* at the early infection stage, probably a crucial step to initiate/establish a compatible plant-fungus interaction in its host. Furthermore, we report that upon *V. longisporum* infection, *AtPDF2.2* intervenes in the expression of responsive genes for JA, ET, and SA pathway as well.

### 3 Materials and methods

#### 3.1 Plant material and growth conditions

*Arabidopsis* ecotype Columbia (Col-0) was the genetic background for all wild type and transgenic plants. The mutant *jar1*, *coi1-16*, *acs1-9*, *ein2-1*, *ein2-1coi1-16*, and *sid2* were obtained from the Nottingham Arabidopsis Stock Centre. The specification of the mutant is listed in Table S4. Genotyping was performed according to the instructions of the SALK-Institute website (<http://signal.salk.edu/tdnaprimers.2.html>). Primers used for genotyping are listed in Supplementary Table S5.

Seeds were surface-sterilized with 5% sodium hypochlorite/Tween 20 solution and 70% ethanol followed by three times washing with sterile water and placed on Petri dishes with half-strength MS media supplemented 0.8% (w/v) sucrose, 0.1% (w/v) 2-(*N*-morpholino) ethanesulfonic acid (MES), and 0.8% (w/v) agar (Murashige and Skoog 1962). After cold treatment at 4°C for 48 h, plates were incubated vertically for 14 days at 22°C under short day conditions (8h light/16h dark, 100  $\mu\text{moles m}^2 \text{sec}^{-1}$ ). EMS mutant plants were grown for 30 days at 16°C under short-day conditions (8h light/16h dark, 100  $\mu\text{moles m}^2 \text{sec}^{-1}$ ).

#### 3.2 *In-silico* analysis

The Arabidopsis Information Resource (TAIR) used for genes hits (*AtPDFs*) blast. The DNA sequences. The “ClustalW2” program in EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) was used for construction of the multiple sequences alignment and Maximum-Likelihood phylogenetic analysis using a distance-based tree that was inferred with the BioNJ algorithm (Gascuel 1997) using PhyML v20160115 (Guindon et al. 2010) ran with model GTR and parameters: -f m --pinv e -o lr --alpha e --nclasses 4 --bootstrap -2.

Expression patterns of *AtPDF2.2* gene in different stages of development (in different tissues) and in response to various abiotic and biotic stresses was analyzed with the help of a free version of the GENEVESTIGATOR (Hruz et al. 2008) online portal.

### 3.3 Trypan blue staining

To investigate disease symptoms associated with fungal growth, infected tissues were stained with trypan blue to visualize the extent of fungal growth (Fernández-Bautista et al. 2016). Control and infected tissues were immersed in trypan blue staining solution (30 mg trypan blue, 10 ml glycerol, 10 ml phenol equilibrated in 10 mM Tris, 1 mM EDTA buffer, 30 ml distilled water) for 60 min at room temperature. Tissues were destained several times with 90% ethanol until all chloroform was completely removed. Stained tissues were photographed using the AxioVision rel. 4.8 microscope (Carl Zeiss Microscopy GmbH).

### 3.4 Pathogen infection procedures and plant inoculation

*Verticillium longisporum* isolate VI43 (kindly provided by Dr. Elke Diederichsen (FU Berlin, Germany) was maintained on potato dextrose agar (PDA, Merck, Darmstadt, Germany) in petri dishes. For the inoculation, 22% glycerol conidia stocks were thawed, centrifuged for 8 min at 6000g and re-suspended in tap water (“*in-vivo*” infection) or sterile Czapek-Dox medium (“*in-vitro*” infection) to a final concentration of  $2 \times 10^6$  conidia / ml. *In-vitro* and *in-vivo* root inoculation experiments were conducted (Behrens et al. 2019). The plants were allowed to grow for up to a further two-three weeks followed by fungal DNA quantification and root length measurement at different time points in infected plants in relation to the mean of mock-treated controls.

The *S. sclerotiorum* isolate used throughout this work was obtained from Professor W. Qian (Mei et al. 2011). Infection of Arabidopsis leaves with *S. sclerotiorum* was performed either by agar-plug infection or by drop infection to test for differences in plant resistance between genotypes. *S. sclerotiorum* liquid culture was prepared as described by Rietz et al. (2012) with minor modifications. 5 weeks old Arabidopsis plants were equally spray inoculated with mock (10mM MgSO<sub>4</sub>), or *Sclerotinia* suspension using a standard spray bottle with 2 g of homogenized mycelium suspended in 50ml 10mM MgSO<sub>4</sub>. Plants were kept under the hood throughout the experiment and grown in short-day conditions (8h light, 22°C). Lesions were classified for all fully expanded leaves individually from 6 to 48 hpi and disease severity index were summarized for each plant after 72 hours. Photos of infected plants were taken at 7 dpi.

*P. syringae* (Pst DC3000), was cultivated on plates containing NYGA medium (5 g/l tryptone/peptone, 3 g/l yeast extract, 20 ml/l glycerol, 15 g/l agar, 50 mg/l Kanamycin, 100 mg/l Rifampicin) and incubated for 2 days at 28°C. Afterward, the bacteria were washed from the plate with 10mM MgCl<sub>2</sub> and the concentration was adjusted to OD<sub>600</sub>= 0,2 for infection. 5 week old Arabidopsis plants grown in individual pots were equally spray inoculated with mock (10mM MgCl<sub>2</sub>), or *Pseudomonas* suspension mixed with 0.04% Silwet Gold using a standard spray bottle. Plants were kept under the hood for 2 days and grown in short-day conditions (8h light, 22°C). Photos of infected and mock-treated plants were taken at 1 and 3 dpi. Bacterial abundance was quantified by a DNA based method proposed by Ross et al. (2016). Samples were collected from two plants (3 leaf discs each).

### **3.5 RNA Isolation**

Total RNA was extracted from 100 mg leaves of the WT and transgenic lines TRIzol® (Thermo Fisher Scientific) reagent according to the manufacturer's recommendation. The quality and quantity of RNA samples were analyzed by agarose gel electrophoresis and NanoVue Plus Spectrophotometer (GE Healthcare Life Science).

### **3.6 Expression analysis by quantitative Real-Time PCR**

The RNA (n=3) was isolated as described above. One µg of RNA was transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) into the first-strand cDNA according to supplier's instructions using oligo-dT primers for mRNA transcription. 2µl of a 1:10 diluted cDNA preparation was mixed with 18 µl master mix as per the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) manual. PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 3 min 95°C, 45 x 15 sec 95°C, 15 sec 58°C, 20 sec 72°C, 10 sec 95°C. Gene expression was determined using the delta C<sub>T</sub> Method to calculate the relative expression according to Pfaffl (2001). Relative expression was normalized to *ACTIN 2* (AT3G18780) as a reference gene. Each data point is based on three independent biological replicates measured with two technical replicates each. Relative expression was log<sub>2</sub> transformed and, to display the differential regulation, the induction

was calculated as the difference between *V. longisporum* and mock-treated samples. All the primer pairs utilized are listed in Table S1.

### 3.7 Gene constructs and plant transformation

A 1519 bp genomic fragment immediately upstream of the *AtPDF2.2* start codon was PCR amplified using the primer pair for Pro::AtPDF2.2 (Table S1). Promoter-PCR product cloned into pDONR201 entry vector followed by a recombination reaction using the Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific) and subcloned into pGWB433 (NAKAGAWA 2007) binary vector to generate Pro::AtPDF2.2::GUS construct (Fig. 3c).

The knockdown mutants for *AtPDF2.2* was generated by PCR amplification of 293 bp long coding region with the stop codon, in an antisense orientation under the control of the CAMV-35S promoter (Fig. 5a). Primer pair for *KD-pdf2.2* are listed in (Table S1). This product was then sub-cloned into pGWB402 (NAKAGAWA 2007) using the Gateway binary vector. The binding of antisense deoxy oligonucleotide and target mRNA leads to the formation of a DNA-RNA duplex, which is unstable and is recognized by RNase- H that selectively degrades the RNA strand in a DNA-RNA duplex, thus inhibiting translation.

For overexpression of *AtPDF2.2* in Arabidopsis, the full-length *AtPDF2.2* coding region was PCR amplified from the cDNA prepared from the leaves of Arabidopsis wild-type (ecotype Columbia-0) (Fig. 5a) with gene-specific primers (listed in table S1) using Pfu Polymerase (Thermo Fisher Scientific). The cDNA was cloned into pDONR201 entry vector followed by a recombination reaction using the Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific) and subcloned into pGWB433 (NAKAGAWA 2007), in the sense orientation.

All the constructs were after re-sequencing brought into *Agrobacterium tumefaciens* GV3101:pMP90RK to facilitate plant transformation. Followed by transformation into Arabidopsis using the floral dip method (Clough and Bent 1998). The seeds were harvested from the infiltrated plants and positive plants were selected on half Murashige and Skoog's (MS) medium supplemented with 50 mg L<sup>-1</sup> kanamycin (Sigma-Aldrich, United States). The kanamycin-resistant plants were transferred to the soil after 10 days of germination and were grown in a growth chamber.

### 3.8 gDNA isolation and PCR analysis of transgenic Arabidopsis plants

For gDNA isolation, the tissue was immediately frozen in liquid nitrogen and grounded using a mortar and pestle. Total genomic DNA was isolated using CTAB (cetyltrimethylammonium bromide) buffer (Rogers and Bendich, 1985) supplemented with  $\beta$ -Mercaptoethanol. For PCR, the reaction mixture contained 100 ng gDNA, 2 mL 10 Taq buffer, 1 mL of 10 pmol each of forward and reverse gene-specific primers, 1 mL of 10 mM dNTP mixture (Thermo Scientific, Germany), and 0.4 mL of Taq DNA polymerase (3 U/mL) (Thermo Scientific, Germany), and the volume was made up to 20 mL using autoclaved MilliQ water. The PCR was performed under the following conditions: initial denaturation at 95°C x 10 min followed by 30 cycles at 95°C x 45 secs, 60°C x 30 secs, and 72°C x 40 secs, and a final extension at 72°C x 7 min. The PCR product was visualized by electrophoresis on a 1% agarose gel. The expression of *AtPDF2.2* in the transgenic plants was assessed using RT-qPCR.

### 3.9 Expression, GUS histochemical analysis and subcellular localization

Arabidopsis seedlings were grown in half-strength MS medium until 2 weeks of age before being subjected to the indicated treatments, and then sampled. Total RNA was extracted and cDNA was prepared followed by RT-qPCR as described above. The primers utilized are listed in Table S1, and the expression level of *AtPDF2.2* was normalized to that of *ACTIN 2*.

To confirm the histological expression pattern of glucuronidase (GUS) driven by *AtPDF2.2* promoter, was performed as described by Rietz (2010) in the Prom::*AtPDF2.2*::GUS plants.

### 3.10 Mutant identification and plant genotyping

Some mutants used in this analysis, *jar1*, *acs1-7*, *ein2*, and *sid2* had T-DNA insertions in exon region. Plant genomic DNA was extracted using the earlier described gDNA isolation method. The T-DNA insertions were checked using LBb, LP and RP primers, which were obtained using T-DNA Primer Design (<http://signal.salk.edu/tdnaprimers.2.html>). The mutant line was in the Arabidopsis background. Table S5 shows a list of PCR primers used for the genotyping of mutations. The genotypes of plants were determined by PCR with allele-specific primers.

For other EMS mutants, *coi1-16* and *ein2-1coi1-16* plants, the *COI1* gene was amplified from genomic DNA using Pfu Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. The amplified PCR products were sequenced as described previously by Ellis et al. (2002).

### 3.11 VI43 gDNA detection in plant tissue

Arabidopsis leaves and roots of six infected plants were sampled at the indicated time points and were sampled individually at indicated time points post-inoculation. gDNA isolation was performed as described above. For real-time qPCR quantification of fungal DNA in infected plants, the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used according to the manufacturer's instructions. Primers used are listed in Table S1. PCR was performed using the following conditions: 3 min x 95°C, 35 x 20 sec 95°C, 20 sec 58°C, 30 secs 72°C. Error bars indicate the standard deviation ( $\pm$ SD) of three different biological repetitions and statistic was performed using the student's t-test (\* $p \leq 0.05$ ). For each sample, the amount of fungal DNA in relation to plant DNA was calculated in ng/mg.

### 3.12 Morphology analysis

Wild-type Arabidopsis plants (Col-0) and transgenic knockdown (*KD-pdf2.2*) and overexpression (OE-PDF2.2) Arabidopsis plants were used for the measurement of differences in plant's morphology. Around 15 seedlings from each line were grown on single 120x120 mm petri dishes similarly like *in-vitro* infection assay that is stated above. The morphological differences between the 20 days old vertically growing transgenic lines and wild types were recorded after the transference on the 120x120 mm petri dishes containing ½ MS (0.5% sucrose) and pictures were taken. These pictures were later analyzed on the Image J software (<https://imagej.nih.gov/ij/download.html>) for measuring root length and number of lateral roots for each plant using the provided reference measurement to the software.

After two days of cold stratification, the Col-0 wild-type, *KD-pdf2.2*, and OE-PDF2.2 seeds were sown and germinated in pot soil. Plants were grown under short-day conditions till the flowering stage and were monitored each day and the number of plants that flowered



(bolted) was recorded. These plants were photographed as well on a particular day to measure the rosette area using Image J software.

### **3.13 Statistical analysis**

All experiments were conducted using a completely randomized design. Four samples used as replicates and two technical replicates were used for each treatment. The data were analyzed using a two-tailed Student's t-test and the differences were considered significant at  $P < 0.05$  and extremely significant at  $P < 0.01$ . Multiple comparisons of statistical significance were carried out using a two-way ANOVA. Graphs and plots were generated with Microsoft® Office 2016. Phenotypic observations were monitored for two to three generations for consistency.

### **3.14 Accession number**

The sequence data of *AtPDF2.2* can be found in the Arabidopsis Information Resource (TAIR) database with the accession number AT2G02100. Additional sequence data are shown in Table S1 and S5.

## 4 Results

### 4.1 Transcript analysis indicates suppression of *AtPDF2.2* in Arabidopsis roots at the early infection stage

Multiple sequence alignment and phylogenetic tree analysis of Arabidopsis *AtPDF* genes (Fig. 1A, S1) revealed the relationships among three classes of *AtPDF* proteins and the conserved cysteine residues, which were shaded in black. These cysteine residues are engaged in four intra-chain disulfide bridges that are believed to be responsible for the stabilization of the three-dimensional structure. These intrachain disulfide bridges form the CS $\alpha$  $\beta$  motif that is well documented in peptides that are endowed with antimicrobial activity (Andre and Valdirene 2009). The phylogenetic tree suggested that, *AtPDF2.2* shared high identity (75 and 71% respectively) with two other closely related *AtPDF* genes, described as *AtPDF2.3* and *2.1* from Arabidopsis Class-II *AtPDF* proteins (Thomma et al. 2002; Fig. S2; Tab. S2 and S3). Class-II *AtPDF* members exist in close proximity where *AtPDF2.1*, *2.3* and *2.6* occur in a tandem array while *AtPDF2.2* is separated from this array just by one gene.

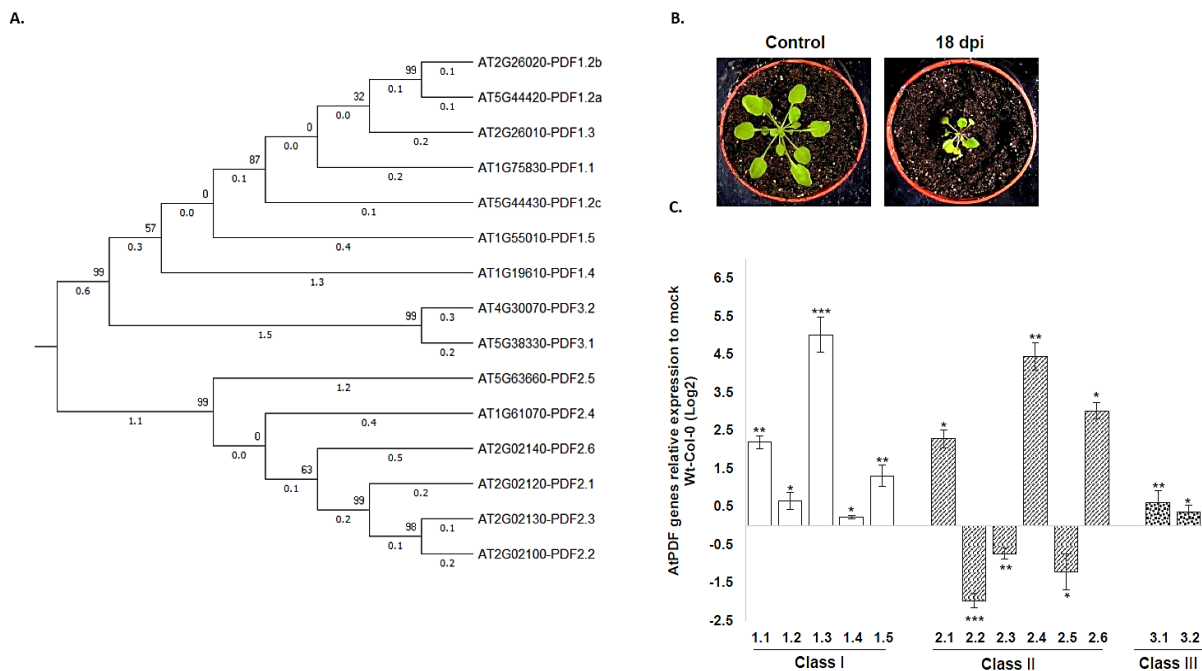


Figure 1 | *AtPDF* genes expression analysis to *V. longisporum* infection in Arabidopsis. (A) Phylogenetic tree based on DNA sequences indicating the relationships among three classes of *AtPDF* proteins using CLUSTAL W using a distance-based tree. (B) Infection of 3 weeks old Arabidopsis plants (ecotype Col-0) with *V. longisporum*. Infected Arabidopsis plants at 18 dpi. (C) Quantification of the relative abundance of the Arabidopsis *AtPDF* genes Class I, II and III (*AtPDF1.1*, *AtPDF1.2*, *AtPDF1.3*, *AtPDF1.4*,

*AtPDF1.5*, *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.4*, *AtPDF2.5*, *AtPDF2.6*, *AtPDF3.1* and *AtPDF3.2*) transcripts in Control- and/or *V. longisporum* inoculated wild-type (Col0). The *AtPDFs* transcript levels were normalized to the expression of *ACTIN 2* measured in the same samples and expressed logarithmically. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

In the next step, the expression of *AtPDFs* in Arabidopsis plants in response to the *V. longisporum* infection was investigated, in which non-infected plant served as a control. As expected, the infected plants at 18dpi displayed severely retarded growth and necrosis symptoms relative to non-infected control (Fig. 1B). Different plant parts at 3 dpi (Fig. 2) stained with trypan blue were examined for the presence of endophytic fungal structures. Intra and intercellular hyphae were seen in the leaves, stems and roots cortex parallel to the longitudinal axis of the plant parts (Fig. 2A, C, E) and intra-cellular, conidia and hypae-like structures were observed (Fig. 2B, D, F), suggesting a successful infection progression of *V. longisporum*.

We examined the transcriptional response of *AtPDFs* to the *V. longisporum* infection in the 6 dpi roots. Contrast to most *AtPDFs*, the expression of *AtPDF2.2* was drastically downregulated by *V. longisporum* infection (Fig. 1C). The expression levels of two other members of Class-II, *AtPDF2.3* and *2.5* were also reduced, whereas other *AtPDF* genes of all the three classes were significantly upregulated. These results indicate a specific suppression of the *AtPDF2.2* expression in the roots by the *V. longisporum* infection.

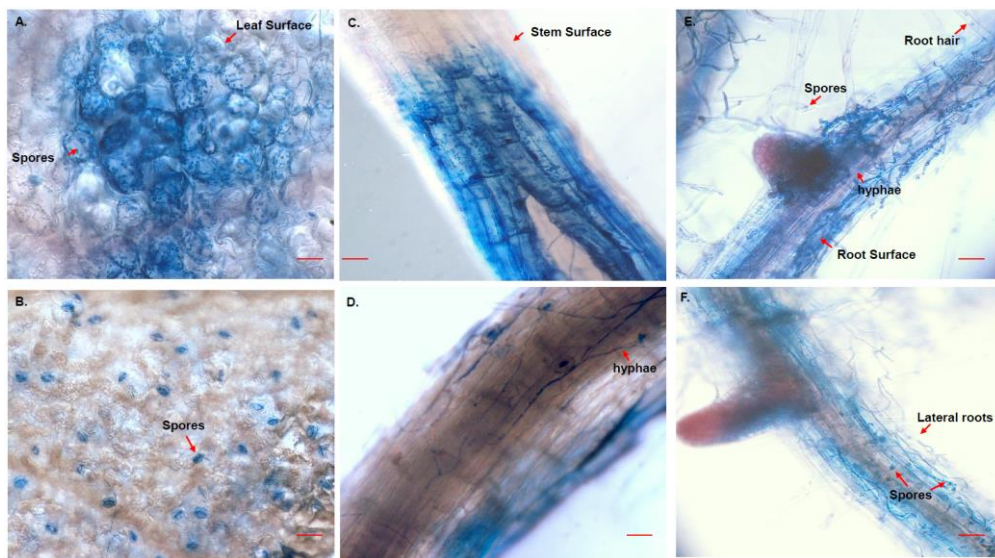


Figure 2 | Confirmation of successful *V. longisporum* colonisation in Arabidopsis using Trypan blue staining. Trypan blue staining of *V. longisporum* infected Arabidopsis plants (Wt) at 4 dpi (A, B) *V. longisporum* colonisation in the leaves; (C, D) in stem; (E, F) in the roots. Roots were heavily colonised at indicated time

point. The trypan blue staining experiments were repeated at least three times. Scale bars: a, c, e = 50  $\mu\text{m}$  and b, d, f = 20  $\mu\text{m}$ .

#### **4.2 *AtPDF2.2* is constitutively expressed in plants and suppressed by the *V. longisporum* infection at the early infection stage**

To gain insight into the *AtPDF2.2* gene regulation, a 1519 bp long *AtPDF2.2* promoter was fused to the GUS reporter gene resulting in the gene construct Prom::*AtPDF2.2*::GUS. In addition, the *AtPDF2.2* promoter sequences were subjected to a motif search (Fig. 3A, B). Transgenic plants carrying the Prom::*AtPDF2.2*::GUS construct was employed for GUS assessments in different tissues of non-infected plants, including seedlings, leaves, roots, flowers, siliques, and after wounding. At 20 DAG (days after germination) the GUS staining in non-infected seedlings showed strong GUS staining (Fig. S3B: i-vi) in cotyledons, leaves, trichomes, and roots. Within root tissues, the GUS staining was more intense in the central cylinder and root tips as well. On the other hand, at 40 DAG, the GUS expression was observed at the outer edges of the leaves, flowers, roots, and siliques but not observed in the root tip, trichomes and seeds any more (Fig. S3B: vii-xvi). Moreover, the GUS expression in the non-infected plant was observed after wounding on intact leaves or stem. These data suggest a high steady-state expression level of *AtPDF2.2* in Arabidopsis plants.

For confirmation, *AtPDF2.2* expression pattern was investigated by microarray data from Genevestigator (Hruz et al. 2008), including six development stages and different plant tissues and organs of Arabidopsis plants. A constitutive expression of *AtPDF2.2* across all developmental stages and tissues/organs were shown (Fig. S3C, D, E). During development, *AtPDF2.2* shows almost constant expression levels, primarily ranging from the germination to mature siliques/seed stage, with maximal or near-maximal expression intensity in mature siliques/seeds. Also, *AtPDF2.2* shows nearly similar expression patterns in the different tissues, ranging from seedling hypocotyl-radicle through most floral tissues/organs as well as siliques and seeds, but with higher expression levels in floral stamens and seeds. However, GUS staining was not detected for seeds in the *AtPDF2.2* promoter analysis. Only one organ displayed medium-range expressions in some tissues of roots like root epidermis and quiescent center protoplast etc. The high steady-state expression levels of *AtPDF2.2* in all the developmental stages and

tissues/organs strongly suggests the importance of this gene in plant growth and development.

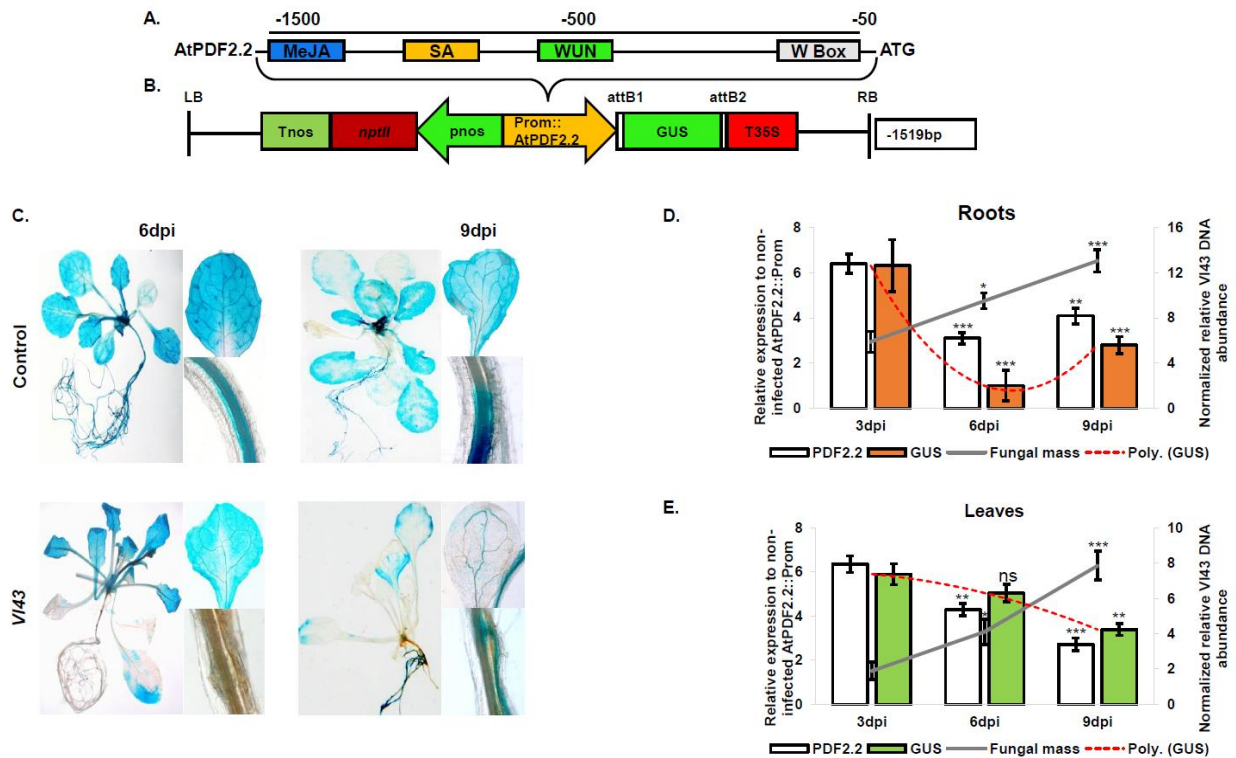


Figure 3 | GUS analysis in the *V. longisporum* infected Prom::AtPDF2.2::GUS plants at different time points. (A) *In-silico* analysis of *cis*-regulatory elements in candidate gene promoter AtPDF2.2 in Arabidopsis using the online tool PLACE. Colored boxes represent specific *cis*-elements; Blue box: MeJA-response CGTCA motif, Yellow box: Element involved in SA response, Green box: Fungal elicitor element, Grey box: TC-rich repeat- involved in defense and stress response. (B) A schematic drawing of Prom::AtPDF2.2::GUS construct used in this study. (C) There was little to no GUS expression in roots at 6 dpi and similarly in leaves at 12 dpi. (D) AtPDF2.2 and GUS transcript levels in the *V. longisporum* infected roots (blank bars) and (E) leaves (green dotted bars) of Prom::AtPDF2.2::GUS seedlings were quantified by RT-qPCR at indicated time points, together with fungal abundance quantification (grey line). Comparatively lower fungal DNA was detectable in the leaf than root at all indicated time points. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

Two-week-old, Prom::AtPDF2.2::GUS plants were challenged with the *V. longisporum* infection. Roots of the infected plants were stained each day from 1 to 17 dpi (Fig. S4) in which the non-infected roots served as a control. Unexpectedly, an extremely faint GUS staining was given at 6 dpi (Fig. 3D), and similarly in the leaves at 9 dpi as compared with the non- infected control plants. But, at 12 dpi the GUS staining was detectable in both the roots and leaves of the AtPDF2.2::GUS-transgenic lines, respectively (Fig. S4, S7).

The transcript levels of *AtPDF2.2* and *GUS* in the Prom::*AtPDF2.2*::*GUS* and Arabidopsis wild-type plants in response to the *V. longisporum* infection was comparatively investigated by RT-qPCR (Fig. 3D, E). The expression of *GUS* gene in Prom::*AtPDF2.2*::*GUS* and *AtPDF2.2* in the wild-type Arabidopsis plants were both considerably downregulated in roots at 6 dpi and a similar downregulated expression was observed at 9 dpi in stems or leaves. Overall, as compared with the control, *V. longisporum*-inoculated plants showed lower *AtPDF2.2* transcript abundance from 5 dpi to 9 dpi, which was however gradually elevated from 10 dpi (Fig. S4). Additionally, fungal biomass gradually increased from 3 dpi to 12 dpi, which was higher in roots relative to stems or leaves (Fig. 3D, E), as determined with qPCR.

### **4.3 Knockdown and overexpression of *AtPDF2.2* alter the plant phenotype and flowering time**

*AtPDF2.2*-overexpression, OE-PDF2.2 and knockdown, *KD-pdf2.2* lines of transgenic Arabidopsis plants were generated, respectively. Transgenic nature and expression of *AtPDF2.2* was measured in T3 individuals of the four independent transgenic Arabidopsis plants of *KD-pdf2.2* and OE-PDF2.2, by using RT-qPCR, respectively. Relative expression of *AtPDF2.2* was reduced by 1-3 folds in the *KD-pdf2.2* while 2-4 folds increased in OE-PDF2.2 plants as compared to wild-type (Fig. 4G, H).

Subsequently, we examined the transgenic effect on the phenotype of *KD-pdf2.2* and OE-PDF2.2 plants as compared to the Col-0 wild-type in respect of their root length, growth, flowering time (bolting days), and rosette size, respectively. A significant difference in growth was observed between the *KD-pdf2.2* and OE-PDF2.2 transgenic plants as compared to the Col-0 wild-type. The seedlings of *KD-pdf2.2* had shorter primary, but more lateral roots than wild-type, whereas OE-PDF2.2 had longer primary and comparable lateral roots to wild-type seedlings (Fig. 4B, D). It was observed when grown under short-day conditions, *KD-pdf2.2* plants flowered 20-25 days earlier than the Col-0 wild-type and OE-PDF2.2 plants (Fig. 4C, E). Mostly all *KD-pdf2.2* plants flowered very early around 30-32 days. In contrast, rarely Col-0 wild type and OE-35S-PDF2.2 plants flowered before 50-60 days. This effect was particularly conspicuous when plants were



grown under long-day conditions, so that *KD-pdf2.2* plants were unable to reach the required flowering stage to produce viable seeds.

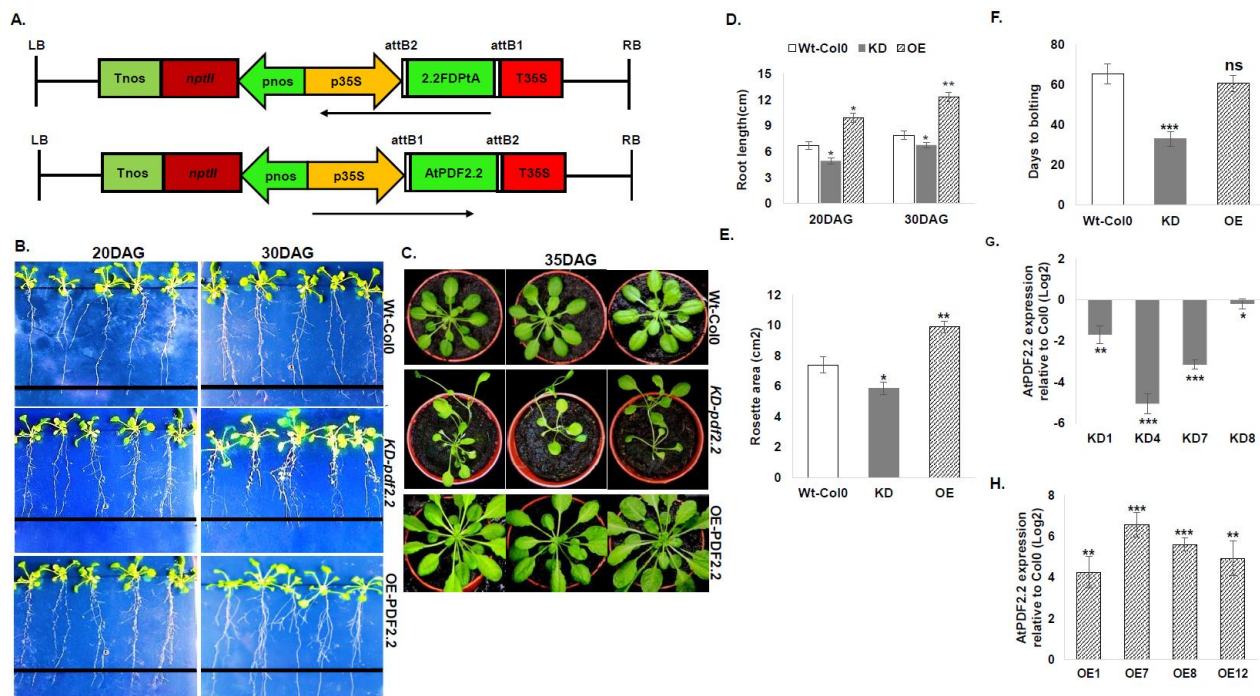


Figure 4 | Phenotyping of *AtPDF2.2* transgenic *Arabidopsis* plants.

(A) A schematic drawing of the constructs used for the knockdown (gene in the antisense orientation) and overexpression (gene in the antisense orientation) analysis. Phenotypes of wild-type (*Wt-Col0*) and *AtPDF2.2* transgenic lines for knockdown (*KD-pdf2.2*) and overexpression (*OE-PDF2.2*). (B, D) Twenty and thirty-day-old seedlings of *Wt-Col0*, *KD-pdf2.2*, and *OE-PDF2.2* promote contrasting primary root growth. Seedlings were grown on vertical 1/2MS agar medium and primary root length was measured for 25-30 seedlings. (C) *Wt-Col0*, *KD-pdf2.2*, and *OE-PDF2.2* rosette at 35 DAG. (E) Number of days to bolt. *KD-pdf2.2* plants bolt early in both long and short days, as measured by days to bolting (F) and rosette diameter measurements at 35 DAG. (G, H) Relative transcript level of *AtPDF2.2* in *KD-pdf2.2* and *OE-PDF2.2* plants. Two-week-old seedlings were used for RT-qPCR expression analysis and were normalized to the expression of *ACTIN 2*. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using a two-tailed Student's t-test.

A visible reduction in the rosette area was observed in *KD-pdf2.2* plants, while, to contrast, it was significantly enlarged in the *OE-PDF2.2* plants when compared with the wild-type (Fig. 4C, F). Thus, the observations that flowering time, root length and rosette area were all significantly affected in both of the *KD-pdf2.2* and *OE-PDF2.2* plants as compared to wild-type indicate a crucial role of *AtPDF2.2* also in plant growth and development. This matches the Genevestigator microarray data showing a constitutive expression of *AtPDF2.2* in various plant tissues and organs (Fig. S3C).

#### 4.4 Knockdown and overexpression of *AtPDF2.2* in transgenic Arabidopsis differentially affects the expressions of JA, SA and ET-associated genes

Expression levels of *AtPDFs* (*AtPDF1.1* - 3.2), JA-associated genes (*LOX3*, *AOC3*, and *OPR3*), SA-responsive genes (*PR1*, *WRKY70*, and *WRKY53*) and ethylene-associated genes (*ETR2* and *EIN3*) were measured in *KD-pdf2.2* and OE-*PDF2.2* transgenic plants and compared them with those in the wild-type (Fig. 5). In *KD-pdf2.2* plants, the expression of JA-associated Class-I, II and III *AtPDF* genes were all downregulated except for *AtPDF1.3*, 1.4, 2.5. However, expressions of two JA biosynthesis-related genes, *AOC3* and *OPR3* were reduced while *LOX3* that works upstream to these two genes was not remarkably altered. Peculiarly, SA-associated genes, *PR1*, *WRKY70*, and *WRKY53* were also significantly induced (Fig. 5A, B). Lastly, expressions of *ETR2*, a negative regulator, and *EIN3*, a positive regulator of the ethylene pathway were examined. We found that the *ETR2* was strongly suppressed transcriptionally, while *EIN3* was significantly induced, thus indicating the upregulation of the ET pathway.

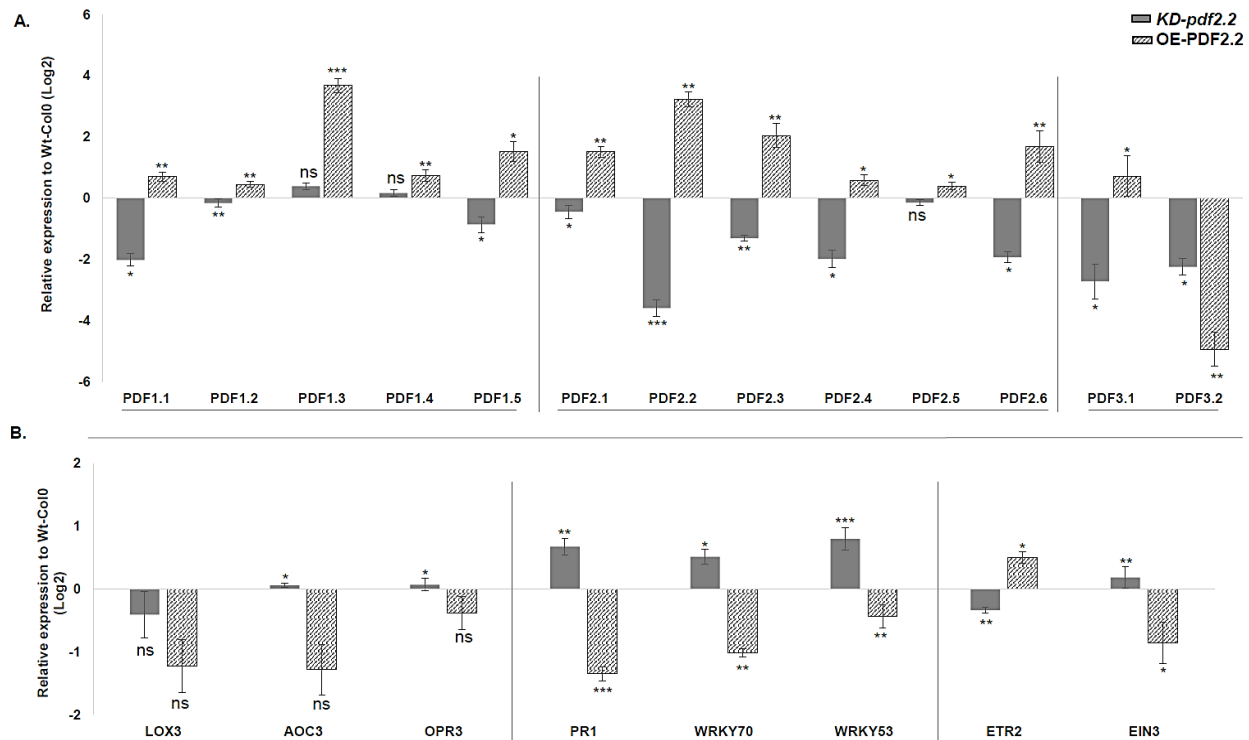


Figure 5 | Defense-related gene expression analysis in the *AtPDF2.2* transgenic plants. Expression analysis in terms of the relative expression levels of defense-related genes in non-infected WT-Col0 compared to the *AtPDF2.2* transgenic line for the knockdown, *KD-pdf2.2* (*KD*) and overexpression,



OE-PDF2.2 (OE) by RT-qPCR. (A) The *AtPDF1.1*, *AtPDF1.2*, *AtPDF1.3*, *AtPDF1.4*, *AtPDF1.5*, *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.4*, *AtPDF2.5*, *AtPDF2.6*, *AtPDF3.1* and *AtPDF3.2* represent fifteen Arabidopsis plant defensin genes from three classes. (B) *LOX3*, *AOC3*, and *OPR3* represent the JA biosynthesis-related gene; *PR1*, *WRKY70*, and *WRKY53* are the major corresponding proteins involved in the SA-synthesis pathway; *ETR2* and *EIN3* are ethylene signaling pathway regulator genes. The gene transcript levels were normalized to the expression of *ACTIN 2* measured in the same samples and expressed logarithmically. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

The *AtPDF2.2* overexpressing plants (OE-PDF2.2) showed an increase in transcript levels of almost all the members of Class-I, II, and III *AtPDF* family (*AtPDF1.1-3.1*), except for *AtPDF3.2*, which was highly downregulated. There were no significant alternations in transcript levels of three JA biosynthesis-related genes. Contrast to the *KD-pdf2.2* plants, SA-associated genes, *PR1*, *WRKY70* and *WRKY53* was all drastically depressed in OE-PDF2.2 plants as compared with the Col-0 wild-type. Strikingly, differing from those in the *KD-pdf2.2* and Col0 wild-type plants, the *EIN3* expression was downregulated while the *ETR2* expression was consequently upregulated in the OE-PDF2.2 plants. These data strongly suggest that *AtPDF2.2* functionally interferes with JA, SA, and ET signaling pathways, respectively.

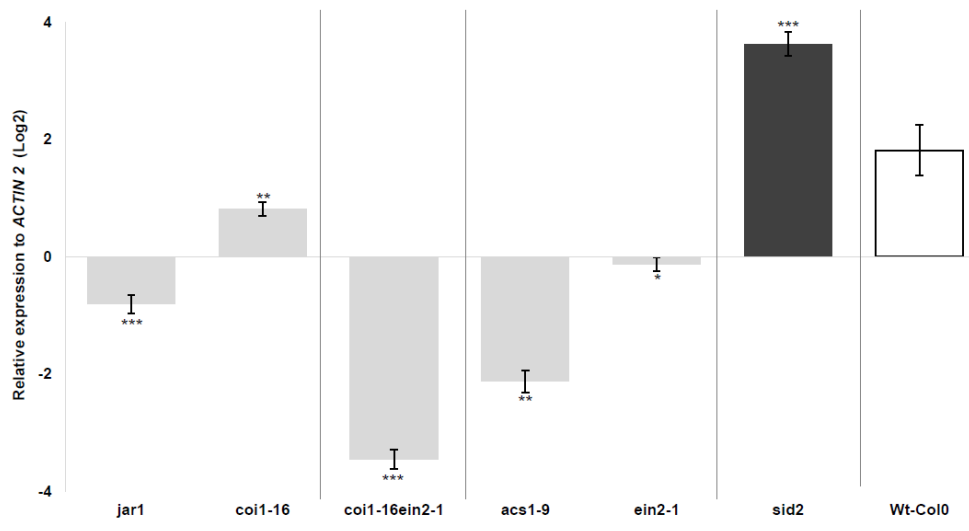


Figure 6 | *AtPDF2.2* gene expression analysis in Arabidopsis defense related mutants. *AtPDF2.2*-induced gene expression in Arabidopsis mutant plants. Wild-type (Wt-Col0) and mutants (*jar1*; *coi1-16*; *coi1-16ein2-1*; *acs1-7*; *ein2-1*; or *sid2*) seedling leaves were harvested at 25 dpi. And, used for RT-qPCR expression analysis and were normalized to the expression of *ACTIN 2*. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using a two-tailed Student's t-test. The experiment was repeated three times with similar results.

Analysis of the Genevestigator microarray data suggested a drastic alteration of *AtPDF2.2* expression in the JA/ET/SA triple mutant (*coin1-16ein2-1sid2-1*). To distinguish the roles of the three pathways in regulating the *AtPDF2.2* expression, we determined the expression levels of *AtPDF2.2* in JA-, ET-, and SA- signaling related Arabidopsis mutants, respectively and compared these with the Col-0 wild-type. As shown in Fig. 6, *AtPDF2.2* was significantly down-regulated in the JA and ET Arabidopsis mutants (*jar1*, *coi1-16*, *acs1-9*, *ein2-1*), respectively and drastically suppressed in the *coi1-16ein2-1* double mutant as compared to the wild-type, whereas, to contrast, a highly elevated *AtPDF2.2* expression was observed in SA-related mutant (*sid2*). Taken together, these data suggest that all three defense-related pathways influence the expression of *AtPDF2.2*, in which *AtPDF2.2* seems to be positively regulated by JA/ET pathways but negatively by SA pathway, respectively.

#### **4.5 *AtPDF2.2* is involved in plant-Verticillium interactions**

To characterize the role of *AtPDF2.2* in the plant-Verticillium interactions, three independent transgenic plants of *KD-pdf2.2* and OE-PDF2.2 with varied expression levels of *AtPDF2.2* were chosen for *V. longisporum* infection experiments. They were *KD4*, *KD5*, and *KD7* for *KD-pdf2.2* and OE1, OE7, OE8 for OE-PDF2.2 (Fig. 7). Four-week-old transgenic and Col-0 wild-type plants were inoculated with *V. longisporum* (VI34). Phenotypic differences were photographically documented every 6 dpi. Fourteen days after inoculation, clear disease symptoms were visible, Col-0 wild-type control leaves began to turn yellow and wilt, and plant growth was visibly stunted. By comparison, *KD-pdf2.2* plants exhibited a higher number of infected leaves and a severely retarded growth while, conversely, OE-PDF2.2 plants displayed enhanced resistance and better plant growth and performance even at 18 and 24 dpi (Fig. 7A). These data indicate that knockdown of *pdf2.2* enhanced plant susceptibility to *V. longisporum* whereas its overexpression (OE-PDF2.2) reduced plant susceptibility to *V. longisporum* as compared with the Col-0 wild-type plants.

An 'In-vitro' infection system has been established in our labor to facilitate the characterization of the early plant-fungus interactions and the generation of equally infected root tissue at the initial phase of infection. As shown in Fig. 7, senescence-like disease symptoms were obvious at 6 dpi seedlings and become much more pronounced

during the infection course (Fig. 7B). While *KD-pdf2.2* plants displayed augmented disease symptoms including the reduction in primary root length and the elevated fungal colonization, OE-PDF2.2 plants showed significantly fewer disease symptoms as compared to Col-0 wild-type, where the fungus was growing along with the root and the root length was significantly reduced (Fig. 7B, C). Again, RT-qPCR analysis showed that the *AtPDF2.2* transcripts were significantly reduced in the *KD-pdf2.2* while being increased in OE-35S-PDF2.2 plants in comparison to the Col-0 wild-type plants upon 6 dpi *V. longisporum* infection in roots (Fig. 7D).

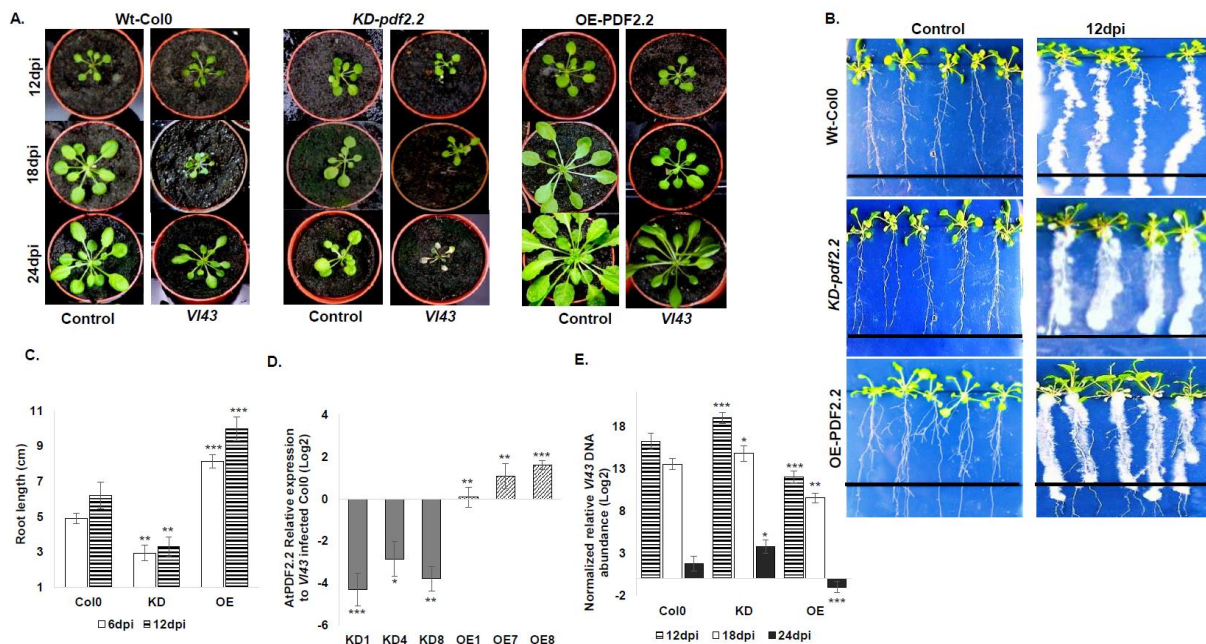


Figure 7 | *V. longisporum* infection analysis on *AtPDF2.2* transgenic plants.

Phenotypic differences between Wt-Col0 and transgenic *AtPDF2.2* lines in response to *V. longisporum* (A) Two weeks old *in-vitro* grown Col-0 wild-type, two knockdown (*KD-pdf2.2*) and three overexpression (OE-PDF2.2) lines were transferred into an individual pot containing a mixture of sand and soil (1:1) and grown for another one week. Followed by mock or *V. longisporum* conidia (2M) inoculation on each plant. Two biological repeats are presented here. To observe consistent phenotypic differences pictures were taken after every 6 dpi. (B) Three weeks old *in-vitro* grown Col-0, *KD-pdf2.2* and OE-PDF2.2 lines on vertical 1/2MS agar medium. The roots were supplemented with mock or *V. longisporum* conidia (2M) inoculation. The fungus can be seen growing along with Col-0 wild-type and *KD-pdf2.2* roots seized to grow after fungal treatment whereas OE-PDF2.2 roots were growing further without fungus growing along with them. (C) The primary root length was measured for mock or *V. longisporum* conidia treated seedlings. (D) 6 dpi mock or *V. longisporum* conidia treated seedling roots were used for the quantification of the relative transcript level of *AtPDF2.2* in *KD-pdf2.2* and OE-PDF2.2 and were normalized to expression of *ACTIN 2*. (E) Fungal quantification using primer pair (olg70/olg71) to amplify a 260bp long fragment of an ITS region from *V. longisporum* from the infected leaf tissues of Col-0, *KD-pdf2.2* and OE-PDF2.2. Data are mean  $\pm$  s.d. (n = 3). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

To confirm successful infection, fungal DNA colonization was quantified by qPCR assay in the leaves of 12, 18, and 24 dpi plants. The fungal biomass significantly increased in infected plants during the infection course as determined by PCR on fungal DNA. The highest amount of fungal DNA was detected in the *KD-pdf2.2* plants, followed by Col-0 wild-type and OE-PDF2.2 plants. The OE-PDF2.2 exhibited less fungal DNA at all indicated time points. At 24 dpi, fungal DNA was barely detectable in the OE-PDF2.2 plants (Fig. 7E).

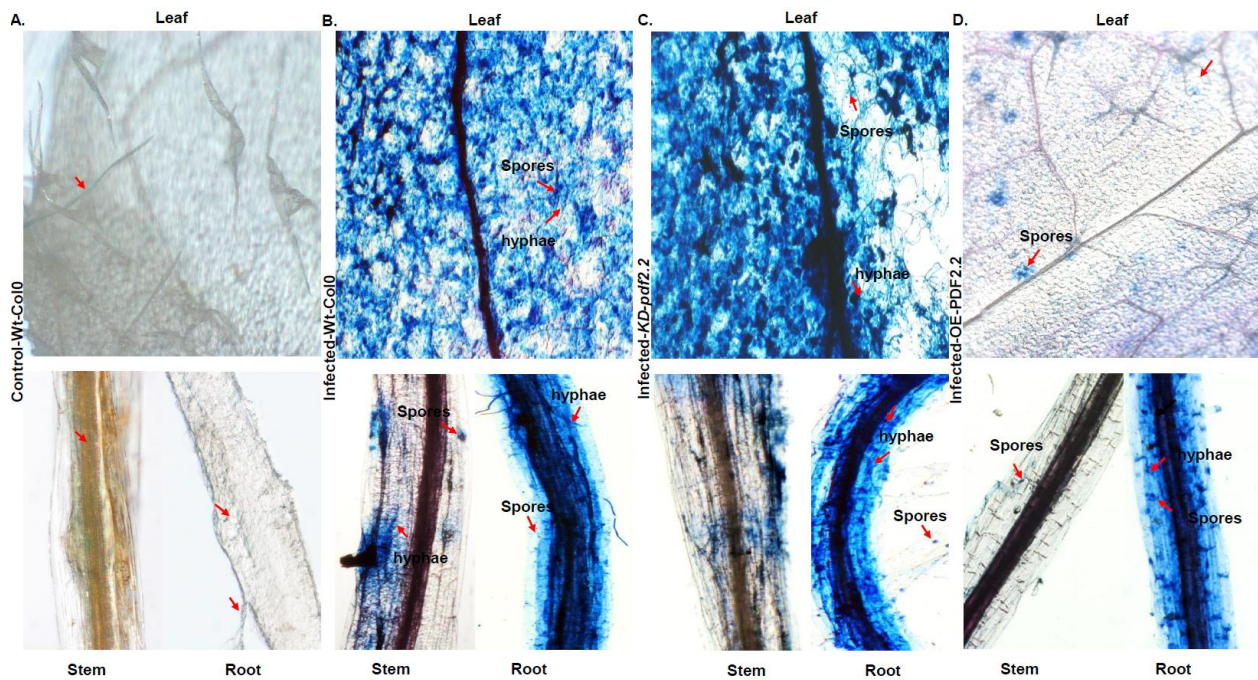


Figure 8 | Trypan blue staining for infection progression in AtPDF2.2 transgenic plants.

Trypan blue staining of Wt-Col0 and transgenic *AtPDF2.2* plants in leaves, stems and roots after 3 days of *V. longisporum* inoculation and destained with 90% ethanol. (A) Non-infected Wt-Col0 plant, (B) infected Wt-Col0 plant. Fungal growth was least in the stem followed by leaves and roots respectively. (C) Comparatively increased colonization in the Infected-*KD-pdf2.2* plant organs, and (D) Reduced growth of *V. longisporum* in OE-PDF2.2 transgenic Arabidopsis plants. Red arrows indicate spores and hyphal growth. The trypan blue staining experiments were repeated at least three times. Scale bars: a, b, c, d = 50  $\mu$ m

Trypan blue staining experiments revealed similar fungal colonization in roots, which however varied in the leaves and stems of the Col-0 wild-type, *KD-pdf2.2*, and OE-PDF2.2 plants. As shown in Fig. 8, in *KD-pdf2.2* plants substantial pathogen aggregates were obvious in the leaves (near their leaf vessels) and stem with hyphal growth, whereas in the OE-PDF2.2 plants extremely fewer spores without hyphal growth were given in the leaves and the stem as well when compared with the Col-0 wild-type plants, even



though the roots of both the transgenic plants of *AtPDF2.2* and Col-0 wild-type plants were heavily colonized.

#### 4.6 *AtPDF2.2* is involved in plant-bacterial interactions

To address whether *AtPDF2.2* is involved in plant-bacterial interactions, we challenged transgenic Arabidopsis plants of *KD-pdf2.2* (*KD4*, *KD5* and *KD7*) and OE-*PDF2.2* (OE1, OE7, OE8) along with Col-0 wild-type with *P. syringae* pv. tomato (DC3000), a gram-negative, biotrophic bacterial pathogen that elicits a wide variety of disease symptoms on plants, including blights, cankers, leaf spots, and galls (David et al. 2003).

At 3 dpi, disease symptoms were visible with clearly reduced growth in both of infected Col0 wild-type and *KD-pdf2.2* plants as compared to OE-*PDF2.2* plants. The difference between infected Col-0 wild-type and *KD-pdf2.2* plants were not less significant as compared with OE-*PDF2.2* plants (Fig. 9B). To evaluate whether *AtPDF2.2* in Arabidopsis is regulated by the *P. syringae* infection (bacterium) similarly as, by *V. longisporum* infection, the expression profiles of *AtPDF2.2* was monitored from 1 dpi and 3 dpi in the infected Col-0 wild-type. The results showed that the expression of *AtPDF2.2* was induced in the Col-0 wild-type plants at 1 and 3 dpi, differing from the *V. longisporum* infection (Fig. 9C).

In the next step, bacterial DNA was quantified from inoculated leaves at 1 and 3 dpi. OE-*PDF2.2* plants showed the least colonization, followed by Col-0 wild-type and *KD-pdf2.2* plants respectively (Fig. 9D). The results revealed that the OE-*PDF2.2* plants exhibited significant tolerance while, to contrast, the *KD-pdf2.2* plants showed enhanced susceptibility to the *P. syringae* infection (Fig. 9A). Also, Prom::*AtPDF2.2*::GUS plants were also infected with *P. syringae* and stained at 3 dpi. In consistence, we observed that the GUS staining intensity increased in the inoculated leaves as compared with those of non-infected plants (Fig. 9E). Taken together, we conclude that *AtPDF2.2* is somehow involved in plant-bacterial interactions and its expression is highly induced by the *P. syringae* infection, which might belong to the plant basal defense mechanism. To understand the underlying mechanisms, further experiments are needed.

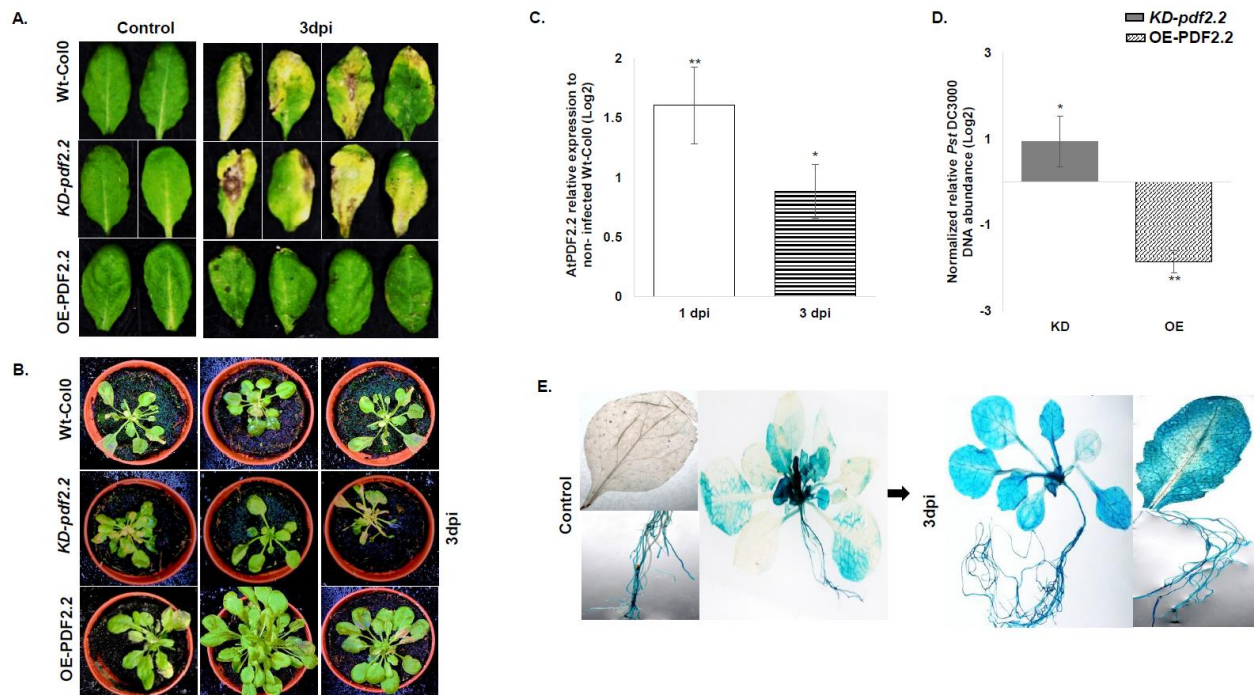


Figure 9 | *P. syringae* infection on transgenic AtPDF2.2 plants.

Susceptibility of plants to *P. syringae* (*Pst* DC3000) when inoculated by infiltration at  $1E + 5$  CFU/ml. **(A)** Leaf appearance of Col-0, *KD-pdf2.2* and OE-PDF2.2 plants 3 dpi. **(B)** Whole plant phenotype at of Col-0, *KD-pdf2.2*, and OE-PDF2.2 plants 3 dpi. **(C)** Quantification of the relative transcript level of *AtPDF2.2* in *Pst* DC3000 treated Wt-Col0 at 1 and 3 dpi and was normalized to the expression of *ACTIN2* and untreated Wt-Col0. **(D)** Bacterial population in *KD-pdf2.2* and OE-PDF2.2 leaves at 3 dpi. **(E)** Leaf, root, and whole plant GUS staining appearance at 3 dpi. Results displayed here (C and D) are means of four different leaves from four different plants, with SD indicated. Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using two-tailed Student's t-test.

#### 4.7 *AtPDF2.2* is involved in plant-*S. sclerotiorum* interactions

Detection of *AtPDF2.2* suppression by the *V. longisporum* infection prompted us to investigate the role of *AtPDF2.2* in plant-*S. sclerotiorum* interactions. To this end, we challenged transgenic Arabidopsis plants of *KD-pdf2.2* (*KD4*, *KD5* and *KD7*) and OE-PDF2.2 (*OE1*, *OE7*, *OE8*) together with Col-0 wild-type and Prom::*AtPDF2.2*::GUS plants with the necrotrophic fungus *S. sclerotiorum*. The disease progression in leaves was monitored from 6 to 24 hpi (hours post-inoculation). As revealed by the GUS staining assays, the GUS activity decreased in the early infection phase (Fig. 10A), and in consistence, the transcript abundance of *AtPDF2.2* in the *S. sclerotiorum* infected plants were significantly suppressed at 1 dpi and elevated at 3 dpi plants as compared with the non-infected control (Fig. 10B).

Interestingly, while *KD-pdf2.2* plants exhibited enhanced susceptibility to *S. sclerotiorum*, OE-PDF2.2 plants showed augmented resistance when compared with the Col-0 wild-type (Fig. 10C). At 7 dpi, approximately 60-70% of the Col-0 wild-type were decayed whereas approximately 90-95% and 33-40% of the *KD-pdf2.2* and OE-35S-PDF2.2, respectively, were decayed (Fig. 10D). At 24 hpi, lesions caused by *S. sclerotiorum* infection were readily visible on *KD-pdf2.2* and Col-0 wild-type but less on the OE-PDF2.2 leaves. Strikingly, the lesions developed more extensively in *KD-pdf2.2* leaves to the entire leaf area (Fig. 10E). To contrast, the OE-PDF2.2 leaves displayed lesions, which were distinctly restricted around the *S. sclerotiorum* inoculated area only (Fig. 10E). The size of lesions caused by *S. sclerotiorum* was approximately two times larger in the *KD-pdf2.2* leaves than those on the Col-0 wild-type leaves (Fig. 10F). Taken together, these data strongly support that *AtPDF2.2* plays a crucial role in regulating plant-fungus interactions, thereby contributing to plant resistance, and that suppression of *AtPDF2.2* expression at the early infection is probably one of virulence strategies of pathogenic fungi to establish/initiate the infection process in its hosts.

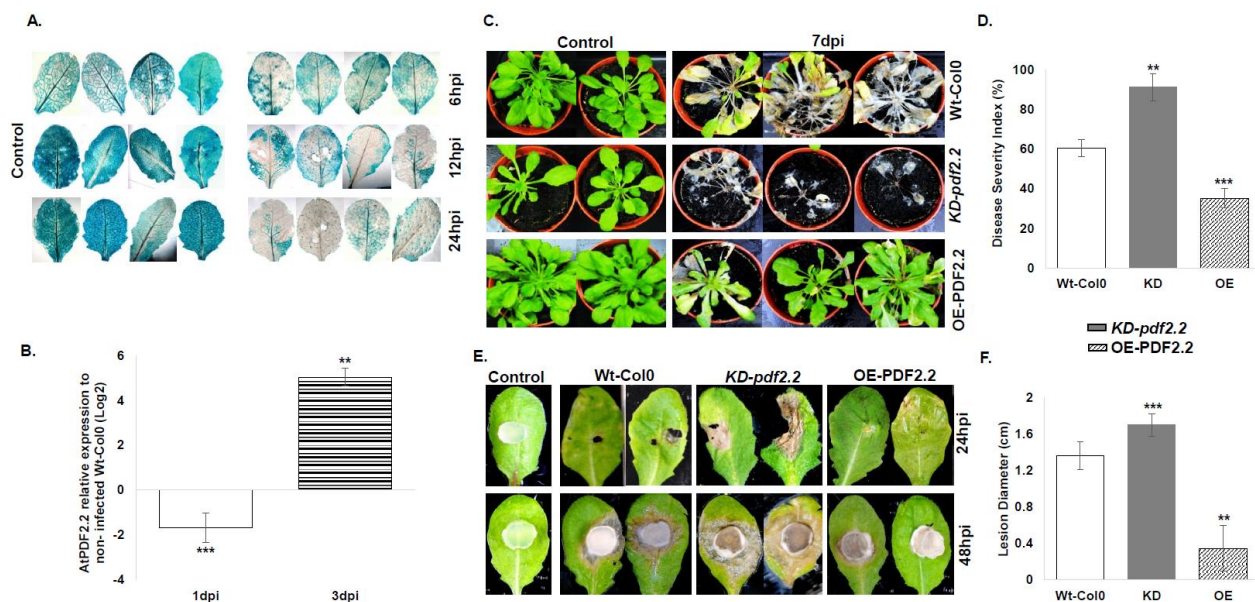


Figure 10 | *S. sclerotiorum* infection on transgenic *AtPDF2.2* plants.

Susceptibility of plants to *S. sclerotiorum* (A) Prom::*AtPDF2.2*::GUS staining in leaves from 6 to 24 hpi. (B) 1 dpi and 3 dpi *S. sclerotiorum* treated Wt-Col0 plant leaves were used for the quantification of the relative transcript level of *AtPDF2.2* and were double normalized to the expression of *ACTIN2* and non-infected Wt-Col0. (C) 4 weeks old Wt-Col0, *KD-pdf2.2* and OE-PDF2.2 plants 7 days after fungal infiltration. (D) Disease severity measurement at 7 dpi in whole plants. (E) Leaf appearance (abaxial sides) 24 and 48 hours after infiltration. (F) Lesion diameter measurement on day 2 after infiltration. Results displayed here (B, D, and F) are means of three different plants. Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using two-tailed Student's t-test.

## 5 Discussion

Plant defensins (PDFs) are endogenous antimicrobial polypeptides that are believed to function as the first line of defense in response to invading pathogens (Wang et al. 1999; Lacerda et al. 2014; Parisi et al. 2018). A transcriptomic study conducted by our group had identified several compatibility factors required for a compatible *B. napus* - *V. longisporum* interaction. Knockout of one of this compatibility factors (CF) resulted in an incompatible interaction between plant-fungus, which was accompanied by the highly elevated transcript level of *AtPDF2.2* (Pröbsting et al. 2020). In this study, *AtPDF2.2* was investigated in detail aiming at the identification of its possible roles in the plant-pathogen interactions, in general. *AtPDF2.2* gene encodes an 8.5-kDa protein containing 77 amino acids. Also known as low-molecular-weight cysteine-rich protein. *AtPDF2.2* was characterized starting with a close analysis of its phylogenetic relationship with other *Arabidopsis AtPDF* gene family members which are divided into three distinguished classes, namely Class-I, II, and III. *AtPDF2.2* (At2g02100) belongs to the Class-II. Most peptides carry a predicted signal peptide and are secreted (Fig. S1). Phylogenetic studies also revealed that *AtPDF2.1* and *AtPDF2.3* shares considerably high sequence homology to *AtPDF2.2* (Fig. S2).

*V. longisporum* is a hemibiotrophic fungal pathogen and causes huge yield losses of *B. napus* (Luo et al. 2014; Jasper et al. 2015; Kroecker; 1970; Novakazi et al. 2015). So far there are no effective strategies to combat *Verticillium* diseases, henceforth, breeding for resistant cultivars is the only strategy to control *Verticillium* stem striping (Depotter et al. 2016).

In our system, at 18 dpi the Col-0 wild-type plants displayed extensive disease symptoms and stunted growth (Fig. 1B), but the expression of *AtPDF2.2* was already found to be substantially suppressed at 6 dpi in roots (Fig. 1C). In the comparative study, infection with *A. brassicicola* has been reported to downregulate the basal expression level of *AtPDF2.2* (Thomma and Broekaert. 1998; Penninckx et al. 1996). Plant pathogens use several strategies to avoid detection by the host plant or to escape the plant defense responses. One such strategy involves the suppression of plant defenses. An increasing body of evidence suggests that fungal pathogens can modulate host gene expression to their benefit, either by suppressing inducible plant defense responses in physical proximity



to infection sites and/or by inducing specific compatibility genes required for infection (Thatcher et al. 2009; Rahman et al. 2012; Campos et al. 2014; Zhang et al. 2017; Schulze-Lefert and Panstruga; 2003; Kunoh et al.1985; 1991).

Expression pattern of *AtPDF2.2* was studied to decipher the transcriptional change of *AtPDF2.2* upon *V. longisporum* with the help of the GUS reporter gene, driven by the endogenous *AtPDF2.2* promoter (Fig. 3C). At 6 dpi, the GUS staining was nearly undetectable in the root, while at 9 dpi a highly decreased GUS staining intensity was observed in the leaves of Prom::*AtPDF2.2*::GUS transgenic plants, whereas in the non-infected control plants, the *GUS* gene was constitutively expressed in both roots and leaves with varying intensities (Fig. 3D, E). Interestingly, an increase in the *GUS* expression could be observed at the later stages of the infection (Fig. S4). To illustrate whether this gradual suppression from roots to leaves is a consequence of the fungal colonization, fungal quantification was performed by qPCR at 3, 6 and 9 dpi in both roots and leaves (Fig. 3D, E). Fungal colonization increased from 3 to 9 dpi and was always measured higher in the roots than in the leaves (Fig. 3D, E). This gradual increase and difference in fungal colonization between leaves and roots are consistent with the observation made in tomato and *Arabidopsis* infected by *V. dahliae* (Buhtz et al. 2015). The fungal biomass kept growing even after 9 dpi but, interestingly, *GUS* and *AtPDF2.2* gene expression in the Prom::*AtPDF2.2*::GUS and Col-0 wild-type plants also started to increase after 9 dpi (Fig. S4, S5, S7) and recovered later on. Therefore, the suppression effect is not merely because of increased fungal colonization but *AtPDF2.2* is targeted by *V. longisporum* in the initial stages to establish the infection.

Knockdown and overexpression of *AtPDF2.2* in *Arabidopsis* resulted in an alteration of phenotypes of transgenic plants. *KD-pdf2.2* plants displayed a phenotype including, reduction in primary root length, smaller rosette area, early bolting, and below-average seed generation while OE-PDF2.2 plants exhibited phenotype with increased growth including primary root length and rosette size as compared to the Col-0 wild-type plants (Fig. 4B-F). Furthermore, Genevestigator expression profiles suggested a constitutive expression of *AtPDF2.2* in almost all the developmental stages and different tissue/organs (Fig. S4C, D, E). Generally, tissue-specific gene expression is associated

with specific physiological and developmental functions (Zhang et al. 2012). Increasing evidence suggests that PDFs play a role in plant growth and development as well. A possible function of PDFs on root development was reported by Allen et al. (2008). Number of PDFs and PDF-like peptides were reported to be expressed specifically in the cells of the female gametophyte of *Arabidopsis* (Punwani et al. 2007; Jones-Rhoades et al. 2007; Steffen et al. 2007; Wuest et al. 2010); *Zea mays* (Cordts et al. 2001; Amien et al. 2010) and *Torenia fournieri* (Okuda et al. 2009). For example, in *Z. mays* the plant defensin, *ZmES4* was expressed in the synergid cells and required for pollen tube burst (Amien et al. 2010). Following this, we speculated that *AtPDF2.2* possibly plays a similar role and function in regulating plant growth and developmental processes. This can explain the drastic change in phenotype resulted from the knockdown of *AtPDF2.2*. It is well known that the JA pathway is involved in plant growth and reproduction process. For instance, mutants impaired in JA biosynthesis or perception genes display insufficient filament elongation, non-viable pollen, male sterility, and delayed anther dehiscence (Mandaokar et al. 2006; Wasternack and Hause 2013). In support of this, we also demonstrated that *AtPDF2.2* is a JA responsive gene.

To shed light on underlying molecular mechanisms, expressions of SA, JA, and ET associated genes in the non-infected *KD-pdf2.2* and OE-PDF2.2 plants were compared to Col-0 wild-type control plants (Fig.5). When Class-I *AtPDF* genes, *AtPDF1.1*, *1.2* and *1.5* were quantified in the *KD-pdf2.2* plants, their expressions were significantly downregulated relative to the Col-0 wild-type. However, the expression levels of Class-I *AtPDF* genes were significantly induced in the OE-PDF2.2 plants. Interestingly, the transcript levels of Class-II *AtPDF* genes, *AtPDF2.1*, *2.2*, *2.3*, *2.4* and *2.6* were drastically reduced in the *KD-pdf2.2* plants except for *AtPDF2.5*, where it was at a comparable level to that of Col-0 wild-type plants. As anticipated, Class-II *AtPDF* genes were considerably induced in the OE-PDF2.2 plants. Antagonistic regulation of *Arabidopsis* Class-I and II genes in the OE-PDF2.2 and the *KD-pdf2.2* transgenic plants, respectively, imply that these two classes work in a dependent manner. Although two members of Class-III, *AtPDF3.1*, and *3.2* displayed declined expression in the *KD-pdf2.2*, *AtPDF3.1* was induced in the OE-PDF2.2 plants where the expression of *AtPDF3.2* was highly suppressed. In accordance, these two proteins do not share significant homology to any of the *AtPDF* proteins (Fig. 1A). Besides that, they have chemical structure and pls

similar to the C-terminal domains of *PDF* genes that are involved in neutralizing the toxicity of the basic effector domain and mitigates the toxicity during synthesis (Thomma et al. 2002; Sels et al. 2008). Therefore, these two proteins were assumed to be fusion proteins or precursors (Thomma et al. 2002). Probably, they are not directly involved in the defense responses, unlike other *AtPDF* genes. Our data indicate that the expressions of nearly all the JA-responsive *AtPDF* genes, including *AtPDF1.1-3.2* were suppressed in the *KD-pdf2.2* while being induced in the OE-PDF2.2 transgenic plants. Plausibly, as *AtPDF2.2* is co-regulated with diverse *AtPDF* genes, its knockdown can negatively affect the expression of these *AtPDF* genes.

JA biosynthesis-related genes were inspected to understand the impact of *AtPDF2.2* on its upstream hormone-signaling pathway. Three JA- biosynthesis-related genes, *LOX3*, *AOC3*, and *OPR3* were analyzed for their expression in the transgenic *AtPDF2.2* plants. In OE-PDF2.2 plants, expression levels of all three genes were not significantly altered as compared with the Col-0 wild-type plants. It had been reported that JA biosynthesis can work independently from changes in gene expression (Wasternack 2007; Browse 2009a; b). Moreover, in resting state, JAZ proteins can act as transcriptional repressors of JA biosynthesis by binding to positive transcriptional regulators (Chini et al. 2007; Doares et al. 1995; McConn et al. 1997; Ziegler et al. 2001; Strassner et al. 2002).

Unexpectedly, *AOC3* and *OPR3*, but not *LOX3* transcript levels increased in the *KD-pdf2.2* plants. *AOC3* and *OPR3* functions downstream to the *LOX3* gene for JA biosynthesis (Zimmerman and Feng 1978; Wasternack 2007; Browse 2009a; b; Schaller and Stintzi 2009). Many studies demonstrated that *AOC3* and *OPR3* could be induced exclusively or together with other JA biosynthesis genes upon pathogen attack (Pratiwi et al. 2017; Li et al. 2009). Pathogens often try to alter the expression of downstream pathway-responsive genes to bypass the defense response (Chini et al. 2018; Chehab et al. 2011; Chehab et al. 2012). It is, therefore, reasonable to speculate that knockdown of *AtPDF2.2* in plants can elicit a similar response that consequently induces JA biosynthesis gene expressions.

SA-associated genes, *PR1*, *WRKY70*, and *WRKY53* were highly induced in the *KD-pdf2.2* and downregulated in the OE-PDF2.2 plants. SA signaling and JA biosynthesis gene expression were remarkably induced in the *KD-pdf2.2* plants

simultaneously. Despite the proven antagonism between SA- and JA-dependent pathways, transcript-profiling analysis revealed a high number of genes co-induced or co-repressed by the two hormones, pointing to a certain degree of overlap between the two pathways (Glazebrook et al. 2003; Schenk et al. 2000). However, the SA associated genes had extensively reduced expressions in the OE-PDF2.2 plants. It is known that under high JA signaling, few NAC and other transcription factors act as SA repressor (Zheng et al. 2012; Bu et al. 2008). Generally, this antagonism is achieved by expression changes in the pathway-associated downstream responsive genes (Mur et al. 2013; Leon-Reyes et al. 2010; Ning et al. 2019). Therefore, it is conceivable that *AtPDF2.2* mediates robust downstream JA signaling and in absence of ongoing infection, conceivably antagonizes to SA signaling.

Lastly, the expression levels of ET-associated genes, *EIN3*, a positive and *ETR2*, a negative regulator of ethylene biosynthesis was investigated. The transcript abundance of *EIN3* was highly suppressed while *ETR2* was substantially induced in the OE-PDF2.2 plants. In contrast, *EIN3* was induced while *ETR2* was decreased in the *KD-pdf2.2* plants. Considering the synergistic regulation of JA and ET biosynthesis, in resting state, where JAZ proteins repress the JA biosynthesis which results in deactivated downstream ERF branch, responsible for the ET response (Zhu et al. 2011). Thus, this crucial mechanism governing the JA/ET synergistic interactions could explain the downregulation of ET pathway in the OE-PDF2.2 plants and upregulation in the *KD-pdf2.2* plants.

Apart from defense responses, many of the JA/ET and SA responsive genes play crucial role in the growth and development process as well. For example, elevated levels of *EIN3* can adversely affect the primary root length, plant growth, low viable pollens, fasten transition from vegetative growth to flowering etc. (Liang et al. 2012; Vasevaa 2018; de Poel et al. 2015). Likewise, *WRKY53* acts as a positive regulator of senescence (Yand and Zentgraf 2010). As these genes were found highly elevated in the *KD-pdf2.2* plants hence, it can be concluded that *AtPDF2.2* directly or indirectly supports for balancing the crosstalk among the hormone signaling pathways, consequently affecting plant growth and development.

For the investigation of the intrinsic mode of interaction of defense pathways to *AtPDF2.2*, the expression level of *AtPDF2.2* was determined in different Arabidopsis genotypes (Wt-

Col-0, *jar1*, *acs1-7*, *ein2*, *coi1-16* and *ein2-1*, *coi1-16* and *sid2*) by RT-qPCR (Fig. 6). *AtPDF2.2* was observed differentially regulated via defense pathways in the absence of ongoing infection. Mutants for JA and ET biosynthesis-related genes (*jar1*, *acs1-7*, *ein2*, *coi1-16*, and *ein2-1coi1-16*) showed a significantly depleted expression of *AtPDF2.2*. In contrast, the *sid2* mutant showed elevated expression levels of *AtPDF2.2*. This fortifies the argument that the SA pathway most likely suppresses the expression of the JA responsive gene, also in the absence of fungal infection. Gupta et al. (2000) demonstrated that in comparison to the Col-0 wild-type plant, mutants impaired in SA accumulation exhibited an increased level of JA-dependent gene expression. Therefore, it might be concluded that *AtPDF2.2* is a vital member of the JA pathway signaling. This can explain why JA biosynthesis substantially influences the expression of *AtPDF2.2*.

To comprehend *AtPDF2.2* involvements in the plant-fungus interaction, the OE-PDF2.2 and *KD-pdf2.2* plants were subjected to the *V. longisporum* infection (Fig. 7). At 18 dpi, *KD-pdf2.2* plants seemed to be more susceptible with pronounced stunted growth as compared to the Col-0 wild-type plants. These disease symptoms further intensified and caused early senescence and death of *KD-pdf2.2* plants at 24 dpi. On the contrary, OE-PDF2.2 plants demonstrated enhanced disease resistance showing fewer disease symptoms in comparison to the Col-0 wild-type plants. So far, many *PDF* genes that have been extensively characterized for their ability to inhibit the growth of fungal and oomycete pathogens *in-vitro* and in plants (Parisi et al. 2018; Kaur et al. 2011; De Coninck et al. 2013; Cools et al. 2017). For instance, overexpression of wasabi PDF (*WT1*) in rice, potato, and orchid has resulted in increased resistance against *Magnaporthe grisea*, *Erwinia carotovora*, and *Botrytis cinerea* (Lay et al. 2005). Expression of Dahlia PDF, *DmAMP1*, in rice directly inhibits the fungal pathogens, *Magnaporthe oryzae*, and *Rhizoctonia solani*. It was observed that the constitutive expression of *DmAMP1* suppresses the growth of *M. oryzae* and *R. solani* by 84% and 72%, respectively (Jha et al. 2009). Results from greenhouse inoculation experiments demonstrate that expressing the *DmAMP1* gene in papaya plants increased resistance against *P. palmivora* and that this increased resistance was associated with reduced hyphae growth of *P. palmivora* at the infection sites (Zhu et al. 2007). It has been

demonstrated that defensins can specifically interact with host membrane compounds, such as bacterial lipid II receptors, fungal sphingolipids, and fungal phospholipids (Wilmes et al. 2011; Poon et al. 2014). Considering their mode of action, it is reasonable to relate the mechanism by which their overexpression can make a highly tolerant plant. There are several examples that *PDF* genes have important potential for effective fungal control in economically important crops (Wong et al. 2007). In light of these evidences, the enhanced disease resistance and susceptibility upon *V. longisporum* infection in the OE-*PDF2.2* and *KD-pdf2.2* plants, respectively, strongly support for a crucial role of *AtPDF2.2* in plant-*V. longisporum* interaction. Thus, it offers a novel opportunity to heighten plant resistance/tolerance against the fungus, e.g. via a genetic engineering approach.

The Arabidopsis-*P. syringae* interaction is mediated by the interplay of both SA and JA (Brooks et al. 2005). So far, mostly plant defensin proteins are known to exhibit defense against fungal pathogens. In this study, *AtPDF2.2* transgenic plants together with wild-type Col-0 wild-type were challenged with the *P. syringae* infection. The OE-*PDF2.2* plants were most tolerant while the *KD-pdf2.2* plants were highly susceptible as compared to the Col-0 wild-type plants (Fig. 9A). The infection severity was approximately in the same range in both the Col-0 wild-type and *KD-pdf2.2* plants, but OE-*PDF2.2* plants showed much less necrotic lesion and decaying plants at 3 dpi (Fig. 9B). MeJA treatment has been shown to be effective *against P. syringae* in Arabidopsis and tomato (Pieterse et al. 1998; Thaler et al. 2002; Wees et al. 1999). Similarly, *AtPDF2.2* overexpression can be linked to increased JA signaling, and thus enhanced resistance in the OE-*PDF2.2* plants. It is well known that JA extensively interacts with other plant hormone signaling pathways. This crosstalk is intriguingly complex and usually dose and time-dependent (Kazan and Manners 2008). *AtPDF2.2* was constantly induced at 1 and 3 dpi in the Col-0 wild-type plants unlike the *V. longisporum* infection (Fig. 9C). Thus, the induction in the expression of *AtPDF2.2* suggests that *AtPDF2.2* is not directly targeted by *P. syringae*. Notably, bacterial DNA was quantified least in the OE-*PDF2.2* plants followed by Col-0 wild-type and *KD-pdf2.2* respectively (Fig. 9D). A plausible explanation of this could be due to the overexpression of *AtPDF2.2*. Though PDFs are considered to be primarily antifungal, some PDFs have been shown to have antibacterial activity (Sathoff et al.

2019). So far, very few PDFs with antibacterial activity have been reported (der Weerden and Anderson 2013). For example, Cp-thionin from cowpea (Franco et al. 2006; Kraszewska et al. 2016); DmAMP1 from *Dahlia merckii*; CtAMP1 from *Clitoria ternatea*, AhAMP1 from *Aesculus hippocastanum* (Osborn et al. 1995), *ZmESR-6* from maize (Balandin et al. 2005), fabatin-2 from broad bean (Zhang and Lewis 1997; Kraszewska et al. 2016); and SOD1-7 from spinach (Segura et al. 1998) have been reported to exhibit antibacterial activity against a range of Gram-positive and Gram-negative bacterial pathogens. Among antibacterial PDFs, only SOD2 from spinach has been demonstrated to confer resistance to Asiatic citrus canker (ACC) and Huanglongbing (HLB) caused by *Xanthomonas citri* and *Candidatus Liberibacter*, respectively, in transgenic citrus (Stover et al. 2013). However, the modes of action of only a few antifungal defensins have been studied in detail in this regard (Cools et al. 2017; Parisi et al. 2018). For example, a unique bi-domain PDF, designated MtDef5, contains two domains, MtDef5A and MtDef5B. MtDef5 and each of its two domains induce distinct morphological changes and cell death in *X. campestris*. They permeabilize the bacterial plasma membrane and translocate across membranes to the cytoplasm. They bind to negatively charged DNA indicating these peptides may kill bacterial cells by inhibiting DNA synthesis and/or transcription. The cationic amino acids present in the two  $\gamma$ -core motifs of MtDef5 that were previously shown to be important for its antifungal activity are also important for its antibacterial activity. MtDef5 and its more potent single domain MtDef5B have the potential to be deployed as antibacterial agents for control of a *Xanthomonas* wilt disease in transgenic crops (Velivelli et al. 2018). Altogether, these examples and our study hint towards a prospect of *AtPDF2.2* in plant resistance response against pathogens with diverse lifestyles, challenging the general notion that PDF-dependent defense responses are predominantly effective against fungal pathogens only.

Upon *S. sclerotiorum* infection, the GUS staining declined greatly from 6-12 hpi in the Prom::*AtPDF2.2*::GUS leaves and relative gene expression of *AtPDF2.2* was found suppressed in the infected Col-0 wild-type plants (Fig. 10A, B). Infection assays exhibited enhanced susceptibility of *KD-pdf2.2* plants, while increased resistance of OE-PDF2.2 plants as compared with the Col-0 wild-type (Fig. 10C - F). Various studies had shown that the knockout and overexpression of JA pathway genes are responsible for the

heightened susceptibility and tolerance, respectively against *S. sclerotiorum* infection (Anderson et al. 2004; Donaldson et al. 2001; Kesarwani et al. 2007). For example, overexpression of a defensin from *Orychophragmus* in *Brassica napus* reduced damage to plants caused by *S. sclerotiorum* (Wu et al. 2009; Zarinpanjeh et al. 2016). PDFs in various model plants and crops exhibited their involvement in the innate immune responses to different fungal pathogens including *F. oxysporum*, *Alternaria alternate*, *Botrytis cinerea*, *Verticillium dahlia* (Gaspar et al. 2014; Graham et al. 2008; Ahmed et al. 2012).

Upon *V. longisporum* infection (Fig. 11), the transcript levels of Class-I and II *AtPDF* genes, was downregulated in the *KD-pdf2.2* plants and were significantly induced in the OE-PDF2.2 plants. Interestingly, Class-I genes displayed drastically elevated expressions in the OE-PDF2.2 plants. But, marginal repression was detected amongst the Class-II *AtPDF* genes in the infected OE-PDF2.2 plants as compared to its non-infected counterpart. Signifying that *AtPDF2.2* is certainly a target upon *V. longisporum* infection. Furthermore, the expression of *AtPDF2.3* and *2.5* was found highly diminished in the infected Col-0 wild-type plants (Fig. 1c). Nevertheless, at the later stage of infection, expression of *AtPDF2.2* together with *AtPDF2.3* and *2.5* was induced (Fig. S6). Highest homology is shared among the *AtPDF2.1*, *2.2* and *2.3*, they can be predicted to behave in the same manner, despite the contrary, *AtPDF2.5* was suppressed along with *AtPDF2.2* and *2.3* instead of *AtPDF2.1*. There have been numerous cases where altered antifungal activity has been detected among structurally related plant PDFs. MsDef1, a seed PDF from *Medicago sativa*, inhibits the growth of *Fusarium graminearum in-vitro*. However, MtDef2 from *Medicago trunculata*, which shares 65% amino acid sequence identity with MsDef1, lacks antifungal activity towards *F. graminarum* (Spelbrink et al. 2004). Lastly, the transcript level in both the *AtPDF* genes from Class-III was induced for the *KD-pdf2.2* plants while only *AtPDF3.1* was upregulated in the OE-PDF2.2 plants. As already discussed before. These two genes are not known to be directly involved in the defense responses. Nonetheless, *AtPDF3.1* was induced in the OE-PDF2.2 plants and *AtPDF3.2* expression was not significantly changed, hinting towards direct interaction of *AtPDF2.2* to only *AtPDF3.1*.



Transcript levels of JA biosynthesis responsive gene, *LOX3*, *AOC3*, and *OPR3* was measured relatively lower in the *KD-pdf2.2* and higher in the OE-*PDF2.2* plants. It is widely accepted that the JA signaling mediates resistance to necrotrophic and hemibiotrophic pathogens (Li and Yen 2008). It can be deduced from the gene expression intensities of different *AtPDF* genes and JA biosynthesis responsive genes in the transgenic *AtPDF2.2* plants that, *AtPDF2.2* is indirectly targeted by *V. longisporum*, which affects its expression and results in suppression of *AtPDF2.2*. In turn, this suppression potentially triggers the JA biosynthesis. During the co-evolution of plants and microbes, pathogens have evolved distinct mechanisms to suppress defense responses by meddling with key pathway regulators, thereby forcing plants to evolve bypass mechanisms (Zhang et al. 2017; McDowell and Dangl 2000). This explanation can further clarify, why JA biosynthesis responsive genes were induced in the non-infected *KD-pdf2.2* plants.

Reasonably, the suppression of *AtPDF2.2*, either by direct or indirect interaction triggers the JA biosynthesis. Nevertheless, plant-primarily recognizes pathogen-associated molecular patterns (PAMPs) to initiate innate immunity and downstream signaling. Hence, in the absence of a pathogen attack, although JA biosynthesis was activated the *AtPDF* genes were repressed in the *KD-pdf2.2* plants. Conversely, the infected *KD-pdf2.2* plants showed substantially reduced expressions of JA biosynthesis-related genes upon infection. In response to *F. oxysporum* f.sp. *lycopersici* (Fol) infection the abundance of specific tomato proteins, including PR proteins, changes in the xylem sap (Rep et al. 2002; Houterman et al. 2007). The appearance of the specific proteins and the abundance of PR proteins decrease, sometimes below the detection level resulting in an apparent disappearance which can lead to non-recognition and enhanced susceptibility to a pathogen (Krasikov et al. 2011) which we have observed in the *KD-pdf2.2* plants. These observations strengthen the argument that *AtPDF2.2* suppression and activation of JA biosynthesis might be a correlated mode of function. SA defense pathways are generally known to work antagonistically to the JA pathway (Thomma et al. 1998).

To comprehend how the SA signaling, which governs local resistance and SAR, reacted to *V. longisporum* infection, the expression of *PR1*, *WRKY70*, and *WRKY53* was investigated responding to *V. longisporum* infection (Fig. 11). Unlike the non-infected OE-

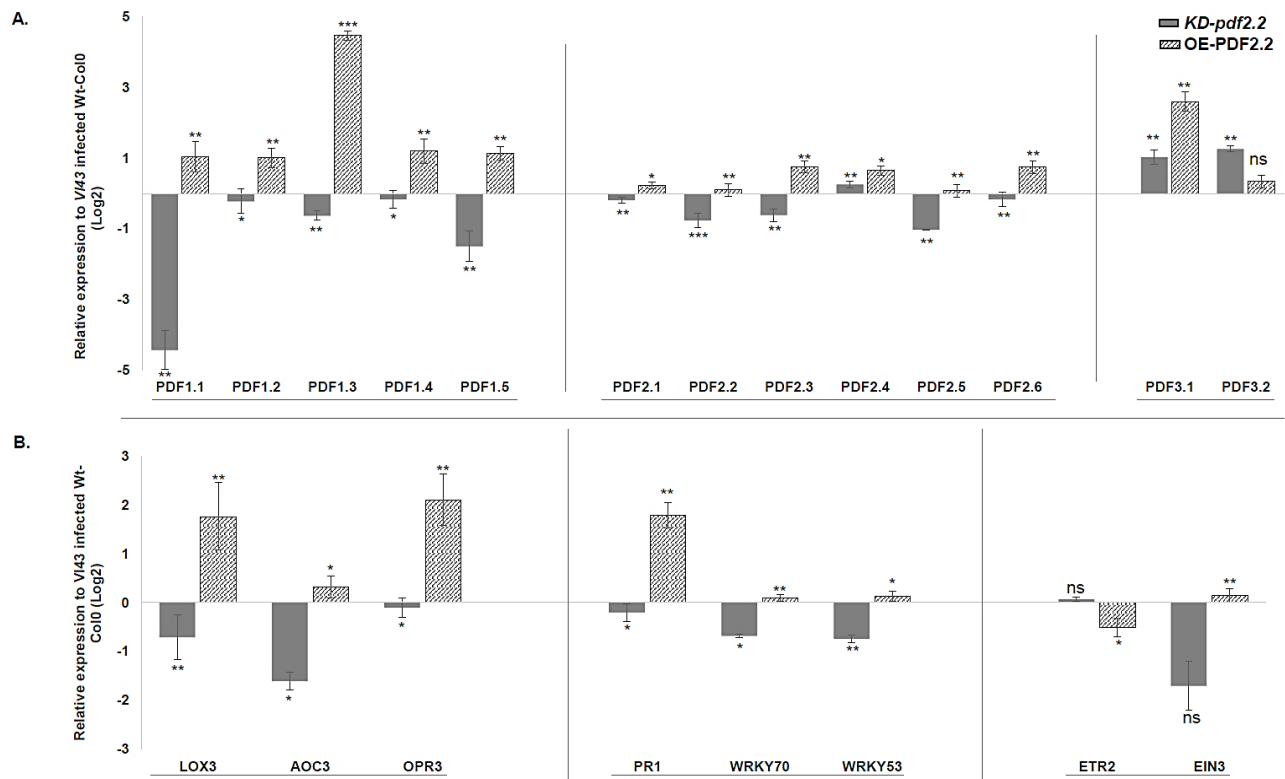


Figure 11 | Defense-related gene expression in *V. longisporum* infected *AtPDF2.2* transgenic plants. Relative expression levels of defence-related genes in the roots of *AtPDF2.2* transgenic plants for knockdown, *KD-pdf2.2* (*KD*) and overexpression, *OE-PDF2.2* (*OE*) at 6 dpi *V. longisporum*. **(A)** The *AtPDF1.1*, *AtPDF1.2*, *AtPDF1.3*, *AtPDF1.4*, *AtPDF1.5*, *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.4*, *AtPDF2.5*, *AtPDF2.6*, *AtPDF3.1* and *AtPDF3.2* represent fifteen Arabidopsis plant defensin gene from three classes. **(B)** *LOX3*, *AOC3* and *OPR3* represent the JA biosynthesis-related gene; *PR1*, *WRKY70* and *WRKY53* are the major corresponding proteins involved in the SA-synthesis pathway; *ETR2* and *EIN3* are ethylene signaling pathway regulator genes. The gene transcript levels were normalized to the expression of *ACTIN 2* measured in the same samples and expressed logarithmically. Data are mean  $\pm$  s.d. ( $n = 3$  to 4). Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using two-tailed Student's t-test.

*PDF2.2* plants, the infected overexpression plants showed highly elevated expressions of SA associated genes. While the SA responsive gene expressions in the *KD-pdf2.2* plants were significantly reduced. Zhu et al. (2012) demonstrated that PR1 protein exhibits antifungal activity. Moreover, emerging evidence also suggests that some of the well-known hemibiotrophic pathogens can induce different defense signaling pathways due to their overlapping biotrophic and necrotrophic lifestyle. Besides, JA dependent defense responses can also contribute to resistance against pathogens with a hemibiotrophic lifestyle, possibly by acting in concert with other defense signaling pathways (Meng and

Zhang 2013; Broekaert et al. 2006; Browse 2009). Besides, a recent study further claims that the early activation of the JA pathway requires SA through SA receptors, instead of the conventional JA pathway (Liu et al. 2016). Considering that both SA and JA pathways were activated in the OE-PDF2.2 and deactivated in the *KD-pdf2.2* plants provides a clue that *AtPDF2.2* might be regulated by both the pathways leading to resistance against *V.longisporum* infection. Correspondingly, our mutant study established that the SA pathway regulates the expression of *AtPDF2.2* in non-infected conditions.

ET biosynthesis-related genes, *EIN3* was not significantly altered in the *KD-pdf2.2* while it was upregulated in the OE-PDF2.2 plants. *ETR2* was significantly increased in the *KD-pdf2.2* while it was downregulated in the OE-PDF2.2 plants. ET commonly acts in cooperation with the JA pathway and activates the expression of defense-related genes (Ding et al. 2011; O'Donnell et al.1996; Penninckx et al.1998; Rojo et al. 2003; Xu et al.1994). Hence, the downregulation of *EIN3* was obvious in the *KD-pdf2.2* plants where JA biosynthesis genes expression was highly reduced. Moreover, in the absence of JA biosynthesis, ET response cannot be initiated.

The results of this study implicate that *V. longisporum* depresses *AtPDF2.2* expression and thereby activates JA, SA, and ET pathways. Differing from previous studies that the SA pathway protects against biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling enhances resistance to necrotrophic pathogens (Thomma et al. 1998), our study however clearly demonstrates that all three defense pathways were involved in defense response against the *V. longisporum* infection.

Following this, we propose a working model for *AtPDF2.2* (Fig. 12), in which (A) in absence of infection; *AtPD2.2* positively mediates the three classes of *AtPDF* genes except for *AtPDF3.2* from Class-III. Together these genes maintain the downstream JA mediated *AtPDF* signaling independent of JA biosynthesis. Moreover, due to active JA signaling, *AtPDF2.2* antagonizes the SA pathway (Thomma et al. 1998). Whereas in the absence of JA biosynthesis, the ERF branch needed to activate the ET response is also inactive. Hence, *AtPDF2.2* does not have direct interaction with either of the pathways, SA or ET. However, JA/ET pathways positively regulate the expression of *AtPDF2.2* and the SA signaling pathway demonstrated a negative regulation. (B) Under *V. longisporum* infection, *AtPDF2.2* is targeted by the fungus to bypass the defense

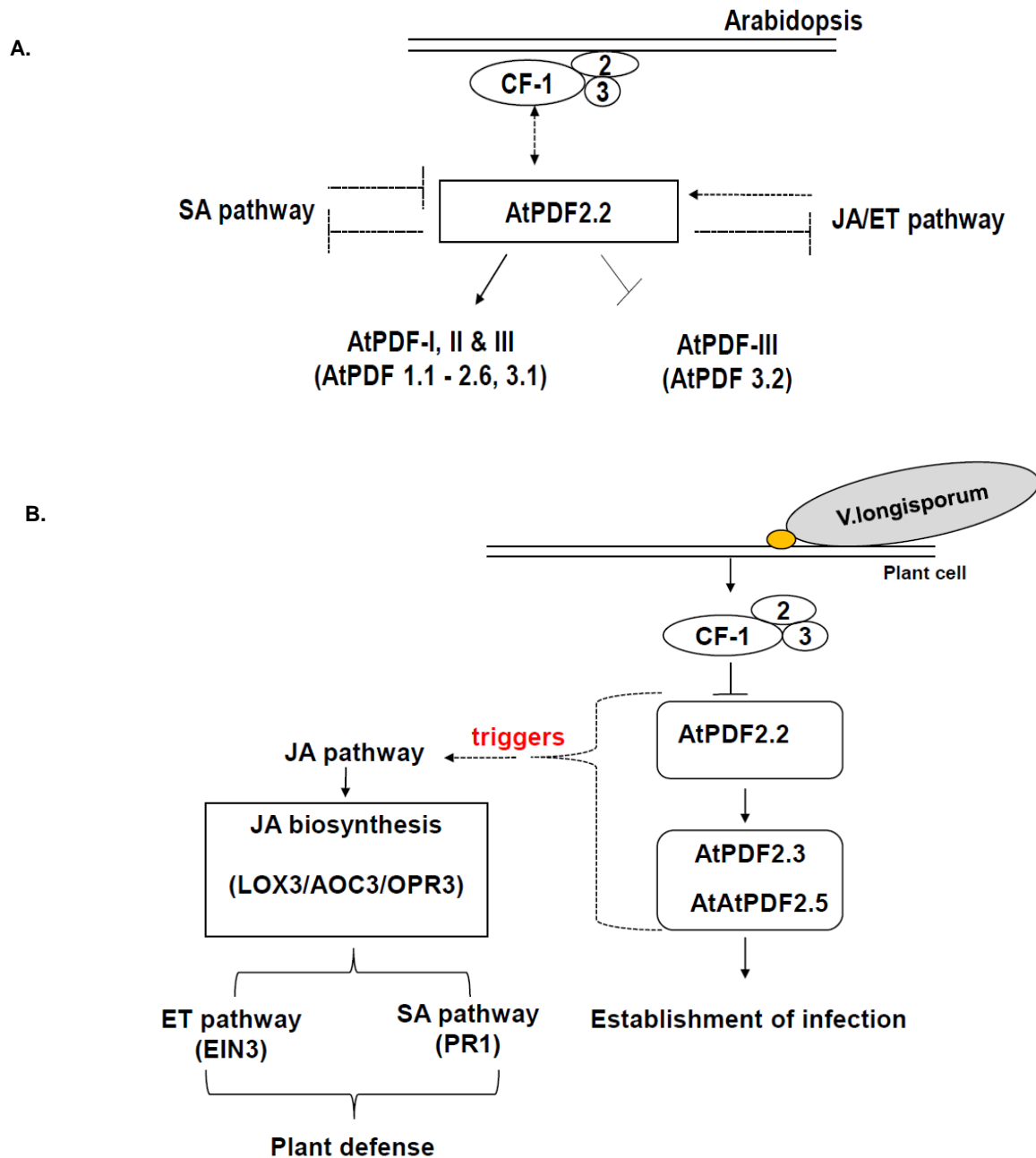


Figure 6 | Proposed functional model of *AtPDF2.2*.

(A) *AtPDF2.2* regulation in absence of infection. (B) *V. longisporum* suppresses the expression of *AtPDF2.2* and that might trigger the JA pathway and synergistic signalling involving SA- and ET- pathway to achieve the plant defense.

response and establish the infection. To attain that, fungus suppresses the expression of *AtPDF2.2*, which in turn, additionally affect the expression of two more *AtPDFs*, *AtPDF2.3* and *2.5*. Jointly, this suppression triggers the JA biosynthesis,

which subsequently activates ET and SA pathways to mediate the defense reprogramming and signaling.

Three important conclusions can be drawn based on this study, (First) *AtPDF2.2* is targeted by *V. longisporum* to establish the infection, (secondly) *AtPDF2.2* prompts the JA biosynthesis response upon suppression by direct or indirect interaction and, (thirdly) it plays an antifungal activity against *V. longisporum* infection with a broad range of resistance against necrotrophic fungal and bacterial pathogens as well. It remains to be determined how *AtPDF2.2* triggers the JA biosynthesis and elicit antifungal activity. Better understanding of each step will help elucidate its mode of action in the detail and can be useful for developing novel strategies against the fungal pathogens.

## **6 Acknowledgments**

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## 7 Supporting Information

## Supplementary Figures:

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PDF2.1 (At2g02120):mkfsmrlisavllfvllfvatgmppvtveaTCASQSRKCKCVSDTNCENVCH---EGFPFGDOR---GFRRCFCTRNC
PDF2.2 (At2g02100):mkfsmrlisavllfvllfvatgmppvtveaTCESQSHRRCCTCVSASNCANVCH---EGFVGCNCR---GFRRCFCTRRC
PDF2.3 (At2g02130):mklsrvfisaallfvllfvatgmppvtveaTCESKSHRRCPCVSTHNCANVCH---EGFGGCKOR---GFRRCFCTRRC
PDF2.4 (At1g61070):MGLVTVVEARTCTSSNLEPCPLSSSNCANVCH---EGFSDGCR---GFRRCFCTRRC
PDF2.5 (At5g63660):menkffaaffllllvlfss--qeihgggeTCCKSKSHRRCVYMCSTNHNCALVCRN---EGFSGCRDH---GFRRCFCTRRC
PDF2.6 (At2g02140):mklsrlisavllfvllfvatgmppvtveaTCESQSRKCKCVSDTNCENVCH---EGFPFGDOR---GFRRCFCTRNC

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Figure S1 | Multiple sequence alignment of AtPDF Class-II proteins using CLUSTAL W.

Consensus sequences (100 %) are exhibited in black shading and (C) denotes conserved cysteine residues in the alignment. The accession numbers of the DNA sequences are given in brackets (AT2G02120-*AtPDF2.1*), (AT2G02100-*AtPDF2.2*), (AT2G02130-*AtPDF2.3*), (AT1G61070-*AtPDF2.4*), (AT5G63660-*AtPDF2.5*) and (AT2G02140-*AtPDF2.6*).

```

*      20      *      40      *      60      *
AT2G02100- : AAAATCGAGAAACTCACACCCTCTTATTCAAATCCAAAGTGAAACTCCAAAAATAAAGTAAATGATATTT : 72
AT2G02130- : -----GAACTGTATAATGGAATAAAAGATATTC : 31
AT2G02120- : -----
AAAATCGAGAAACTCACACCCTCTTATTCAAATCCAAAGTGAAACTCCAAAAATAAAGTAAATGATATTT
*      80      *      100     *      120     *      140
AT2G02100- : ATCCAAAAATATCTAACAAACCGCTAAAATGTGAATATCTTGAACGTTTAAATACCTC-----TTTACTCT : 139
AT2G02130- : GT-----TTTCTTTTA-----GAAAAAGTTCAATTATCTCGAGCGTTCTTATACCTT-----TCCACTCT : 86
AT2G02120- : -----
RTCCAAAAATWTCTWYAAACCGCTAAAARKTSAATTATCTYGARCGTTYTWATACCTY TYYASTCT
*      160     *      180     *      200     *
AT2G02100- : CTTTATATAGTGCCATTTTCCCTACTAGCAG-----CACACACTTGAACACTACTTTCGAACCTCTCTC : 203
AT2G02130- : CTTTATATAGTGCCCACTTTCCCTACTAGCAGTTTACAGTCTGCACACTTT---ATCACTCTCTTCGAAATCTCTC : 157
AT2G02120- : -----TCACCACACACCACATTCACTTTA-----TTCCCACTCTC---TGCTCTCTC---AATTTTCTCT : 54
CTTTATATAGTGCCCACTTTCCCTACTAGCAGTTWACAGTCTDCACACTTTGAWCACTTCTTTCGAAATTCTCTC
*      220     *      240     *      260     *      280
AT2G02100- : TTTATCTCTCTSCAA---TGAAGCTCTCTA---TGCGTTTGA-----TCTCAGCTGTTCTCATCCTGT : 261
AT2G02130- : TCTATCCTCTCAAAAATGAAGCTCTCTS---TGCGTTTTA-----TCTCCCTGCTCTCTCTTTGT : 216
AT2G02120- : TCCTTTCTCTC---AACACA---TGAAGTTCTCTCTA---TGCGTTTGA-----TCTCAGCTGTTCTTTCTCTCTG : 112
TCTATCTCTCTTHAAAATGAAGCTCTCTA TGCGTTTGA TCTCAGCTGTTCTTTCTCTTTGT
*      300     *      320     *      340     *      360
AT2G02100- : TCATGATTTCTGCCCACGGGATGGGCCAGTCACTGTGGAGGCACGCACTTGTGAGTCCAGAGCCATA : 333
AT2G02130- : TCATGCTATTCTCTTTGCCACAGGGATGGGTCCAGTCACCGTCGAGGCACGCCACTGCTGACTCAAAAGCCATA : 288
AT2G02120- : TCATGATTTCTGCCCAAGGGATGGGTCCAGTCACCGTCGAGGCACGCCACTGCTGCTCCAAGCCATA : 184
TCATGATTTCTGCCCAAGGGATGGGTCCAGTCACCGTCGAGGCACGCCACTGCTGAGTCTGCAAGCCATA
*      740     *      760     *      780
AT2G02100- : AATAATCAAAATCTATGTTCTCTATAACAAGCTA---TTTTCTCTCGAAATATT---TAAGCCATCACTTCTCTCTCTC : 710
AT2G02130- : ATCGATCTCAATCTTAAACTTTTTAATAACCGCA---GTCTGCAACTTAATCACAATAATCTCCCAATAGAAT : 663
AT2G02120- : AAGGTTTCGAATTATGTTTCTTTGCAAATA---GCCAATTTTATTCAAGTTACA---ATTAGAAG : 568
AAGGATCHVAATCTATGTTCTTTTACAAAAVTA GTCTGCATHGTAATCAHDWTAAGCGWYSAHTTGAAG
*      800     *      820     *      840     *      860
AT2G02100- : TCTCACTTGAAGAGATCAACTATGATGAACTTAAAAAAAA----- : 753
AT2G02130- : TGAGAGAACGATCGATTATATAGTTAATCTCGTTPAGAAATT----- : 707
AT2G02120- : TATAAAAAACGTTTCTC----- : 584
TVTVVAVAACGDATHGATYAWMTAKRRTKAAWCTKRWWAAAAAVT
*      880
AT2G02100- : ----- : -
AT2G02130- : ----- : -
AT2G02120- : ----- : -

```

Figure S2 | Multiple sequence alignment and phylogenetic analysis of AtPDF2.1, 2.2 and 2.3 proteins using CLUSTAL W.

DNA sequence alignment of the three *AtPDFs* from Arabidopsis. Consensus sequences (100%) are exhibited in black shading and (-) denotes non-homologous sequences in the alignment. The accession numbers of the DNA sequences are given in brackets (AT2G02120-*AtPDF2.1*), (AT2G02100-*AtPDF2.2*), (AT2G02130-*AtPDF2.3*).

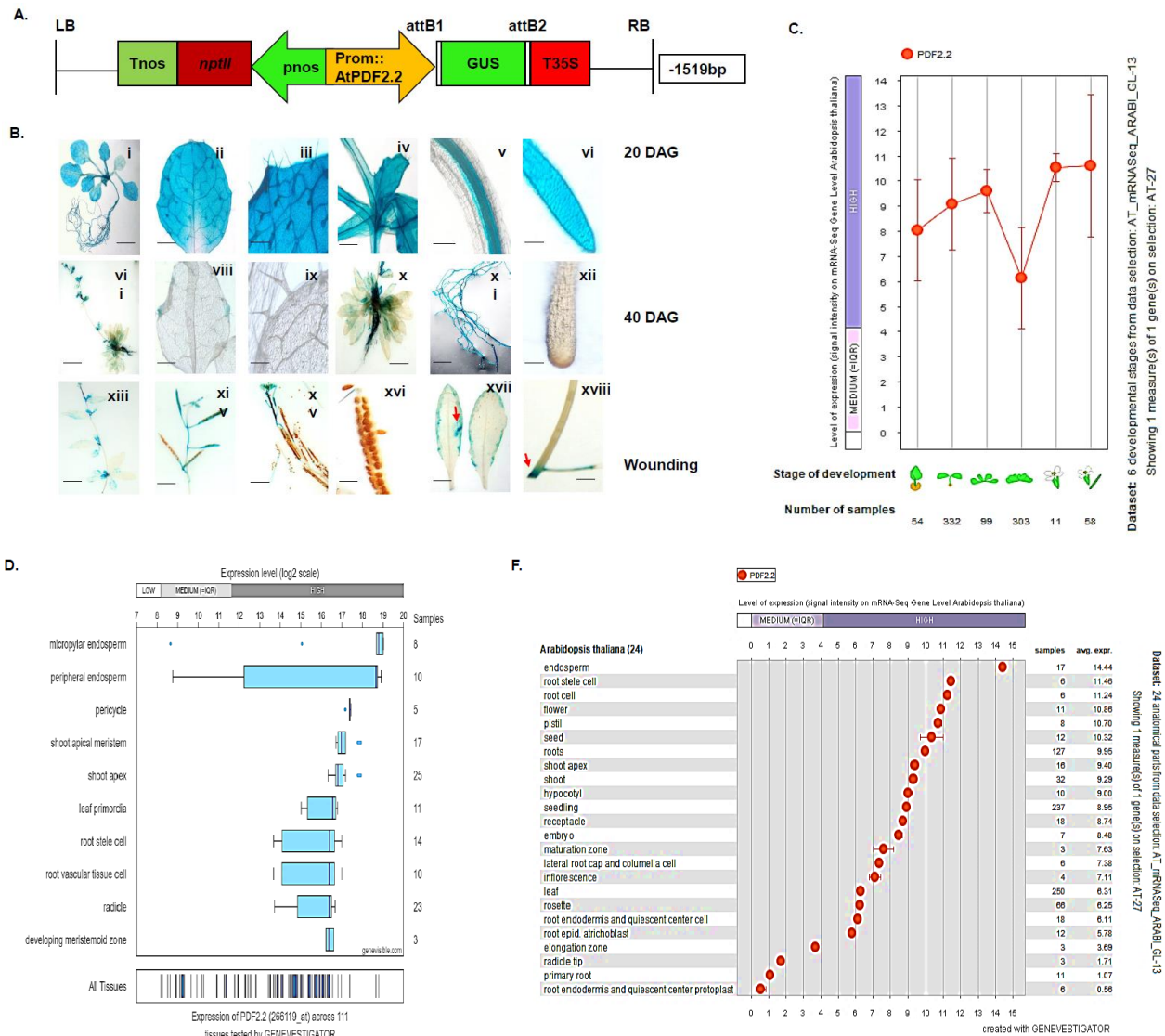


Figure S3 | *AtPDF2.2* promoter driven GUS expression in different plant organs. **(A)** A schematic drawing of the constructs used for the Prom::*AtPDF2.2*::GUS expression analysis. **(B)** Histochemical localization of *AtPDF2.2* promoter-GUS expression in Arabidopsis and shown in the different tissues: seedling of 20 days after germination (i); whole seedling (ii); young leaf (iii); trichoms (iv); shoot apex and hypocotyl (v); enlarged root (vi); and the root tip of primary root in vascular tissue. GUS activity shown in 40 days old plant (vii); whole plant (viii); limited GUS expression in mature leaf (ix); no expression in trichomes of aged leaves (x); flower buds and axillary buds in the reproductive stage (xi); mature roots (xii); root tip (xiii-xvi); internodes, intermediate development stages, anthers but not in pollen, (xvii, xviii); and, at the wound site in mature leaf and steam (indicated by red arrow). **(C)** *In-silico* analysis of *AtPDF2.2* expression via GENEVESTIGATOR for six developmental stages, **(D)** for different plant tissues and, **(E)** organ in Arabidopsis respectively. Scale bars: i, ii, iv, vii, viii, x, xvii, xviii = 0.5mm, iii, ix, xi, xiv = 2 mm and v, vi, xii, = 25  $\mu$ m.



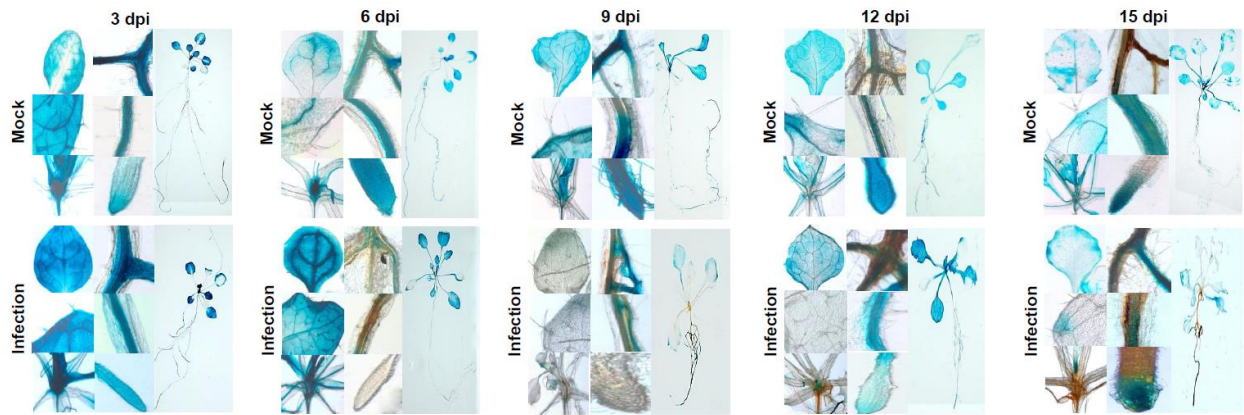


Figure S4 | GUS kinetic study in Prom::*AtPDF2.2*::GUS plants for *V. longisporum* infection. GUS Staining was performed from 1 dpi to 20 dpi. Only interesting and fluctuating time points are shown.

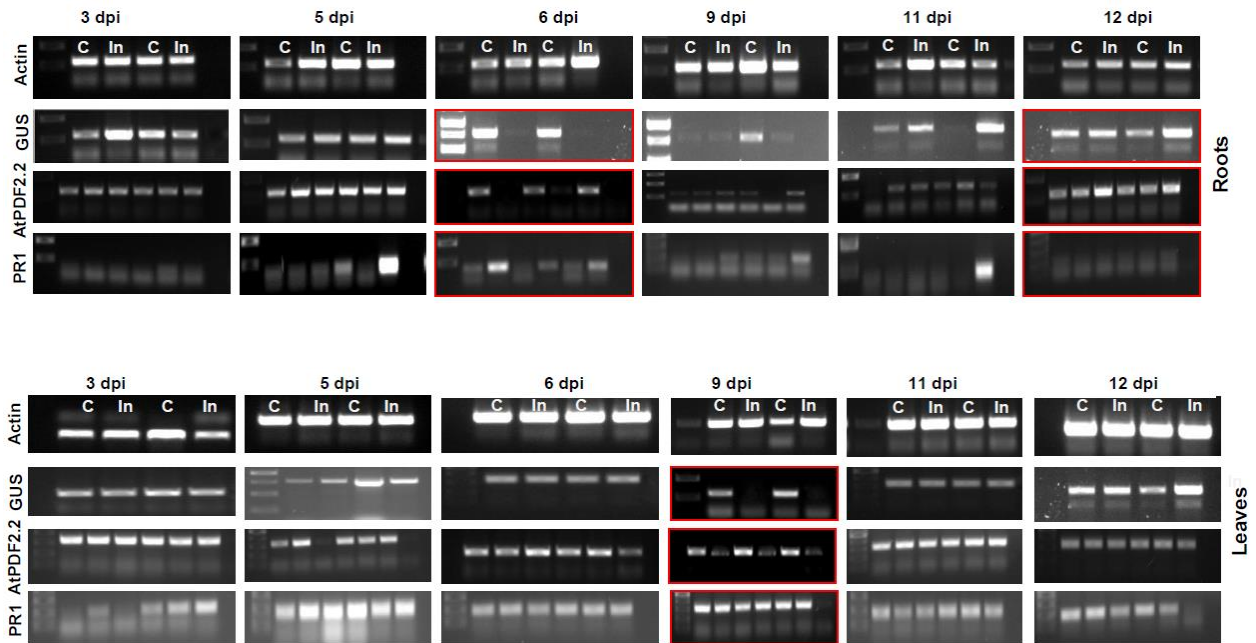


Figure S5 | Quantification of varying GUS expression in Prom::*AtPDF2.2*::GUS plants. The expression of *AtPDF2.2* in the Prom::*AtPDF2.2*::GUS plant was studied by semi-quantitative RT-PCR. RNA was isolated from infected roots and leaves. RT-PCR was performed with indicated primer pairs. Expression of *GUS* and *AtPDF2.2* was extremely faint in roots at 6 dpi while same was observed for the leaves at 9 dpi, both relative to non-infected control. *GUS* and *AtPDF2.2* gene expression was detected again from 11 dpi in both roots and leaves. PR1 expression was also detected in the infected root till 9 dpi and remained constant in the leaves. Primers for the *ACTIN 2* gene were used as a control. **C = control; In = infection.**



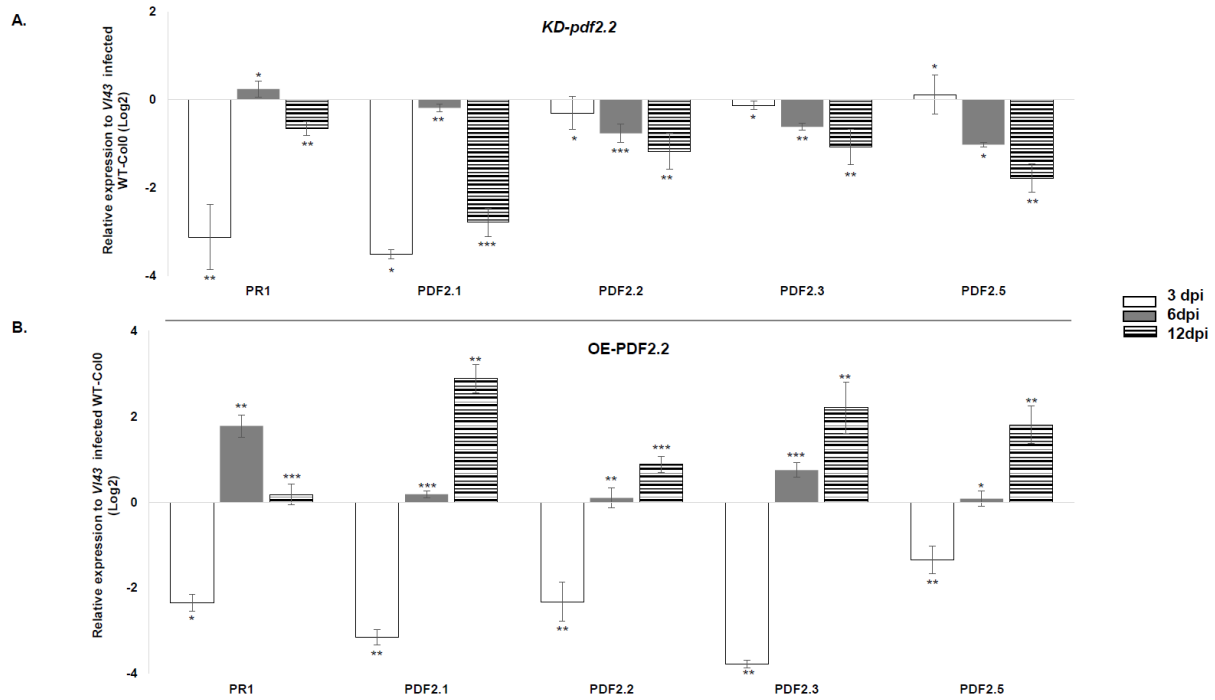


Figure S6 | Time-shift expression of defense related genes in *AtPDF2.2* transgenic plants. Relative expression levels of defence-related genes in the roots of *AtPDF2.2* transgenic *Arabidopsis* plants for knockdown (a) *KD-pdf2.2* (*KD*) and overexpression (b) *OE-PDF2.2* (*OE*) at 3, 6, 12 dpi *V. longisporum*. The *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.5* and *PR1* represent JA and SA synergistic interaction at 3 and 6 dpi and antagonism at 12 dpi or later time points. The gene transcript levels were double normalized to the expression of *ACTIN 2* and mock *Wt-Col0* and expressed logarithmically. Data are mean  $\pm$  s.d. ( $n = 3$  to 4). Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using two-tailed Student's t-test.

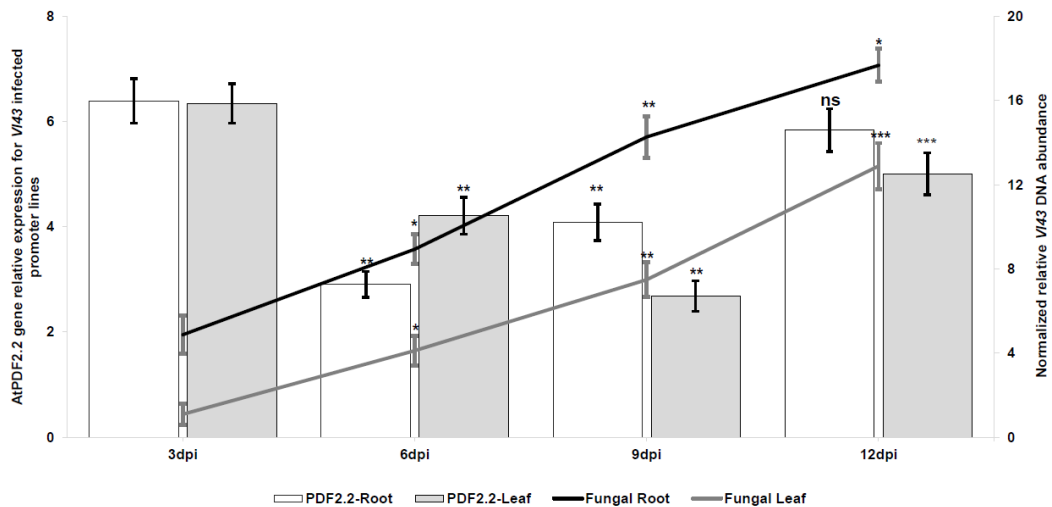


Figure S7 | Kinetics of *AtPDF2.2* and fungal quantification in *Prom::AtPDF2.2::GUS* plants from 3 dpi to 12 dpi.

Combined graph to show *AtPDF2.2* gene expression in roots (grey bar) and leaves (green patterned bar) along with *V. longisporum* colonisation quantification from 3 dpi to 12 dpi in the roots (black line) and leaves (grey line). Comparatively lower fungal DNA was detectable in the leaf than root. Data are mean  $\pm$  s.d. ( $n =$

3 to 4). Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using two-tailed Student's t-test.

## Supplementary Tables:

Table S1 | Primer table.

Accession Numbers starting with 'AT' were taken from Arabidopsis database (TAIR) and others were taken from NCBI database.

Gene	Identifier	Forward	Reverse	(bp)	Tm (°C)
<i>AtPDF2.2</i>	AT2G0210	GCACCAGACATTGCTGATCT	AACCGGAAACACACAAATCC	159	58
OE-PDF2.2	AT2G0210	TCTCTTGCAATGAAGCTCTCTATG	ATGGGTAGATCAGCAATGTCTGGT	252	58
<i>KD-pdf2.2</i>	AT2G0210	TCTCTTGCAATGAAGCTCTCTATG	ATGGGTAGATCAGCAATGTCTGGT	252	58
Prom:: <i>AtPDF2.2</i>	AT2G0210	CTTCTACGCATGTAAATTATAACC	CAAGTGTGTTGCTGCTAGTGA	1519	60
<i>GUS</i>	EG11055	GTTGACTGGCAGGTGGTGG	GGTAGATATCACACTCTGTCTGG	252	57
<i>AtPDF1.2</i>	AT5G44420	CGCTGCTCTTGTTCTCTTTGC	TCCATGTTTGGCTCCTTCAA	154	57
<i>AtPDF1.4</i>	AT1G19610	AGTCATGGCTTCTTCTTACACACT	TGAAGTAGCAGAAACATGCGAA	233	58
<i>AtPDF2.1</i>	AT2G02120	GATCGACGATTGAGTCATGC	CTCAGCCGTTCTTTTCTTGG	250	58
<i>AtPDF2.3</i>	AT2G02130	CACACACAACGTGCAAACG	CGGAAACACACAAACCAATG	234	59
<i>AtPDF2.5</i>	AT5G63660	TGGAGAACAAGTTTTTCGCTGC	AAACTAGTTAGCAAAGGCGGG	228	60
<i>PR1</i>	AT2G14610	TCAGGTTGTTGGAGAAAGTC	CATTAGTAAGGCTTCTCGTTCACA	126	57
<i>ETR2</i>	AT3G23150	CGTTGGAATTTACAGGTCGATGAG	CGTCTTCGAGTTACATCGTGGGA	213	59
<i>EIN3</i>	AT3G20770	TGGTGGCCTCAACTTGGTTT	CGAGTTTCTGCTGGGACTT	459	60
<i>ACO3</i>	AT3G25780	GAATTGGGGCAAGAAGACCGAAA	TTGCCGAGTTTAAGAAGTCTGGA	111	62
<i>WRKY70</i>	AT3G56400	CCGCCGTTGAGGGATCT	GGACGAACCATGATGACGAT	479	58
<i>WRKY53</i>	AT4G23810	GCACGACTTAGAGAAGCTC	ACTCCTTGGGAATTTGGCG	450	56
Verticillium ITS	OLG70/71	CAGCGAAACGCGATATGTAG	GGCTTGTAGGGGGTTTAGA	261	58
Pst biomass	oprF	AACTGAAAAACACCTTGGGC	CCTGGGTTGTTGAAGTGGTA	304	62
Plant biomass	AT4G2641	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC	81	56

Table S2 | *AtPDF2.2* protein homologs present in Arabidopsis.

Bit score for *AtPDF2.3* and *AtPDF2.1* indicates high level of similarity with *AtPDF2.2*.






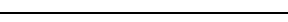



Similar proteins in Arabidopsis thaliana:	Similar proteins anywhere in STRING:	Similar proteins in a taxon of choice:	
		Similarity (Bit Score)	Orthology (level)
LCR69- AT2G02100- <i>AtPDF2.2</i>			
LCR68- AT2G02130- <i>AtPDF2.3</i> →		136.3	(paralog)
LCR70- AT2G02120- <i>AtPDF2.1</i> →		133.3	(paralog)
LCR66- AT1G61070- <i>AtPDF2.4</i> →		108.6	(paralog)
LCR72- AT2G02140- <i>AtPDF2.6</i> →		87.4	-
LCR74- AT5G63660- <i>AtPDF2.5</i> →		70.9	-
LCR73- AT2G02147 →		63.2	-
LCR75- AT2G31957 →		60.1	-
LCR76- AT2G31953 →		59.3	-

Table S3 | STRING-based networks for the Arabidopsis interactors experimentally determined or from curated databases.

Sequence similarity shared by the three Class-II *AtPDF* genes in reference to *AtPDF2.2*, *AtPDF2.3* and *AtPDF2.1* respectively.

	<b>At2G02100-<i>AtPDF2.2</i></b>	<b>At2G02130-<i>AtPDF2.3</i></b>	<b>At2G02120-<i>AtPDF2.1</i></b>
<b>At2G02100-<i>AtPDF2.2</i></b>	100%	71%	55%
<b>At2G02130-<i>AtPDF2.3</i></b>	75%	100%	59%
<b>At2G02120-<i>AtPDF2.1</i></b>	71%	71%	100%

Table S4 | Mutants used for genotyping to obtain homozygous knockout plants.

Line	Database name	Origin	ID	Features
<i>coi1-16</i>	coi1-16	NASC	N67817	EMS mutant
<i>acs1-7</i>	acs1-1acs2-1acs4-1acs5-2acs6-1acs7-1acs9-1	NASC	N16650	T-DNA insertion in the exon
<i>sid2</i>	SALK_088254	NASC	N65675	T-DNA insertion in the exon
<i>jar1</i>	SALK_059774C	NASC	N658259	T-DNA insertion in the exon
<i>ein2-1</i>	SALK_086500C	NASC	N654757	T-DNA insertion in the exon
<i>ein2-1coi1-16</i>	ein2-1, coi1-16	NASC	N67818	EMS mutant

Table S5 | Primers used in mutant genotyping and their detail.

Name	Sequence (5' → 3')	Description
COI1_R	ACCACAAATAAAGTTTCAGATGCCA	Flanking Primer for COI1 (Ellis et al. 2002)
COI1_R	CCCCGAAAACATATGTCGGCT	Flanking Primer for COI1 (Ellis et al. 2002)
ACS2_F	GCAGTTTTGTCTGAAGATAGCG	T-DNA LP Primer for <i>acs2-1</i> (Tsuchisaka et al. 2009)
ACS2_R	ATCTGTACCTGATTCTCTGCAA	T-DNA RP Primer for <i>acs2-1</i> (Tsuchisaka et al. 2009)
SID2_F	TCTCCGCAAGGTCTTTTT	SALK_088254 LP Primer for SID2
SID2_R	TCAGAGACGGCGGAGATTAG	SALK_088254 RP Primer for SID2
JAR1_F	ACGTCATCAAGTCCAGAAACA	SALK_059774C LP Primer for JAR1
JAR1_R	GGAACAGGGTGACCAGTGAG	SALK_059774C RP Primer for JAR1
EIN2_F	ACAAAGAGGTTTCGTCACCGT	SALK_086500C LP Primer for EIN2
EIN2_R	TGACGGATCCCAGTATCCACT	SALK_086500C RP Primer for EIN2

## 8 Reference (see page 146)

**Chapter III: Overexpression of *AtPDF1.2a* confers resistance to fungal pathogens in *Arabidopsis thaliana***

To be submitted to the journal *Frontiers in Plant Science*

Shailja Singh, Wanzhi Ye, Daguang Cai

Institute of Phytopathology, Department of Molecular Phytopathology and Biotechnology, Christian-Albrechts-University of Kiel, Hermann Rodewald Str. 9, D-24118 Kiel, Germany

**Running head:** Plant PDF *AtPDF1.2a* in plant-fungus interactions

**Keywords:** Plant defensins, PDFs, *AtPDF1.2a*, *Verticillium longisporum*, plant-fungus interactions

## 1 Abstract

Plant defensin *AtPDF1.2a* is known to accumulate in leaves/roots of *Arabidopsis thaliana* upon attacks of various fungal pathogens. As expected, the infection with *Verticillium longisporum* (VI43) resulted in a pronounced induction of the *AtPDF1.2a* expression in Arabidopsis plants, differing from *AtPDF2.2*. To exclusively investigate the antifungal activity of *AtPDF1.2a*, we generated *AtPDF1.2a*-overexpressing and -knockout Arabidopsis plants and challenged them first with VI43 infection in which the wild type Col-0 plants served as control. We report that transgenic Arabidopsis plants overexpressing *AtPDF1.2a* exhibited strong resistance towards *V. longisporum* infection while *AtPDF1.2a* knockdown plants suffered severely from the fungal infection. In addition, we report that the *AtPDF1.2a* expression modulates antifungal activity towards both of *V. longisporum* and *S. sclerotiorum*, but not bacterial pathogen *Pseudomonas syringae* (*Pst* DC3000). Furthermore, analysis of signaling and transcriptional processes underlying the mode of *AtPDF1.2a* functions identified that JA, ET, and SA pathway responsive genes were upregulated only in the *AtPDF1.2a* knockout mutant plants, but not in its overexpression plants. This might reflect possible feedback to maintain the JA-mediated downstream signaling *via AtPDF1.2a* limiting expression. In addition, preferentially augmented ET and SA pathway responsive gene expressions could be a consequence of the highly up-regulated JA pathway induced by the fungal infection. A functional model is discussed.

## 2 Introduction

Achieving effective and durable control on fungal pathogens is one of the major challenges in modern agriculture (Collinge et al. 2010). Despite the continued release of resistant cultivars and chemical fungicides, the estimated 10 % of crop yields are lost due to fungal diseases (Strange and Scott 2005). Fungal diseases are often catastrophically resulting in massive destruction of crop yields and food shortage. Successful pathogens are able to cause disease because of their ability to thwart the surveillance and defense mechanisms of their host plants.

One such pathogen is *Verticillium longisporum*, a soil-borne fungal pathogen, which especially thrives in northern Europe where it particularly attacks Brassica oil crops (Fahleson et al. 2003; Johansson et al. 2006; Zeise and Tiedemann 2002). To date, the economic importance and acreage of oilseed rape is increasing because of the growing demand for oil crops for nutrition and bio-fuels (Nath et al. 2016; Robson et al. 2002). This is accompanied by a spread of *Verticillium* diseases, which may cause yield losses as high as 10 to 50% (Dunker et al. 2008). Conventional fungicides cannot control diseases caused by *Verticillium* spp., the use of resistant cultivars is one of the most promising alternatives (Depotter et al. 2016). *Verticillium* species infect their hosts by root penetration and subsequently colonize the xylem, where they cause partial clogging of the vessels (Depotter et al. 2016; Eynck et al. 2007; Schnathorst 1981). These obstructions are expected to affect water and nutrient transport. Therefore, typical disease symptoms such as wilting, stunting, chlorosis, and premature senescence have been observed to occur as consequences of water limitations and insufficient nutrient supply (Johansson et al. 2006). In contrast to other target plants, wilting symptoms were not observed in oilseed rape or *Arabidopsis* plants infected with *V. longisporum* (Floerl et al. 2008). However, the *V. longisporum*-infected plants exhibited severe stunting indicating that the plant-pathogen interaction resulted in extensive re-modeling of plant architecture (Floerl et al. 2008; Floerl et al. 2010; Ratzinger et al. 2009). Therefore, the disease is termed as 'Verticillium stem striping' (Depotter et al. 2016). Fine-tuning of defense responses to fungal pathogens allows plants to combat fungal pathogens without compromising their normal growth and development. Molecular breeding and transgenic approaches are being pursued aggressively for the development of disease-resistant crops.

To fight against pathogenic microorganisms, plants have evolved an innate immune system. This ancient defense system provides nonspecific broad-spectrum resistance against microbial invasion. The innate immunity of plants comprises fortification of the cell wall, hypersensitive response, and production of antimicrobial compounds and antimicrobial peptides (AMPs). AMPs, which are usually cysteine-rich molecules, possess a potential and broad range of antimicrobial activity. AMPs serve as one of the first lines of defense against pathogen invasion and are one of the key contributors to innate immunity in plants (Goyal et al. 2014).

Plant defensins (PDFs) are small cysteine-rich proteins of 45-54 amino acids that are closely related to insect and mammalian PDFs (Lay and Anderson 2005; Thomma et al. 2002; Zasloff 2002). PDF genes encode a precursor protein that possesses an amino-terminal endoplasmic reticulum targeting signal peptide followed by the mature PDF domain and an optional C-terminal propeptide (CTPP). Based on the presence or absence of CTPP, PDFs are classified into two subgroups: class 1 and class 2. Class-2 PDFs have an approximately 27-33 amino acid long CTPP rich in aspartic acid and glutamic acid, imparting a net negative charge to counterbalance the positive charge on the PDF domain (Lay et al. 2014). Most of the seed PDFs are class1 type, whereas most class 2 PDFs are found in leaves, flowers, and fruits (Lay et al. 2003). The class 2 PDFs are expressed in most plants. Although abundant in seeds, they are expressed in almost all organs of a plant. The majority of PDFs are synthesized as precursor proteins and post-translational processing cleaves out the C-terminal mature PDF peptide from the secretory signal peptide. Although most major PDFs are secreted to the extracellular space, a few floral PDFs are targeted to the vacuole. PDFs have a compact shape and share an identical backbone structure stabilized by four, occasionally five, intramolecular disulfide bonds. The three-dimensional structures of several PDFs have been determined and are each characterized by the presence of a single  $\alpha$ -helix and three antiparallel  $\beta$ -strands (Bloch et al. 1998; Fant et al. 1998; Fant et al. 1999; Almeida et al. 2002; Janssen et al. 2003; Lay et al. 2003a; b). The  $\alpha$ -helix is connected to the second  $\beta$ -strand through a cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet ( $\alpha/\beta$ ) motif. Despite their structural similarity, amino acid sequences of mature PDFs are highly variable indicating a rich diversity of variants (Thomma et al. 2002). This variation in primary sequences may account for different biological roles attributed to PDFs, which include antibacterial activity (Zhang and Lewis

1997; Segura et al. 1998; Chen et al. 2005; Aerts et al. 2008), zinc tolerance (Mirouze et al. 2006), proteinase inhibitory activity (Wijaya et al. 2000),  $\alpha$ -amylase inhibitory activity (Bloch and Richardson 1991), ion channel blocking activity (Kushmerick et al. 1998; Spelbrink et al. 2004; Amien et al. 2010) and pollen tube growth arrest, burst, and sperm discharge (Amien et al; 2010). Increasing data suggest a role for PDFs in the resistance of plants against pathogens, especially fungi. However, their mode of action are not yet fully understood, which but differs among PDFs (De Coninck et al. 2013; Sagaram et al. 2012; Vriens et al. 2014).

To date, more than 300 PDF-like (DEFL) genes have been identified in the model plant *Arabidopsis* (Silverstein et al. 2005). By performing a TBLASTN search on the fully sequenced genome of *Arabidopsis*, 15 putative *AtPDF* genes were identified (Thomma et al. 2002; Penninckx et al. 1996; Epple et al. 1997; Thomma and Broekaert 1998). The deduced putative proteins of *AtPDF* sequences can be classified into three families, Class-I, II, and III.

Class-I contains seven *AtPDF* genes, *AtPDF1.1*, *1.2a*, *1.2b*, *1.2c*, *1.3*, *1.4* *1.5*. Five genes show high sequence similarity (*AtPDF1.1* to *AtPDF1.3*). Three genes (*AtPDF1.2a*, *b* and *c*) encode the same mature peptide (*AtPDF1.2*) with the difference in their corresponding signal peptide sequences. *AtPDF1.2a* and *AtPDF1.2c* gene sequences are located in tandem repeat on chromosome 5, while *AtPDF1.2b* and *AtPDF1.3* are clustered in tandem repeat on chromosome 2 (Thomma et al. 2002; Sels et al. 2008). The first family members *AtPDF1.1*, *1.2a*, *1.2b*, *1.2c*, *1.3* and to some extent *1.4* were found induced in the non-host resistance response of *Arabidopsis* to the barley powdery mildew fungus (Sels et al. 2008). Overexpression of *AtPDF1.1* resulted in enhanced resistance of *Arabidopsis* plants against *Cercospora beticola* and *P. carotovorum* (De Coninck et al. 2010; Pao-Yuan et al. 2017). *AtPDF1.2* gene was induced upon pathogen challenge, both locally and at the site of inoculation with incompatible fungal pathogens and systemically in non-inoculated regions of the plant (Su et al. 2018; Penninckx et al. 1996).

*AtPDF1.2* has been purified from *Arabidopsis* leaves infected with the fungus *Alternaria brassicicola* (Padovan et al. 2010; Thomma et al. 2002). Expressed sequence tags



(Broekaert et al. 1995) have identified the cDNAs corresponding to *AtPDF1.1* and *AtPDF1.2*. In contrast to most systemically pathogen-induced genes described so far, *AtPDF1.2* was not activated by exogenous salicylic acid (Broekaert et al. 1995), a signal molecule that plays a central role in many induced defense responses in plants (Delaney et al. 1994). However, *AtPDF1.2* was induced by treatment with methyljasmonate (MeJA), ethylene (ET) and reactive oxygen-generating compounds (Broekaert et al. 1995). Regarding a possible role in defense *in-vivo*, there are several Arabidopsis mutants, with altered *AtPDF1.2a* expression levels, which often show altered pathogen sensitivity. For instance, there are several reports on mutants with diminished *AtPDF1.2a* levels, which show a higher sensitivity against necrotrophic pathogens like *B. cinerea*, including the JA signaling mutant *coi1* and the ET signaling mutant *ein2* (Thomma et al. 1999) and *bos2*, *bos3*, and *bos4* mutants (Veronese et al. 2004). However, these effects are not considered to be solely caused by altered *AtPDF1.2a* expression, for example, the *bos1* mutant shows increased sensitivity against *B. cinerea* while having normal *AtPDF1.2a* expression levels (Mengiste et al. 2003). Although *AtPDF1.2* shows *in-vitro*, antifungal activity (Penninckx et al. 1996), its precise role in plant defense remains unclear. Yet, no direct evidence for a role in plant defense *in-vivo* has been shown for any AtPDF member.

It was observed that transgenic Arabidopsis plants, overexpressing *AtPDF2.2* exhibited strong resistance towards *V. longisporum* infection while *AtPDF2.2*-knockdown plants suffered severely from the fungal infection. Here, we report that overexpression of *AtPDF1.2a* in transgenic plants enhanced plant resistance against the *V. longisporum* infection, while knockout of *AtPDF1.2a* to contrast greatly enhanced the plant susceptibility to the *V. longisporum* infection. Interference of different signaling pathways with the *AtPDF1.2a* and vice versa was analyzed.

### **3 Materials and methods**

#### **3.1 Plant material and growth conditions**

Arabidopsis ecotype Columbia (Col-0) was the genetic background for all wild type and transgenic plants. The Arabidopsis mutants; *KO-pdf1.2a*, *jar1*, *coi1-16*, *acs1-9*, *ein2-*

1, *ein2-1coi1-16* and *sid2* were obtained from the Nottingham Arabidopsis Stock Centre. The specification of the mutants are listed in Tab. S1. Genotyping was performed according to the instructions of the SALK-Institute. Primers used for genotyping are listed in Supplementary Table S3.

Seeds were surface-sterilized with 5% sodium hypochlorite/Tween 20 solution and 70% ethanol followed by three times washing with sterile water and placed on Petri dishes with half-strength MS media supplemented 0.8% (w/v) sucrose, 0.1% (w/v) 2-(*N*-morpholino) ethanesulfonic acid (MES), and 0.8% (w/v) agar (Murashige and Skoog, 1962). After cold treatment at 4°C for 48 h, plates were incubated vertically for 14 days at 22°C under short-day conditions (8h light/16h dark; 100  $\mu\text{moles m}^2 \text{sec}^{-1}$ ). EMS mutant plants were grown for 30 days at 16°C under short-day conditions (8h light/16h dark; 100  $\mu\text{moles m}^2 \text{sec}^{-1}$ ).

### **3.2 *In-silico* analysis**

The Arabidopsis Information Resource (TAIR) used for genes hits (AtPDFs) blast. The DNA sequences. The “ClustalW2” program in EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) was used for the construction of the multiple sequences alignment and Maximum-Likelihood phylogenetic analysis using a distance-based tree that was inferred with the BioNJ algorithm (Gascuel, 1997) using PhyML v20160115 (Guindon et al., 2010) ran with model GTR and parameters: -f m --pinv e -o lr --alpha e --nclasses 4 --bootstrap -2.

Expression patterns of *AtPDF* genes in different stages of development (in different tissues) and in response to various abiotic and biotic stresses were analyzed with the help of a free version of the GENEVESTIGATOR (Hruz et al. 2008) online portal.

### **3.3 Pathogen infection procedures and plant inoculation**

*Verticillium longisporum* isolate VI43 (kindly provided by Dr. Elke Diederichsen (FU Berlin, Germany) was maintained on potato dextrose agar (PDA, Merck, Darmstadt, Germany) in petri dishes. For the inoculation, 22% glycerol conidia stocks were thawed, centrifuged for 8 min at 6000g and re-suspended in tap water (“*in vivo*” infection) or sterile Czapek-

Dox medium (“*in vitro*” infection) to a final concentration of  $2 \times 10^6$  conidia / ml. *In vitro* and *in vivo* root inoculation experiments were conducted as described by Behrens (Behrens et al. 2019). The plants were allowed to grow for up to a further two-three weeks followed by fungal DNA quantification and root length measurement at different time points in infected plants in relation to the mean of mock-treated controls.

The *Sclerotinia sclerotiorum* isolate used throughout this work was obtained from Prof. W. Qian (Mei et al. 2011). The infection of Arabidopsis leaves with *S. sclerotiorum* was performed either by agar-plug infection or by drop infection to test for differences in plant resistance between genotypes. *S. sclerotiorum* liquid culture was prepared as described by Rietz et al. (2012) with minor modifications. 5 weeks old Arabidopsis plants were equally spray inoculated with mock (10mM  $\text{MgSO}_4$ ), or *S. sclerotiorum* suspension using a standard spray bottle with 2 g of homogenized mycelium suspended in 50ml 10mM  $\text{MgSO}_4$ . Plants were kept under the hood throughout the experiment and grown in short-day conditions (8h light; 22°C). Lesions were classified for all fully expanded leaves individually from 6 to 48 hpi and disease severity index were summarized for each plant after 72 hours. Photos of infected plants were taken at 7 dpi.

*P. syringae* (Pst DC3000) was cultivated on plates containing NYGA medium (5 g/l tryptone/peptone; 3 g/l yeast extract; 20 ml/l glycerol; 15 g/l agar; 50 mg/l Kanamycin; 100 mg/l Rifampicin) and incubated for 2 days at 28°C. Afterward, the bacteria were washed from the plate with 10mM  $\text{MgCl}_2$  and the concentration was adjusted to  $\text{OD}_{600} = 0.2$  for infection. 5 week old Arabidopsis plants grown in individual pots were equally spray inoculated with mock (10mM  $\text{MgCl}_2$ ), or *Pseudomonas* suspension mixed with 0.04% Silwet Gold using a standard spray bottle. Plants were kept under the hood for 2 days and grown in short-day conditions (8h light; 22°C). Photos of infected and mock-treated plants were taken at 1 and 3 dpi. Bacterial abundance was quantified by a DNA based method proposed by Ross et al. (2016). Samples were collected from two plants (3 leaf discs each).

### **3.4 RNA Isolation**

Total RNA was extracted from 100 mg leaves of the WT and transgenic lines TRIzol® (Thermo Fisher Scientific) reagent according to the manufacturer’s recommendation. The

quality and quantity of RNA samples were analyzed by agarose gel electrophoresis and NanoVue Plus Spectrophotometer (GE Healthcare Life Science).

### **3.5 Expression analysis by Real-Time quantitative PCR**

The RNA (n=3) was isolated as described above. One  $\mu\text{g}$  of RNA was transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) into the first-strand cDNA according to supplier's instructions using oligo-dT primers for mRNA transcription. 2  $\mu\text{l}$  of a 1:10 diluted cDNA preparation was mixed with 18  $\mu\text{l}$  master mix as per the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific) manual. PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following conditions: 3 min 95°C; 45 x 15 sec 95°C, 15 sec 58°C, 20 sec 72°C; 10 sec 95°C. Gene expression was determined using the delta  $C_T$  Method to calculate the relative expression according to Pfaffl (2001). Relative expression was normalized to *ACTIN 2 (AT3G18780)* as a reference gene. Each data point is based on three independent biological replicates measured with two technical replicates each. Relative expression was  $\log_2$  transformed and, to display the differential regulation, the induction was calculated as the difference between *V. longisporum* and mock-treated samples. All the primer pairs utilized are listed in Table S2.

### **3.6 Gene constructs and plant transformation**

For overexpression of *AtPDF1.2a* in Arabidopsis, the full-length *AtPDF1.2a* coding region was PCR amplified from the leaves of Arabidopsis wild-type (ecotype Columbia-0) with gene-specific primers (listed in table S2) using Pfu Polymerase (Thermo Fisher Scientific). The cDNA was cloned into pDONR201 entry vector followed by a recombination reaction using the Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific) and subcloned into pGWB433 (NAKAGAWA 2007), in sense orientation.

Consequently, the overexpression construct was brought into *Agrobacterium tumefaciens* GV3101:pMP90RK to facilitate plant transformation. Followed by transformation into Arabidopsis using the floral dip method (Clough and Bent 1998). The seeds were harvested from the infiltrated plants and positive plants were selected on half Murashige and Skoog's (MS) medium supplemented with 50 mg L<sup>-1</sup> kanamycin (Sigma-

Aldrich, The United States). The kanamycin-resistant plants were transferred to the soil after 10 days of germination and were grown in a growth chamber.

### **3.7 gDNA isolation and PCR analysis of transgenic Arabidopsis plants**

For gDNA isolation, the tissue was immediately frozen in liquid nitrogen and grounded using a mortar and pestle. Total genomic DNA was isolated using CTAB (cetyltrimethylammonium bromide) buffer (Rogers and Bendich, 1985) supplemented with  $\beta$ -Mercaptoethanol. For PCR, the reaction mixture contained 100 ng gDNA, 2 mL 10 Taq buffer, 1 mL of 10 pmol each of forward and reverse gene-specific primers, 1 mL of 10 mM dNTP mixture (Thermo Scientific, Germany), and 0.4 mL of Taq DNA polymerase (3 U/mL) (Thermo Scientific, Germany), and the volume was made up to 20 mL using autoclaved MilliQ water. The PCR was performed under following conditions: initial denaturation at 95°C x 10 min followed by 30 cycles at 95°C x 45 secs, 60°C x 30 secs, and 72°C x 40 secs, and a final extension at 72°C x 7 min. The PCR product was visualized by electrophoresis on a 1% agarose gel. The expression of *AtPDF1.2a* in the transgenic plants was assessed using RT-qPCR.

### **3.8 Mutant identification and plant genotyping**

The Arabidopsis mutants; *KO-pdf1.2a*, *jar1*, *acs1-7*, *ein2*, and *sid2* had T-DNA insertions in exon region. Plant genomic DNA was extracted using the above described gDNA isolation method. The T-DNA insertions were checked using LBb, LP, and RP primers, which were obtained using T-DNA Primer Design tool using SALK website (<http://signal.salk.edu/tdnaprimers.2.html>). The mutant line was in the Col-0 background. Table S3 shows a list of PCR primers used for the genotyping of mutations. The genotypes of plants were determined by PCR with allele-specific primers. For EMS mutants, *coi1-16* and *ein2-1coi1-16* plants, the COI1 gene was amplified from genomic DNA using Pfu Polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. The amplified PCR products were sequenced as described by Ellis et al. (2002).

### **3.9 V. longisporum (VI43) gDNA detection in plant tissue**

Arabidopsis leaves and roots of six infected plants were sampled individually at the indicated time points post-inoculation. gDNA isolation was performed as described above.

For real-time qPCR quantification of fungal DNA in infected plants, the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) was used according to the manufacturer's instructions. Primers used are listed in Table S2. PCR was performed using the following conditions: 3 min x 95°C; 35 x 20 sec 95°C, 20 sec 58°C, 30 secs 72°C. Error bars indicate the standard deviation ( $\pm$ SD) of three different biological repetitions and statistics was performed using the student's t-test (\* $p \leq 0.05$ ). For each sample the amount of fungal DNA in relation to plant DNA was calculated in ng/mg.

### 3.10 Morphology analysis

Arabidopsis wild-type plants (Col-0) and Arabidopsis transgenic plants of *AtPDF1.2a* (*KO-pdf1.2a*) and overexpression (OE-PDF1.2a) were used for the measurement of differences in plant's morphology. Around 15 seedlings from each line were grown on single 120x120 mm petri dishes similarly like in *in-vitro* infection assay that is stated above. The morphological differences between the 20 days old vertically growing transgenic lines and wild types were recorded after the transference on the 120x120 mm petri dishes containing  $\frac{1}{2}$  MS (0.5% sucrose) and pictures were taken. These pictures were later analyzed on the Image J software (<https://imagej.nih.gov/ij/download.html>) for measuring root length and number of lateral roots for each plant using the provided reference measurement to the software.

After two days of cold stratification, the Col-0 wild-type, *KO-pdf1.2a* and OE-PDF1.2a seeds were sown and germinated in pot soil. Plants were grown under short-day conditions till the flowering stage and then transferred to the long-day conditions (16h light/8h dark). These plants were photographed as well on a particular day to measure the rosette area by the Image J software.

### 3.11 Statistical analyses

All experiments were conducted using a completely randomized design. Four samples used as replicates and two technical replicates were used for each treatment. The data were analyzed using a two-tailed Student's t-test and the differences were considered significant at  $P < 0.05$  and extremely significant at  $P < 0.01$ . Multiple comparisons of

statistical significance were carried out using a two-way ANOVA. Graphs and plots were generated with Microsoft® Office 2016. Phenotypic observations were monitored for two to three generations for consistency.

### **3.12 Accession number**

Sequence data of *AtPDF1.2a* can be found in the Arabidopsis Information Resource (TAIR) database with the accession number AT5G44420. Additional sequence data and mutant information are shown in Table S1 and S3 respectively.

## 4 Results

### 4.1 Upregulation of *AtPDF1.2a* gene in Arabidopsis roots at the early infection stage

The response of Arabidopsis (Col-0) wild-type to infection with *V. longisporum* (VI43) at 18 dpi was investigated, in which non-infected Col-0 wild-type served as a control. The infected Col-0 wild-type plants showed severely retarded growth and necrosis symptoms relative to non-infected control (Fig. 1B). We further examined the response of *AtPDF* genes to infection with *V. longisporum* (VI43) at 6 dpi at the transcript level in the roots. As shown in Fig. 1c, *AtPDF1.2a* expression level was elevated upon VI43 infection (Fig. 1C). The expression intensities of most members of *AtPDF* gene families, Class-I, II, and III were also significantly induced.

Multiple sequence alignment and phylogenetic tree analysis of Arabidopsis Class-I *AtPDF* genes revealed that *AtPDF1.2a* shared high identity (85 and 80% respectively) with two other closely related *AtPDF* genes, described as *AtPDF1.2b* and *1.2c* followed by *AtPDF1.3* and *AtPDF1.1* respectively (Fig. 2A, B, Table S4).

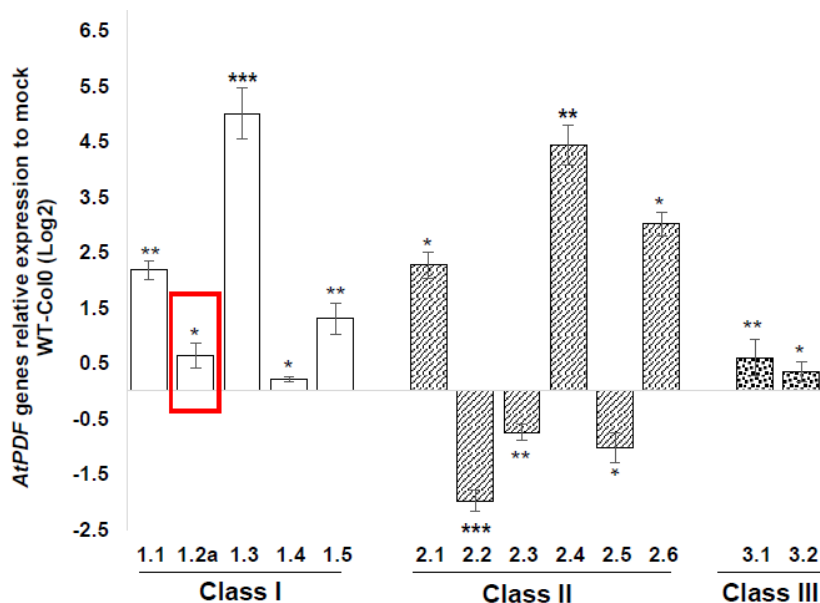


Figure 1 | Induction of *AtPDF1.2a* expression upon *V. longisporum* infection at 6dpi in Arabidopsis roots. Relative abundance of the Arabidopsis *AtPDF* genes from Class- I, II and III (*AtPDF1.1*, *AtPDF1.2*, *AtPDF1.3*, *AtPDF1.4*, *AtPDF1.5*, *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.4*, *AtPDF2.5*, *AtPDF2.6*, *AtPDF3.1* and *AtPDF3.2*) was quantified in Control- and/or *V. longisporum* inoculated Col-0 wild-type plants. The *AtPDFs* transcript levels were normalized to the expression of *ACTIN 2* measured in the same samples and expressed logarithmically. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.



The possible interaction network of AtPDF1.2a proteins was constructed *in-silico*, to identify the putative functions as well as interactions between AtPDF1.2a and other Arabidopsis proteins (Fig. 2C). By applying the STRING database, the network of AtPDF1.2a were given with a high confidence (score>0.9), involving 20 interactive proteins, including protein PATHOGENESIS-RELATED PROTEIN 1 (PR1), ENHANCED DISEASE SUSCEPTIBILITY 16 (EDS16), NONEXPRESSER OF PR 1 (NPR1), etc. The largest part of the network contains ten proteins (PR1, NPR1, EDS1, EDS16, TGA1, TGA6, PAD4, etc.) are all associated with the SA signal pathway, suggesting its involvement/interaction in/with the SA pathway. However, AtPDF1.2a directly interacts with OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59), an essential integrator of the JA and ethylene signal transduction pathways along with few other JA and ET pathway proteins. Noticeably, all the proteins interacted with AtPDF1.2a are involved in plant defense responses.

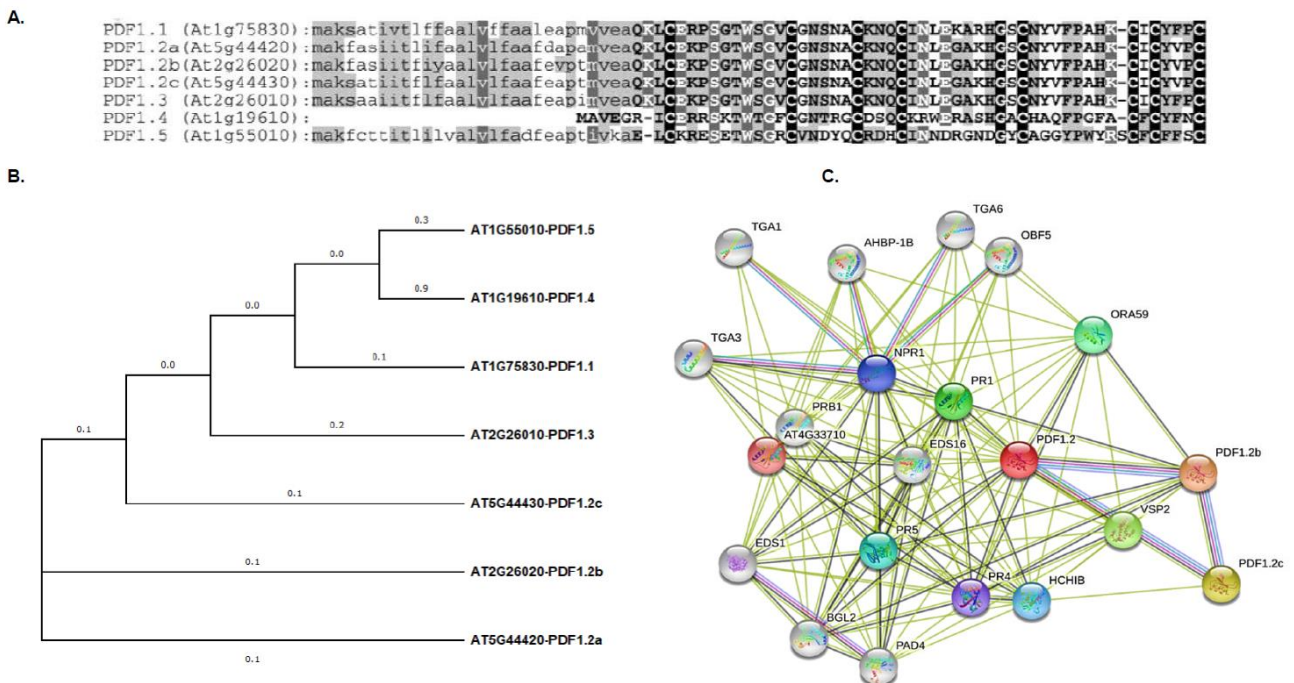


Figure 2 | *AtPDF1.2a* Phylogenetic analysis and *in-silico* protein interactions. **(A)** Multiple sequence alignment of the AtPDF Class-I proteins using CLUSTAL W. Consensus sequences (100 %) are exhibited in black shading and (C) denotes conserved cysteine residues in the alignment. The accession numbers of the gene sequences are given in brackets (AT1G75830-*AtPDF1.1*), (AT5G44420-*AtPDF1.2a*), (AT2G26020-*AtPDF1.2b*), (AT5G44430-*AtPDF1.2c*), (AT2G26010-*AtPDF1.3*), (AT1G19610-*AtPDF1.4*) and (AT1G55010-*AtPDF1.5*). **(B)** Phylogenetic tree based on amino acid sequences indicating the relationships among Class-I of AtPDF proteins using CLUSTAL W using a distance-based tree that was inferred with the BioNJ algorithm (Gascuel, 1997) using PhyML v20160115 (Guindon et al. 2010) ran with model GTR and parameters: -f m --pinv e -o lr --alpha e --n-classes 4 --bootstrap -2. **(C)** *AtPDF1.2a*

interaction observed by String (<https://string-db.org/cgi/network.pl?taskId=LhjXqVQq9t5z>). Network: shows current interactions. Co-expression: proteins whose genes are observed to be correlated in expression, across a large number of experiments.

## 4.2 *In-silico* analysis of expression of Class- I *AtPDF* genes

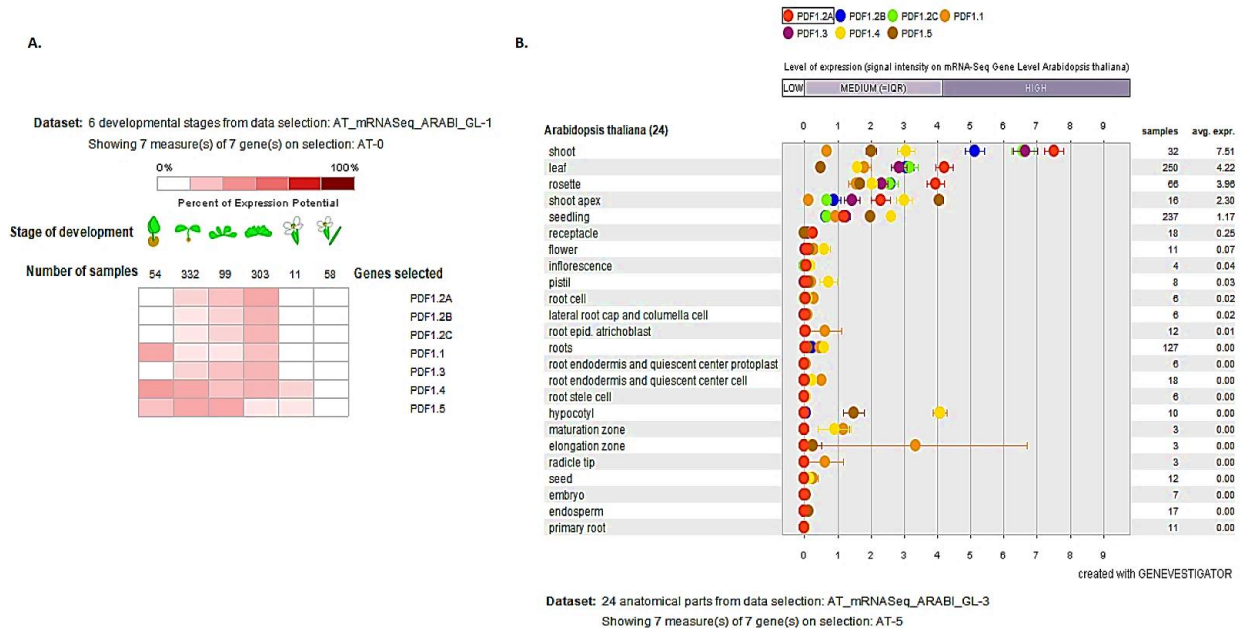


Figure 3 | *In-silico* analysis of *AtPDF1.2a* expression via GENEVESTIGATOR for six developmental stages and different organs/tissues in Arabidopsis respectively.

(A) *AtPDF1.2a* expression was limited in seedling, young rosette developed rosette stage. Maximum expression was in developed rosette which was observed similarly in other *AtPDF* Class-I genes as well except *AtPDF1.5*. (B) Microarray expression data for different plant organs/tissues of *AtPDF1.2a* from At mRNASeq\_ARABI\_GL-3 platform database. Red dot on scatter plot represent it. *AtPDF1.2a* expression was detected maximum in shoot, medium in leaf, rosette, shoot apex, seedling and minutely induced in receptacle, flower, inflorescence and root cell. Other Class-I *ATPDFs* also showed the similar expression intensity in different tissues.

The *AtPDF1.2a* expression, along with other members of Class- I *AtPDF* genes were evaluated for six development stages and in different plant tissues/organs of Arabidopsis plants using microarray data from Genevestigator (Hruz et al. 2008). The analysis in different developmental stages revealed that the expression of *AtPDF1.2a* was found to be 20 to 30% in the young rosette stage, followed by 40 to 50% in the mature rosette stage (Fig. 3A). Other Class-I *AtPDF* genes displayed up to 40% expression in all specified stages. The fact that all Class- I *AtPDF* genes express during the rosette stage, indicating a synchronized role during the vegetative development of the rosette. While *in-silico* analysis in different plant tissues demonstrated, that *AtPDF1.2a* exhibits differential expression patterns in various organs. The *AtPDF1.2a* expression level is highest in the

shoot and becoming lower in the upper plant tissues, and nearly null expression in the root-related tissues. Similarly, other *AtPDF* genes of Class- I also exhibited an inclination as described above (Fig. 3B). This analysis supports that *AtPDF1.2a* plays a definite role in plant vegetative growth and development.

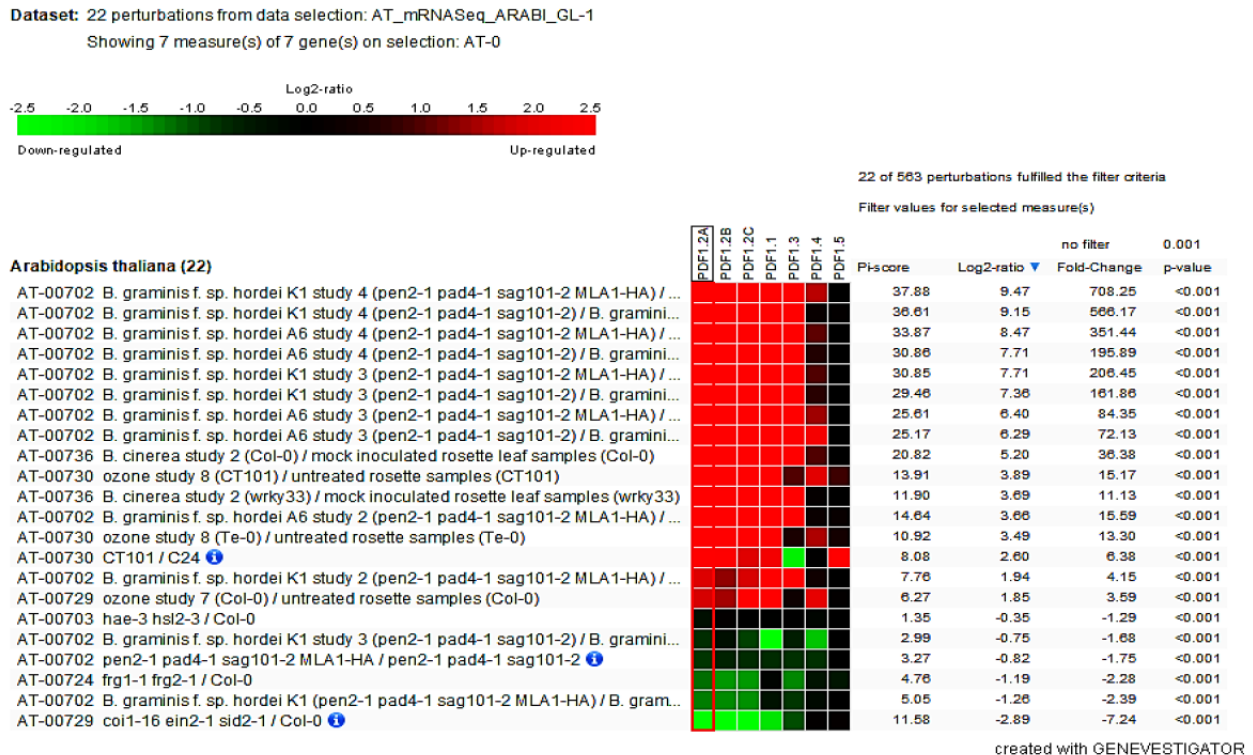


Figure 4 | Clustering analysis conducted via Genevestigator software.

Microarray's gene expression data from abiotic and biotic treatments and experiments were obtained from the plant model *Arabidopsis* and AT\_mRNASeq\_ARABI\_GL-1 platform database. Gene expression patterns are represented in a log2 ratio ranging from - 2.5 (green colour denotes down-regulated genes) to +2.5 (red colour denotes up-regulated genes). Fungal and ozone treatments presented upregulation of *AtPDF1.2a* along with other Class-I *AtPDF* genes except *AtPDF1.5* whereas SA/JA/ET pathway mutant experiments exhibited downregulation of *AtPDF1.2a*. Again, it was similar for most of the other Cass-I *AtPDF* genes as well.

Genevestigator analysis suggested distinct expression patterns of *AtPDF1.2a* in response to abiotic and biotic stresses (Fig. 4). The *AtPDF1.2a* expression was greatly upregulated by fungal pathogens, *Blumeria graminis*, and *B. cinerea*, which meets the observations made in many studies on fungal pathogens. However, De Coninck et al. (2010) presented that transgenic plants overexpressing *AtPDF1.2a* did not affect the plant disease resistance response against the host-pathogen *B. cinerea*. In regarding abiotic stresses, *AtPDF1.2a* was induced only by ozone stress, while a drastic reduction in *AtPDF1.2a* expression was observed in triple mutants of JA/ET/SA pathways. Some of

the PDFs have also been recognized to be expressed during abiotic stress conditions such as drought, cold, and salt, implying its role in stress-responsive signal transduction (Lay and Anderson 2005). However, the suppression in triple mutants is probably an indication of the simultaneous regulation of *AtPDF1.2a* by all three pathways.

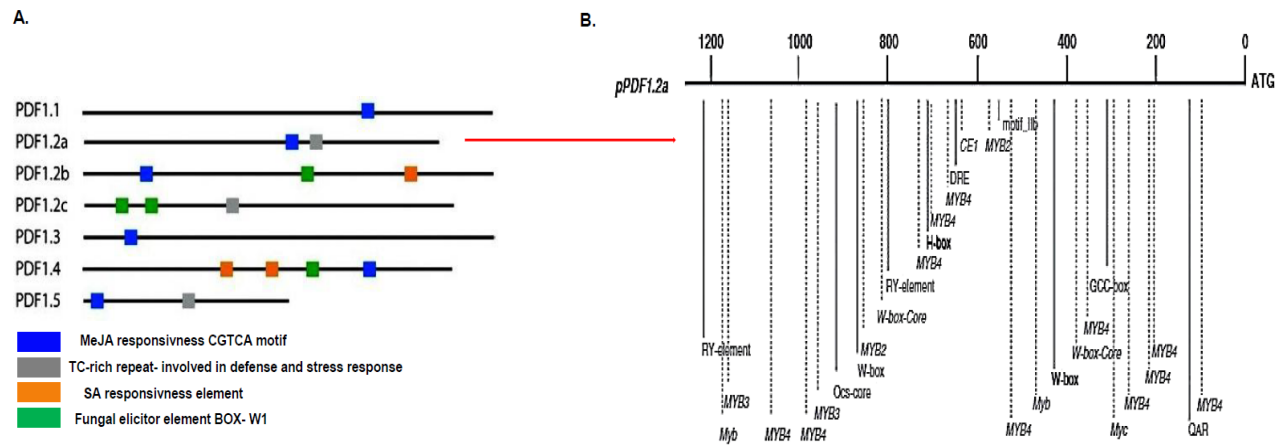


Figure 5 | *In-silico* analysis for the presence of *cis*-acting elements involved in gene expression upon biotic or abiotic stress using PlantCARE.

(A) Some important *cis*-elements were detected on the promoter region of all the Class-1 *AtPDFs*. (B) Detailed motif searched was performed in the promoter region of *AtPDF1.2a* and important *cis*-elements were identified like stress responsive W-Box (binding site for WRKY transcription factors), several MYB-responsive elements (MYB2: drought and low oxygen induced element; MYB3: phenylpropanoid biosynthesis gene expression etc), DRE- (cold- and dehydration-responsiveness), H-Box (chalcone synthase) etc.

Investigation of the 1183 bp promoter region upstream of the transcription start site of the *AtPDF1.2a* revealed several putative motifs with homology to known *cis*-elements, including those with particular relevance for jasmonate-mediated expression (Fig. 5). Two of these motifs, located between -277 and -495 of the *AtPDF1.2a* promoter, included the jasmonate-responsive element, TGACG (-392 to -396) and a 9-bp sequence, AAATGTTGT (-410 to -419). GCC-boxes were also detected. Analysis of *AtPDF1.2a* promoter indicated the existence of more interactive *cis* elements necessary for a jasmonate-mediated regulation of *AtPDF1.2a* expression during plant defense responses.



### 4.3 Knockdown and overexpression of the *AtPDF1.2* gene alters the phenotype and rosette size

With the help of transgenic *Arabidopsis* plants of *AtPDF1.2* knockout (*KO-pdf1.2a*) and overexpression (OE-PDF1.2a), the role of *AtPDF1.2a* in plant growth and development was examined.

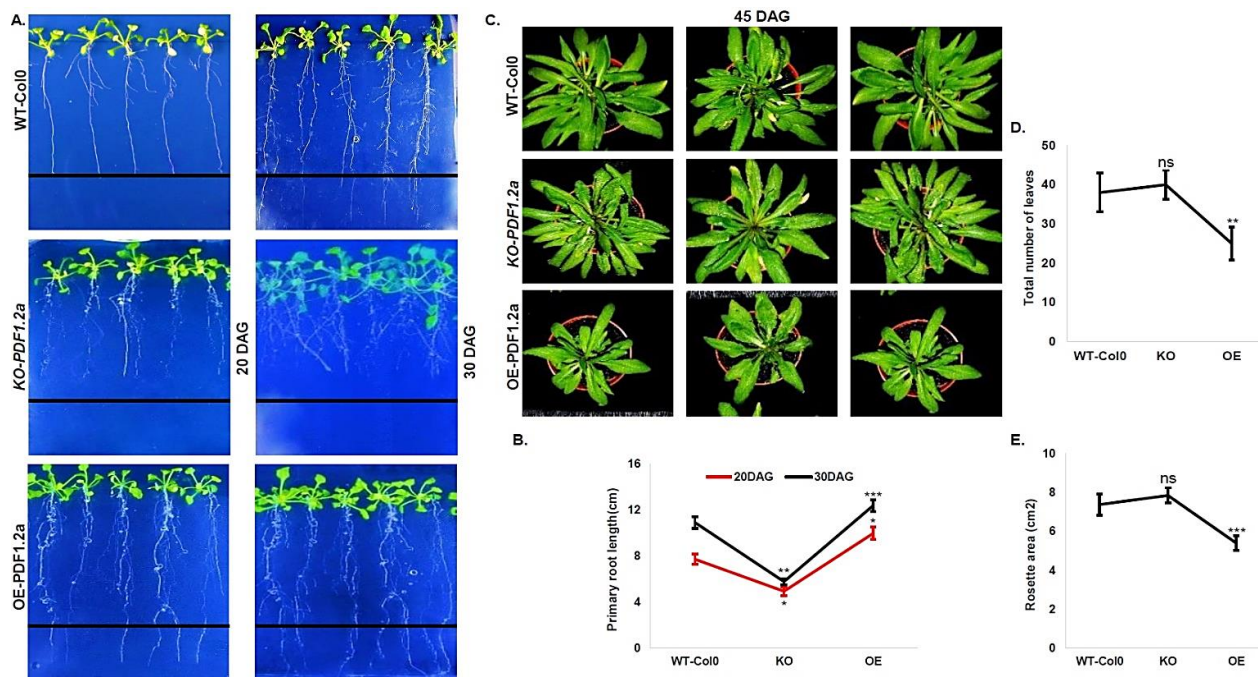


Figure 6 | Phenotyping of *AtPDF1.2a* transgenic plants.

Phenotypes of wild-type (Wt-Col-0) and *AtPDF1.2a* transgenic lines for mutant (*KO-pdf1.2a*) and overexpression (OE-PDF1.2a). (A, B) Thirty-day-old seedlings of Wt-Col-0, *KO-pdf1.2a*, and OE-PDF1.2a promotes contrasting primary root growth. Seedlings were grown on vertical 1/2MS agar medium and primary root length was measured for 25-30 seedlings. (C) Wt-Col-0, *KO-pdf1.2a*, and OE-PDF1.2a rosette at 45 DAG. (D) Average leaf count (E) and rosette diameter measurements at 45 DAG. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

The presence and expression intensity of *AtPDF1.2a* in *KO-pdf1.2a* and OE-PDF1.2a were confirmed and measured using RT-qPCR (Fig. 9C). We examined the phenotypic changes in the three consecutive T2, T3 and T4 generations of *KO-pdf1.2a* and OE-PDF1.2a, including primary root length, the total number of leaves and rosette area and compared them with the Col-0 wild-type. A significant difference in plant growth was visualized between the *KO-pdf1.2a* and OE-PDF1.2a plants. The seedlings of *KO-pdf1.2a* showed shorter primary but more lateral roots, whereas OE-PDF1.2a seedlings at the indicated time points (Fig. 6A). To confirm the difference between

the primary root length of *KO-pdf1.2a* and OE-PDF1.2a seedlings, ImageJ software was utilized (Fig. 6B).

In addition, at 20 days after germination (DAG), the total number of leaves counted in the OE-PDF1.2a plants was significantly lesser as compared with the Col-0 wild-type and *KO-pdf1.2a* plants as well (Fig. 6C, D). In addition, the rosette area of OE-PDF1.2a plants was reduced, whereas *KO-pdf1.2a* showed a comparable rosette area size as that of Col-0 wild-type plants (Fig. 6C, E). The modulation of the *AtPDF1.2a* expression affects the leaves number and overall rosette size, indicating a substantial role of *AtPDF1.2a* in plant growth and development.

#### **4.4 Knockout of *AtPDF1.2a* prompts differential expressions of defense related genes**

Expression levels of JA pathway responsive genes (*AtPDF1.1 - 3.2*, *LOX3*, *AOC3*, and *OPR3*), SA pathway responsive genes (*PR1*, *WRKY70*, and *WRKY53*) and ethylene-associated genes (*ETR2* and *EIN3*) were measured in *KO-pdf1.2a*, OE-PDF1.2a and Col-0 wild-type in the roots of same samples (Fig. 7).

The expression intensities of *AtPDF1.1*, *1.4*, *1.5*, *2.1*, *2.2*, *2.3*, *3.1* and *3.2* genes were found to be down-regulated in the *KO-pdf1.2a* plants where the expression abundance of *AtPDF1.3*, *2.4*, *2.5* and *2.6* were not affected. However, JA biosynthesis responsive genes, *LOX3*, *AOC3*, and *OPR3* in the absence of infection did not demonstrate any transcript change in the *KO-pdf1.2a* plants. Afterward, we examined the ethylene response genes *ETR2*, a negative regulator, and *EIN3*, a positive regulator of the ethylene pathway (Wang et al. 2006). The transcript levels of *ETR2* and *EIN3* in *KO-pdf1.2a* remained unchanged as in the Col-0 wild-type, where *ETR2* was notably upregulated while consequently *EIN3* was suppressed, significantly. These data demonstrate an inactive ET pathway signaling. In similar, expressions of SA-associated *PR1*, *WRKY70*, and *WRKY53* genes were found all with comparable levels as those observed in the Col-0 wild-type plants (Fig. 7 A, B), respectively.

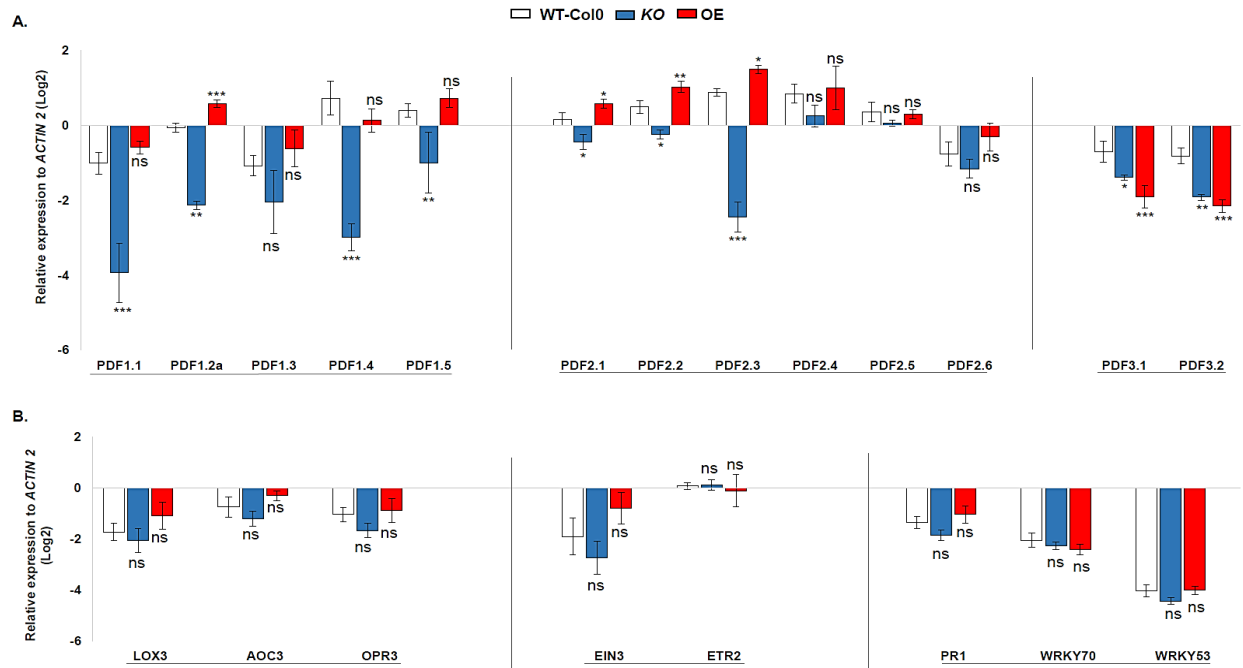


Figure 7 | Defense related gene expression in *AtPDF1.2a* transgenic plants.

Expression analysis in terms of the relative expression levels of defence-related genes in non-infected WT-Col-0 compared to the *AtPDF1.2a* transgenic line for *KO-pdf1.2a* (*KO*) and OE-*PDF1.2a* (*OE*) by RT-qPCR. **(A)** *AtPDF1.1*, *AtPDF1.2a*, *AtPDF1.3*, *AtPDF1.4*, *AtPDF1.5*, *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.4*, *AtPDF2.5*, *AtPDF2.6*, *AtPDF3.1* and *AtPDF3.2* represent fifteen Arabidopsis *AtPDF* genes. **(B)** *LOX3*, *AOC3* and *OPR3* represent the JA biosynthesis-related gene; *ETR2* and *EIN3* are ethylene signalling pathway regulator genes; *PR1*, *WRKY70* and *WRKY53* are the major corresponding genes involved in the SA-synthesis pathway. The gene transcript levels were normalized to the expression of *ACTIN 2* measured in the same samples and expressed logarithmically. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

Interestingly, the OE-*PDF1.2a* plants exhibited strongly induced expressions of *AtPDF2.1*, 2.2 and 2.3, while OE-*PDF1.2a* also showed repressed expressions of Class-III genes, *AtPDF3.1*, and 3.2, which was but downregulated in the *KO-pdf1.2a* plants. Again, the transcriptional changes in three JA biosynthesis genes were insignificant. Similarly, the expression intensities for ET and SA associated genes remained unaffected in OE-*PDF1.2a* plants like in Col-0 wild type plants.

#### 4.5 SA-signaling pathway deficient mutants show impeded *AtPDF1.2a* expression

Analysis of the Genevestigator microarray data indicates a strong down-regulation of *AtPDF1.2a* in the JA/ET/SA triple mutants (*coin1-16ein2-1sid2-1*) even without any infection. To evaluate how the *AtPDF1.2a* expression is dependent on the three defense pathways, respective mutants of defense pathways were analyzed. As shown in Fig. 8, without fungal infection *AtPDF1.2a* expression was found to be constitutive in the JA and

ET single Arabidopsis knockout mutants (*jar1*, *coi1-16*, *acs1-9*, *ein2-1*) and their double knockout mutant (*coi1-16/ein2-1*) as well. However, SA related single mutant (*sid2*) showed a markedly up-regulated expression of *AtPDF1.2a*. These data suggest that the *AtPDF1.2a* expression is independent of JA or ET pathways, however repressed by SA-signaling pathway. The underlying mechanism remains unsolved.

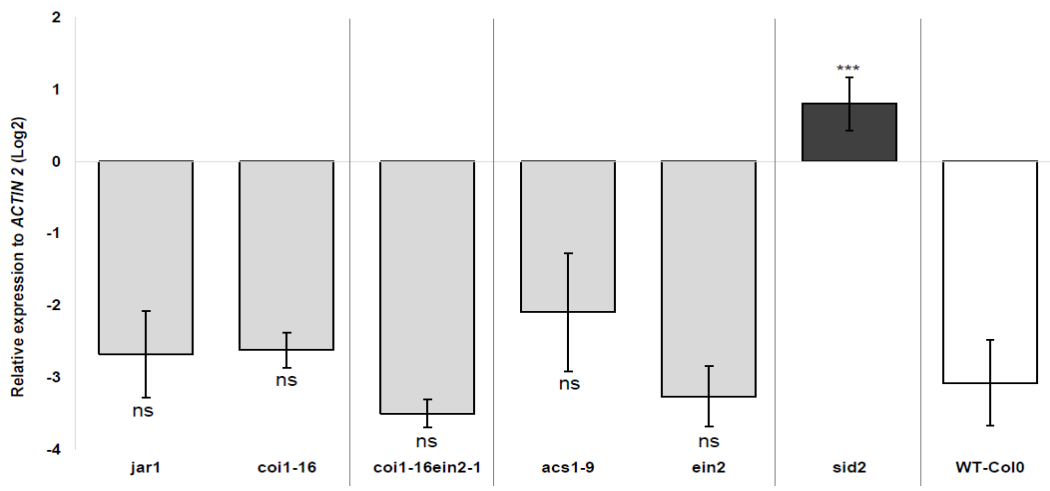


Figure 8 | *AtPDF1.2a* gene expression analysis in different defence related Arabidopsis mutants. Wild-type (WT-Col-0) and mutants (*jar1*, *coi1-16*, *coi1-16ein2-1*, *acs1-7*, *ein2-1*, *sid2*) leaves were harvested at 25 dpi. Harvested samples were used for RT-qPCR expression analysis and were normalized to the expression of *ACTIN 2*. Data are mean  $\pm$  s.d. (n = 3 to 4). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test. The experiment was repeated three times with similar results.

#### 4.6 *V. longisporum* (VI43) infection inhibits the primary root length of *KO-pdf1.2a*

To characterize the role of *AtPDF1.2a* in the plant-pathogen interaction, transgenic Arabidopsis plants of *KO-pdf1.2a* (*KO4* and *KO5*) and OE-PDF1.2a (*OE1*, *OE2*, *OE3*) were chosen for *V. longisporum* infection experiments (Fig. 9). The *in-vitro* infection system was established to facilitate the characterization of the early plant-fungus interactions, which allows additional monitoring of the infection process and facilitated the generation of equally infected root tissue during the initial phase of infection.

Senescence-like disease symptoms became apparent at 6 dpi in wild type Arabidopsis seedlings and were drastically pronounced during infection development (Fig. 9A). Germination of fungal conidia on root surfaces and colonization of roots followed by typical disease symptoms at 12 dpi could be observed visually for all the infected seedlings. Nevertheless, *KO-pdf1.2a* plants displayed clear disease symptoms including, inhibited



primary root length and severe fungal colonization, while on the other hand, OE-PDF1.2a showed a similar disease progression, nonetheless, the primary root was growing underneath the application area of fungal inoculation (shown as a black horizontal line) as compared with the Col-0 wild-type, where the fungus was growing lengthways with the root (Fig. 9a). However, the primary root length was considerably reduced in the *KO-pdf1.2a* seedlings, but not in the OE-PDF1.2a and Col-0 wild-type plants (Fig. 9B).

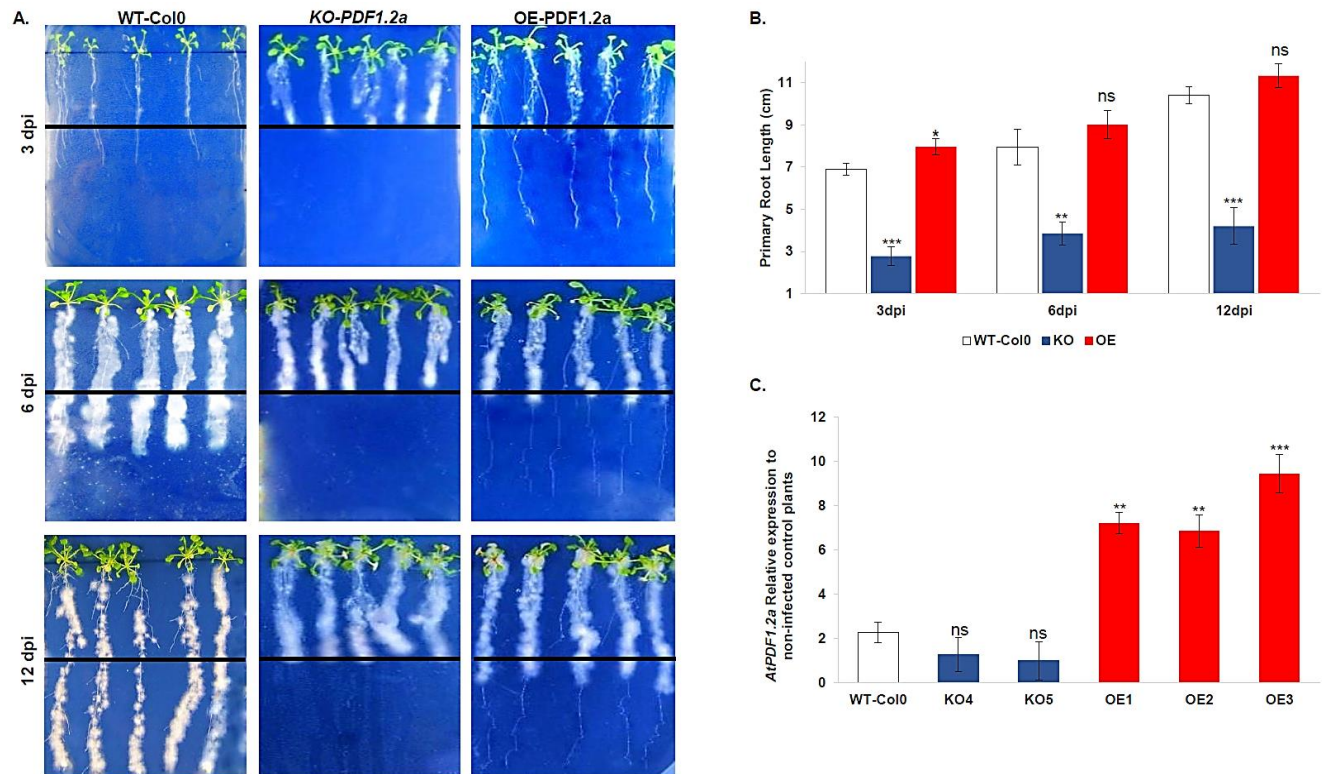


Figure 9 | *In-vitro* *V. longisporum* infection analysis of *AtPDF1.2a* transgenic plants.

(A) Three weeks *in-vitro* grown WT-Col0, two independent *KO-pdf1.2a* and three independent OE-PDF1.2a lines on vertical 1/2MS agar medium. The roots were supplemented with mock or *V. longisporum* conidia (2M) inoculation. The fungus can be seen growing along with WT-Col0 and *KO-pdf1.2a* roots seized to grow after fungal treatment whereas OE-PDF1.2a roots were growing further without fungus growing along with them. (B) The primary root length was measured for *V. longisporum* conidia treated seedlings at indicated time points. (C) 6 dpi mock or *V. longisporum* conidia treated seedling roots were used for the quantification of the relative transcript level of *AtPDF1.2a* in *KO-pdf1.2a* and OE-PDF1.2a were normalized to the expression of *ACTIN2*. Data are mean  $\pm$  s.d. (n = 3). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using a two-tailed Student's t-test.

#### 4.7 Overexpression of *AtPDF1.2a* enhanced plant resistance to *V. longisporum* infection

Infection experiments were performed with four-week old seedlings including, *KO-pdf1.2a*, OE-PDF1.2a, Col-0 wild-type plants and inoculated with *V. longisporum*.

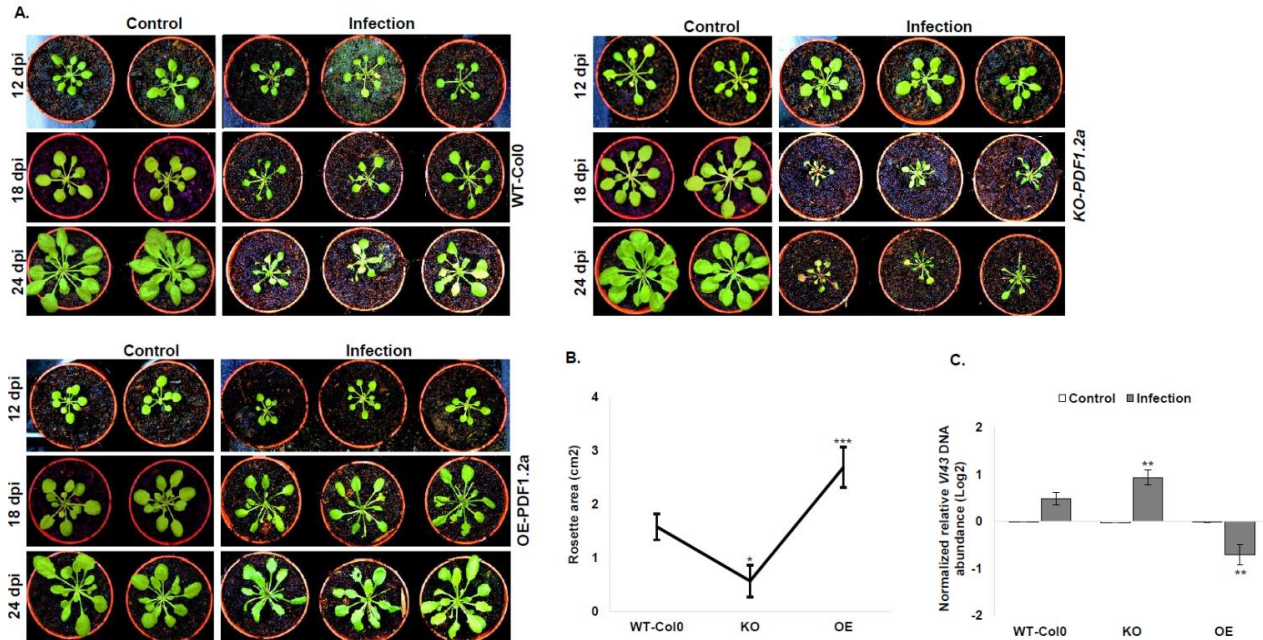


Figure 10 | Phenotyping of *AtPDF1.2a* transgenic plants for *V. longisporum* infection. Phenotypic differences between WT-Col0 and transgenic *AtPDF1.2a* lines in response to *Verticillium longisporum* (A) Two weeks old *in-vitro* grown WT-Col0, knockout (*KO-PDF1.2a*) and overexpression (*OE-PDF1.2a*) lines were transferred into an individual pot containing a mixture of sand and soil (1:1) and grown for another one week. Followed by mock or *V. longisporum* (VI43) conidia (2M) inoculation on each plant. Two biological repeats are presented here. To observe consistent phenotypic differences pictures were taken after every 6 dpi. (B) Rosette diameter measurements at 24 dpi. (C) Fungal quantification using primer pair (olg70/olg71) to amplify a 260bp long fragment of an ITS region from *V. longisporum* from the infected leaf tissues of WT-Col0, *KO-PDF1.2a* and *OE-PDF1.2a* lines. Data are mean  $\pm$  s.d. (n = 3). Asterisks indicate statistically significant difference at \*\*\*P < 0.01 and \*p < 0.05 using two-tailed Student's t-test.

To observe consistent phenotypic differences pictures were taken every 6 dpi. Around twelve to fourteen days after inoculation, Arabidopsis leaves began to yellow and wilt, and the growth of the plants was stunted as expected. *KO-pdf1.2a* plants exhibited increased vulnerability to *V. longisporum*, whereas *OE-PDF1.2a* offered heightened resistance as compared to Col-0 wild-type plants. Besides, at 18 and 24 dpi, *KO-pdf1.2a* plants exhibited a significantly higher number of diseased leaves and severely retarded growth, but in contrast, *OE-PDF1.2a* plants displayed strong resistance and better performance than the Col-0 wild-type plants (Fig. 10A). At 24 dpi, the rosette area was measured for both the *KO-pdf1.2a* and *OE-PDF1.2a* plants. The affected rosette size is additionally indicative that *KO-pdf1.2a* suffered severely from fungal infection, followed by Col-0 wild-type plants. Noticeably, *OE-PDF1.2a* plants sustained its growth regardless of the ongoing infection (Fig 10B).

To confirm successful infection, fungal DNA colonization was quantified by qPCR assay with the leaves at 24 dpi. A higher abundance of the fungal DNA was detected in the *KO-pdf1.2a* plants, while it was nearly negligible in the OE-PDF1.2a plants when compared with the Col-0 wild-type (Fig. 10C). These observations strongly support that in *Knockout of pdf1.2a* enhances plant susceptibility and its overexpression increases plant resistance to the *V. longisporum* infection.

#### **4.8 *Pseudomonas syringae* (Pst DC3000) induces the expression of *AtPDF1.2a***

To investigate the antibacterial role of *AtPDF1.2a*, we challenged independent *KO-pdf1.2a* (*KO4*, *KO5*) and OE-PDF1.2a (*OE1*, *OE2*, *OE3*) lines and Col-0 wild-type plants with *Pseudomonas syringae* (*Pst* DC3000) a gram-negative, biotrophic bacterial pathogen that elicits a wide variety of symptoms on plants, including blights, cankers, leaf spots, and galls (David et al. 2004).

*P. syringae* infection revealed that at 3 dpi, a slightly reduced infection progression in OE-PDF1.2a plants was visible, and *KO-pdf1.2a* plants like Col-0 wild-type plants were similarly affected with clear disease symptoms (Fig. 11A). However, all the plants including the OE-PDF1.2a were dead at a later time points (not shown). Detached leaf assays were also performed to further assess the infection progression and compared among the plants. It was observed that *KO-pdf1.2a* leaves displayed around 85-90% necrosis symptoms, and likewise the leaf margins of OE-PDF1.2a plants were also infected, and was covered in approximately 70% of necrosis as appeared in the Col-0 wild-type leaves (Fig. 11B).

The expression profiles of *AtPDF1.2a* was monitored at 1 dpi and 3 dpi in the infected Col-0 wild-type plants. The results indicated that the expression of *AtPDF1.2a* was not affected at 1 dpi but considerably induced at 3 dpi (Fig. 11C). In the next step, bacterial DNA was quantified from inoculated plant leaves at 3 dpi. OE-PDF1.2a and *KO-pdf1.2a* displayed bacterial biomass in a similar range as the Col-0 wild-type plants (Fig. 11D). All these data indicate that *AtPDF1.2a* does not participate in defense response to *P. syringae*.



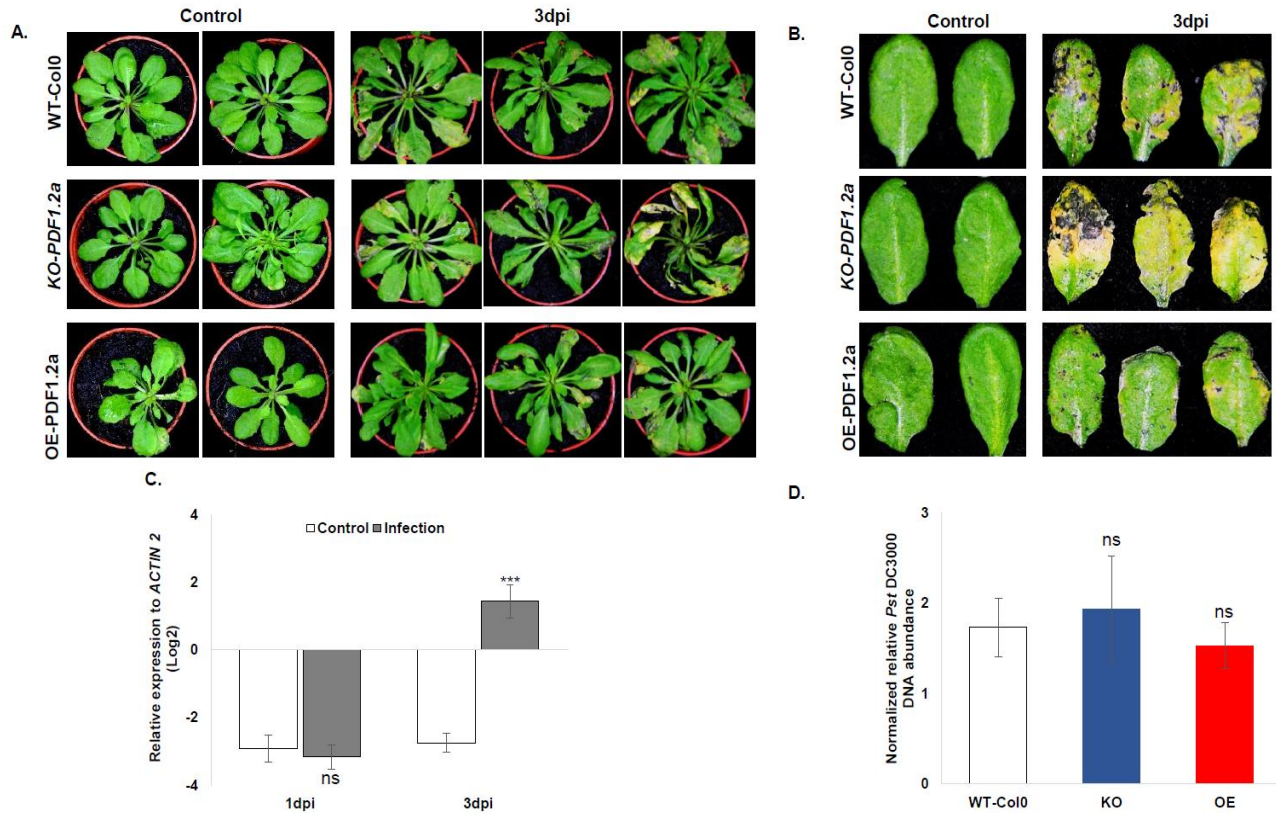


Figure 11 | *P. syringae* infection analysis of *AtPDF1.2a* transgenic plants. Susceptibility of *AtPDF1.2* transgenic plants to *P. syringae* (*Pst* DC3000) when inoculated by infiltration at  $1E + 5$  CFU/ml. (A) Whole plant phenotype of WT-Col0, *KO-PDF1.2a* and OE-*PDF1.2a* plants at 3 dpi. (B) Leaf appearance of WT-Col0, *KO-PDF1.2a* and OE-*PDF1.2a* plants at 3 dpi. (C) Quantification of the relative transcript level of *AtPDF1.2* in *P. syringae* treated WT-Col0 at 1 and 3 dpi and was normalized to the expression of *ACTIN 2*. (D) Bacterial biomass in WT-Col0, *KO-PDF1.2a*, and OE-*PDF1.2a* leaves at 3 dpi. Results displayed here (C and D) are means of four different leaves from four different plants, with SD indicated. Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using two-tailed Student's t-test.

#### 4.9 OE-*PDF1.2a* plants displayed improved resistance to *S. sclerotiorum* infection

To test whether *AtPDF1.2* is active in plant defense response against the necrotrophic fungus *S. sclerotiorum* infection was performed. OE-*PDF1.2a* and *KO-pdf1.2a* plants were inoculated with mycelium of *S. sclerotiorum* and the disease progression was monitored from 1 to 21 dpi. (Fig. 12A). As documented at 7 and 21 dpi, OE-*PDF1.2a* plants displayed significantly improved resistance against the *S. sclerotiorum* infection as compared with *KO-pdf1.2a* and Col-0 wild-type plants, respectively. Strikingly, around 50% of OE-*PDF1.2a* plants survived even at 21 dpi, to contrast, 80% of *KO-pdf1.2a* plants were decayed and nearly 100% of Col-0 wild-type plants were decayed.

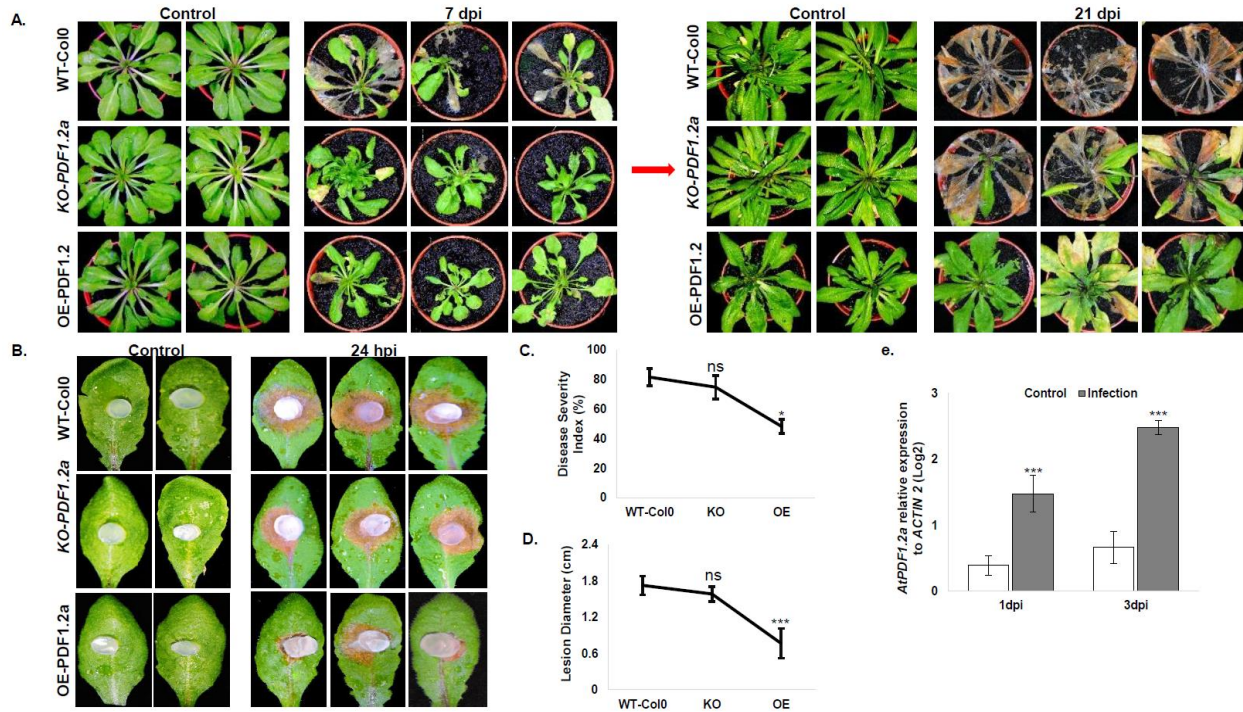


Figure 12 | *S. sclerotiorum* infection analysis of *AtPDF1.2a* transgenic plants. (A) 4 weeks old WT-Col0, *KO-PDF1.2a*, and OE-*PDF1.2a* plants after 7 and 21 days fungal infiltration (dpi). (B) Leaf appearance (abaxial sides) 24 hours after infiltration (hpi). (C) Disease severity measurement at 21 dpi in whole plants. (D) Lesion diameter measurement at day 24 after hours after infiltration. (E) 1dpi and 3dpi *S. sclerotiorum* treated WT-Col0, *KO-PDF1.2a*, and OE-*PDF1.2a* leaves were used for the quantification of the relative transcript level of *AtPDF1.2a* and were normalized to the expression of *ACTIN2*. Results displayed here (B, D, and F) are means of three different plants. Asterisks indicate statistically significant difference at \*\*\* $P < 0.01$  and \* $p < 0.05$  using a two-tailed Student's t-test.

As revealed by disease severity scoring, OE-*PDF1.2a* plants showed the lowest degree of disease symptoms, followed by *KO-pdf1.2a* plants and Col-0 wild-type plants (Fig 12C). In accordance, detached leaf assays confirmed that OE-*PDF1.2a* plants exhibited resistance to *S. sclerotiorum* infection, in which the necrotic lesions were restricted distinctly around the *S. sclerotiorum* inoculated leaf area, while the lesions in the *KO-pdf1.2a* and Col-0 wild-type leaves were encircled the whole leaf area (Fig 12B). For confirmation, size of lesion area was measured and compared to each other, providing data corresponding with the visible observations (Fig 12D). Remarkably, the results obtained demonstrate that the overexpression of *AtPDF1.2a* in transgenic Arabidopsis plants enhanced plant resistance against *S. sclerotiorum* infection. In accordance, the *AtPDF1.2a* expression was significantly induced by the fungal infection in the Col-0 wild-type plants, as revealed by RT-qPCR analysis at 1 dpi and 3 (Fig. 12E).

## 5 Discussion

In the present paper, we demonstrate the effect of the expression modulation of *AtPDF1.2a* can contribute to plant disease resistance or susceptibility in transgenic plants to *V. longisporum* infection. However, few studies (Ferrari et al. 2003; Chassot et al. 2007) have asserted that the expression of antimicrobial proteins via a single gene approach, in general, appears not to be efficient enough against aggressive pathogens such as *B. cinerea*. It might be because the infection is too devastating or the ability of this fungus to neutralize plant-derived antifungal compounds (van Kan, 2006). Interestingly, at the same time, PDFs have been also reported to present broad-spectrum antimicrobial activities. Transgenic plants with the constitutive expression of foreign PDF was reported to lead to plants possessing multiple biological potentialities, such as antibacterial, antifungal, insecticidal activities, protein synthesis inhibition, inhibitors of digestive enzymes, and abiotic stress and heavy metal resistance (de Carvalho and Gomes 2009; de Oliveira and Moreira 2011). Likewise, OE-PDF1.2a plants displayed increased resistance to *V. longisporum* infection. It is to note when different Arabidopsis host pathogens were tested in OE-PDF1.2a plants, for example, *S. Sclerotiorum*, enhanced resistance was given, but failed against bacterial pathogen *P. syringae* (*Pst* DC3000).

*V. longisporum* is a hemibiotrophic pathogen and causes huge yield losses of *B. napus* (Luo et al. 2014; Jasper et al. 2015; Novakazi et al. 2015). So far, there is no effective fungicide treatment to combat Verticillium diseases. Henceforth, using resistant cultivars could be competent management strategy against Verticillium stem striping (Depotter et al. 2016). The Col-0 wild-type plants displayed extensive disease symptoms and stunted growth at 18 dpi (Fig. 1), along with elevation in the *AtPDF1.2a* expression levels as revealed in 6 dpi roots.

PDFs are endogenous antimicrobial polypeptides that are believed to be functional as the first line of defense in response to invading pathogens (Parisi et al. 2018; Lacerda et al. 2014; Wang et al. 1999). *AtPDF1.2a* was analyzed for its phylogenetic relationship within the Arabidopsis Class-I *AtPDF* gene family. *AtPDF1.2a* was found very closely associated with *AtPDF1.2b* and *AtPDF1.2c*, which are known to produce a similar mature peptide (Thomma et al. 2002). Also, *AtPDF1.2a* shares an extremely high homology

with *AtPDF1.3* and *AtPDF1.1*. Many of the JA/ET and SA associated genes, which are well-known to be involved in defense responses, were revealed direct interaction with *AtPDF1.2a* using the STING program (Fig. 2).

Increasing evidence demonstrates that plant PDF-like peptides have evolved specific functions during plant growth and development. Analysis of microarray data revealed that *AtPDF1.2a* expressed primarily only in the upper-most plant parts/tissues (Fig. 3). And, the highest in the shoots, followed by leaf, rosette, shoot apex, and seedlings. Lay et al. (2013) reported that most type-2 (class 2) PDFs are found in leaves, flowers, and fruits. PDFs are usually found close to the site of pathogen entry, such as peripheral cells and stomatal cells, owing to their primary role of protection against any external threat (Broekaert et al. 1995; Terras et al. 1995).

*In-silico* expression study by GENEVESTIGATOR using the microarray dataset from Arabidopsis depicted that in response to biotic and abiotic stresses, *AtPDF1.2a* expressions varied (Fig. 4) from 1-1.5-fold changes in expressional upregulation. Contrastingly, the gene expression data showed that in response to photoperiod shift from the short day (SD) to darkness, the gene expression of *AtPDF1.2a* was down-regulated, although there is no evidence of AtPDFs known yet in photoperiod. However, JA responses are demonstrated to fluctuate upon varying day lengths (Cagnola et al. 2008). There is a tight association between the light environment and plant defense (Ballaré 2014), and light perceived by phyA or phyB increases the responses to JA. Plants exposed to low red/ far-red ratios that reduce phyB activity showed impaired induction of gene expression by either JA (Cerrudo et al. 2012; de Wit et al. 2013). Compared with that under SD, LD mainly extends the high expression of genes involved in JA-dependent defense during the period of additional light exposure (Cagnola et al. 2008). From the present microarray data and earlier literature, it can be enumerated that the JA pathway perhaps modulates the expression of *AtPDF1.2a* to cope up with the changing day lengths ratios. Similar to the abiotic stress, expression study by microarray demonstrated up-regulated gene expression for *AtPDF1.2a* together with other Class-I *AtPDF* genes in response to *B. graminis* and *B. cinerea*. Therefore, these biotic stress responses seemed to be pathogen-specific, as *AtPDF1.2a* level was greatly increased just for mentioned pathogens but not for any bacterial pathogens. Considering

the previous findings and our present study it can be inferred that, *AtPDF1.2a* could be involved in resistance to abiotic and biotic stresses via broad-spectrum resistance or basal mechanism of resistance in plants.

*AtPDF1.2a* promoter is reported to be activated by jasmonate (MeJA) and pathogen (Brown et al. 2003; Manners et al. 1998; Mitter et al. 1998). Moreover, investigation of the 1183 bp region upstream of the transcription start site of the *AtPDF1.2a* revealed several putative motifs with homology to known *cis-elements* involved in the transcriptional regulation of other genes, including those with particular relevance for jasmonate-mediated expression (Fig. 5). Two of these motifs, located between -277 and -495 of the *AtPDF1.2a* promoter, included the jasmonate-responsive element, TGACG (-392 to -396) present in the *ATPDF1* of barley (*Hordeum vulgare*, Rouster et al. 1997), and a 9-bp sequence, AAATGTTGT (-410 to -419), similar to a sequence within the jasmonate-responsive region of the promoter of the soybean (*Glycine max*) gene *VspB* (Mason et al. 1993). Moreover, GCC-box and its surrounding bases can interact with several members of the ERF family of transcription factors, which results in transcriptional activation of *AtPDF1.2a* (Brown et al. 2003). The response of *AtPDF1.2a* to jasmonic acid and ethylene is mediated through the transcription factor ORA59 that binds to GCC boxes in the *AtPDF1.2a* promoter (Pre et al. 2008). In addition to MeJA, *AtPDF1.2a* was presented to be induced during non-host interaction of Arabidopsis plants with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Siddique et al. 2011). Analysis of the *AtPDF1.2a* promoter has also indicated the existence of other, probably interactive, *cis-elements* necessary for high-level jasmonate-mediated regulation of *AtPDF1.2a* expression during plant defense responses.

*KO-pdf1.2a* and OE-PDF1.2a plants displayed altered phenotypes. *KO-pdf1.2a* plants displayed a reduction in primary root length while the OE-PDF1.2a plants exhibited increased primary root length but smaller rosette size as compared to the Col-0 wild-type plants (Fig. 6). A study exclusively reported the presence of *AtPDF1.2a* expression only in a rosette in the absence of any infection (Epple et al. 1997). Additionally, *AtPDF1.2a* has been published to be involved in growth and developmental processes (Schmid et al. 2005) as well as in plant architecture (Epple et al. 1997).



Furthermore, STING program also indicated that *AtPDF1.2a*, directly interacts with many important pathway genes of JA, SA and ET pathway, some of which are highly expressed in flowers, leaves, vegetative shoots, petioles, peduncles, and receptacles of floral organs like *VSP2* (Utsugi et al.1998). *AtPDF1.2a* belongs to the class-2 category of PDFs and it has been reported that overexpression of class-2 PDFs without CTPP has resulted in deleterious effects on the plant growth, indicating phytotoxicity of the mature PDF peptides (Ghag et al. 2013; Lay et al. 2014). Based on these facts, it is reasonable to argue that modification in the expression of *AtPDF1.2a* can lead to changes in plant architecture due to disturbance in the gene homeostasis.

To shed some light on underlying molecular mechanisms of *AtPDF1.2a*, expressions of *AtPDFs*, JA, ET, and SA associated genes were quantified in non-infected *KO-pdf1.2a* and OE-PDF1.2a plants and were compared to Col-0 wild-type (Fig. 7). *AtPDF1.2a* transgenic plants showed variable expression patterns for Class-II, III *AtPDF* genes, and I. OE-PDF1.2a plants showed increased expressions of Class-II, *AtPDF2.1*, *2.2* and *2.3* and *KO-pdf1.2a* displayed decreased expression of *AtPDF2.1*, *2.2* and *2.3*. In our previous study on *AtPDF2.2* (chapter:II), similar results were observed, where OE-PDF2.2 plants showed elevated expressions of *AtPDF1.2a* and *KD-pdf2.2* showed reduced transcript levels of *AtPDF1.2a*. Likewise, *KO-pdf1.2a* plants showed significantly declined expression intensities of few Class-I genes, *AtPDF1.1*, *1.4* and Class-III genes, *AtPDF3.1* and *3.2* while, OE-PDF1.2a plants did not show the altered expressions for Class-I, *AtPDF* genes but markedly reduced expressions for Class-III genes. It seems that *AtPDF1.2a* directly interacts with *AtPDF2.2* and simultaneously regulates the expressions of some other *AtPDF* genes. It gives the impression that, these *AtPDF* genes perhaps interact with each other independent of JA mediated signaling.

When JA biosynthesis responsive genes, *LOX3*, *AOC3*, and *OPR3* were analyzed for their expression in the *KO-pdf1.2a* and OE-PDF1.2a plants, expression levels of all three genes were not significant compared to the Col-0 wild-type plants. It had been reported that JA biosynthesis could work independently from changes in gene expression (Wasternack 2007; Browse 2009a; b). Therefore, although when downstream JA signaling gene, *AtPDF1.2a* was repressed or overexpressed in the *KO-pdf1.2a* and OE-

PDF1.2a plants respectively resulted in no modulation upstream of the JA pathway. Moreover, in resting state, JAZ proteins act as transcriptional repressors of JA biosynthesis by binding to positive transcriptional regulators (Chini et al. 2007; Doares et al. 1995; McConn et al. 1997; Ziegler et al. 2001; Strassner et al. 2002). This further explains the insignificant changes in the expressions of JA biosynthesis responsive genes.

ET responsive genes, *EIN3*, a positive and *ETR2*, a negative, regulators of ethylene biosynthesis were quantified. The transcript intensity of *EIN3* and *ETR2* in the *KO-pdf1.2a* and OE-PDF1.2a plants were similar to Col-0 wild-type plants, where *EIN3* was highly suppressed and *ETR2* was substantially induced. Considering the synergistic regulation of JA and ET biosynthesis, in the resting state, where JAZ proteins repress the JA biosynthesis that results in deactivated downstream ERF branch, responsible for the ET response (Zhu et al. 2011). Thus, this crucial mechanism governing the JA/ET synergistic interactions could explain the repressed ET pathway in the non-infected transgenic and Col-0 wild-type plants. SA associated genes, *PR1*, *WRKY70*, and *WRKY53* were equally repressed in the *KO-pdf1.2a* and OE-PDF1.2a plants as the Col-0 wild-type plants. These genes transcript analysis on non-infected plants indicates that, *AtPDF1.2a* does not mediate interaction with the SA pathway. Altogether, *AtPDF1.2a* was not established to be involved in the regulation of any of the JA, ET, or SA pathways.

To understand the regulation of *AtPDF1.2a* by JA, ET, and SA pathways, expression of *AtPDF1.2a* was evaluated in the Arabidopsis mutant plants of these respective pathways under non-infected conditions (Fig. 8). This analysis could clarify the basic interaction *via* defense pathways to *AtPDF1.2a*. The genotypes utilised were Col-0 wild-type, *jar1*, *coi1-16*, *coi1-16ein2-1*, *acs1-7*, *ein2* and *sid2* by RT-qPCR.

*AtPDF1.2a* expression was not impacted in the JA and ET mutants (*jar1*, *coi1-16*, *coi1-16ein2-1*, *acs1-7*, and *ein2*), which suggests that the basal expression level of *AtPDF1.2a* is independent of JA and ET pathways because the loss of JA and ET biosynthesis-related genes could not contribute to the altered basal expression of *AtPDF1.2a*. Ellis and Turner (2001) showed that the expression of *AtPDF1.2* was constitutive in the Arabidopsis ET mutant (*cev1*). On the other hand, activation of the SA pathway suppresses a large set of JA-responsive genes, including the JA marker genes,

like, *AtPDF1.2a* (Van der Does et al. 2013; Van Wees et al. 1999). Therefore, the transcript level of *AtPDF1.2a* was quantified in the SA mutant, to determine the effect of the non-induced SA pathway on *AtPDF1.2a*. SA mutant, *sid2* displayed greatly increased expression of *AtPDF1.2a*. Many SA mutants display constitutively high expression of *AtPDF1.2* (Glazebrook 2001; Spoel et al. 2003), suggesting either that the block in JA signaling relieves the suppression of SA signaling or that the activation of SA signaling blocks JA signaling. Moreover, many studies to date, have identified antagonistic interactions between the SA and JA/ET mediated signaling pathways (Feys and Parker 2000; Kunkel and Brooks 2002), which possibly plays, even in absence of infection. Therefore, it is reasonable to consider that the SA pathway targets directly or indirectly some key regulatory pathway genes of JA signaling to alter the expression of *AtPDF1.2a*.

Plant-fungus interaction was studied in the *AtPDF1.2a* transgenic and Col-0 wild-type plants, where the plants were subjected to the *V. longisporum* infection (Fig. 9, 10). *In vitro* *V. longisporum* infection affected the length of the primary root of *KO-pdf1.2a* and OE-PDF1.2a seedlings (Fig. 9A). The primary root length was measured consecutively at three-time points from 3 to 12 dpi. Fungi were visibly growing together with the Col-0 wild-type roots while *KO-pdf1.2a* roots seized to grow any further from the point of application and were completely covered in fungi at 12 dpi. On the other hand, OE-PDF1.2a roots were growing in length devoid of fungi (Fig. 9B). This disease resistance ability could be connected to the two steps model for the mechanism of action proposed for PDFs. The first step is the interaction of the PDFs with sphingolipids, GlcCer or M(IP)<sub>2</sub>Con fungal membranes. In the second step, the PDF inserts itself into the membrane causing permeabilization provoking an event of membrane destabilization (Thevissen, François et al. 2003; Thevissen, Ferket et al. 2003). However, after intensive studies, it is still not known whether the arrest of fungal is strictly the result of membrane permeabilization, or whether it is caused by additional effects. Transcript analysis of *KO-pdf1.2a* and OE-

PDF1.2a supports that *AtPDF1.2a* expression was significantly elevated in the OE-PDF1.2a roots at all the indicated time points but quantification data is shown only for 6 dpi (Fig. 9C). Besides, *AtPDF1.2* is already demonstrated to be expressed in Arabidopsis wild-type plants upon pathogen challenge (Penninckx et al. 1996; Thomma et al. 1998, 1999). Hence, it can be concluded that *AtPDF1.2a* plays a role to limit fungal growth that

is why *KO-pdf1.2a* showed limiting root growth and the expression of *AtPDF1.2a* was amplified in the OE-PDF1.2a roots upon infection.

Likewise, the *in-vivo* experiment also demonstrated a weak phenotype for *KO-pdf1.2a* plants (Fig. 10). At 18 and 24 dpi, *KO-pdf1.2a* plants were most susceptible to *V. longisporum* infection including inhibited growth as compared to the Col-0 wild-type plants. On the contrary, OE-PDF1.2a plants demonstrated higher disease resistance and lesser disease symptoms with enhanced recovery at 24 dpi in comparison to the Col-0 wild-type (Fig. 10A). Measurement of rosette diameter additionally validated the observed results (Fig. 10B). Lastly, fungal quantification revealed that the fungus colonization was highest in the mutant plants followed by Col-0 wild-type and it was least in the OE-PDF1.2a plants (Fig. 10C). So far, there have been numerous claims of the successful transformation of different plants with various *PDF* genes that have proven to restrict fungal growth (Zhu et al. 2007). For example, overexpression of wasabi PDF (*WT1*) in rice, potato, and orchid has resulted in increased resistance against *Magnaporthe grisea*, *Erwinia carotovora*, and *Botrytis cinerea* (Lay et al. 2005). Expression of Dahlia PDF, *DmAMP1*, in rice directly inhibits the pathogen, *Magnaporthe oryzae*, and *Rhizoctonia solani*. It was observed that the constitutive expression of *DmAMP1* suppresses the growth of *M. oryzae* and *R. solani* by 84% and 72%, respectively (Jha et al. 2009). Now, it is important to understand the mode of action of these PDFs, whether these PDFs are either internalized by the fungal cell and interact with intracellular targets, or they stay at the cell surface and induce cell death through induction of a signaling cascade (Poon et al. 2014; Wilmes et al. 2011). Considering their mode of action in mind, it is easy to understand why their overexpression could make tolerant plants. There are several examples that *PDF* genes have important potential for effective fungal control in economically important crops (Wong et al. 2007). In the light of these pieces of evidence and enhanced disease resistance against *V. longisporum* in the OE-PDF1.2a plants, it can be assumed that *AtPDF1.2a* has antifungal activity in Arabidopsis-*V. longisporum* interactions.

For the evaluation of the role of *AtPDF1.2a* in resistance against other pathogens, Col-0 wild-type, *KO-pdf1.2a*, and OE-PDF1.2a plants were challenged with a hemibiotrophic bacterial pathogens *P. syringae* and extremely aggressive necrotrophic fungal pathogen *S. sclerotenia*. *P. syringae* interaction with Arabidopsis is mediated by the

interplay of both SA and JA (Brooks et al. 2005). So far, most PDF proteins are known to exhibit defense primarily against fungal pathogens. To date, only a few PDFs are shown to inhibit the growth of bacteria. For instance, the expression levels of *AtPDF1.1* in transgenic *Arabidopsis* plants were positively correlated with resistance to *Pectobacterium carotovorum*, suggesting its involvement in the defense against this bacterium (Hsiao et al. 2017). Therefore, to analyze whether *AtPDF1.2a* can provide tolerance against a bacterial pathogen or not. *KO-pdf1.2a* and OE-PDF1.2a plants along with Col-0 wild-type was challenged with *P. syringae*. The disease symptoms observed were nearly identical for both *KO-pdf1.2a* and OE-PDF1.2a plants. Bacterial biomass for *KO-pdf1.2a* and OE-PDF1.2a plants were also at the same level as the Col-0 wild-type. However, significantly induced expression of *AtPDF1.2a* was detected at 3 dpi (Fig. 11). Since *P. syringae* produces the phytotoxin coronatine (COR). A major aspect of COR function is its ability to mimic a bioactive jasmonic acid (JA) conjugate and thus target the JA-receptor COR-insensitive 1 (COI1). This COR stimulates the JA signaling and consequent suppression of SA-dependent defense through antagonistic crosstalk (Geng et al. 2012; Geng et al. 2014; Kloeck et al. 2001; Brooks et al. 2005). Henceforth, COR, through its ability to mimic active JA-conjugates, could similarly alter gene expression independent of targeting COI, and perhaps that's why we observed induced expression of *AtPDF1.2a*. However, the induction of *AtPDF1.2a* expression did not result in disease resistance. Most likely because the inhibitory effects of JA on SA signaling is there to benefit the bacterial pathogen to advance its infection course (Geng et al. 2014; Uppalapati et al. 2005).

Upon *S. sclerotiorum* infection, the relative gene expression of *AtPDF1.2a* was found induced in the infected Col-0 wild-type plants (Fig. 12). As per the literature, the induction of *AtPDF1.2a* expression comes from the pathogen triggered the JA pathway, not because of direct interaction fungal pathogen to *AtPDF1.2a* (Guo and Stotz 2007). Infection assay on *KO-pdf1.2a* plants presented the comparable disease symptoms as the Col-0 wild-type plants. Intriguingly, OE-PDF1.2a plants showed augmented disease resistance even at 21 dpi, when *KO-pdf1.2a* plants were entirely dead and damaged. One thing to consider is that *AtPDF1.2a* expression is regulated with the AtPep1-6 short peptide elicitors. These elicitors, which participate in defense response

against damage and infection of plants with *S. sclerotiorum*, are formed from their precursors and recognized by the AtPEPR1 and AtPEPR2 receptors of the PRR family (Huffaker and Ryan 2007). It is interesting that *AtPep1* activates a synthesis of not only the *AtPDF1.2a* but hydrogen peroxide as well (Huffaker and Ryan 2007). Farther, PDFs in diverse model plants and crops exhibited their involvement in the innate immune responses to fungal pathogens including *F. oxysporum*, *Alternaria alternata*, *Botrytis cinerea*, *Verticillium dahlia* (Gaspar et al. 2014; Graham et al. 2008; Ahmed et al. 2012). It has been also reported that overexpression of *PDF* genes in transgenic *B. napus* resulted in enhanced resistance to *S. sclerotiorum* (Zarinpanjeh et al. 2016). *PDFs* exhibited antimicrobial activity not only because they bind host intracellular targets triggering defense signaling like cell death induction, but they also are able to interact with various fungal sphingolipids and phospholipids (Tavormina et al. 2015). Consequently, overexpression of *PDFs* can be an effective fungal targeting opportunity to inhibit the infection progression. Finally, to examine the effect of *V. longisporum* infection on the underlying molecular mechanisms of *AtPDF1.2a*, expression patterns of *AtPDFs*, SA, JA and ET associated genes were analyzed at 6 dpi, in *KO-pdf1.2a* and OE-*PDF1.2a* roots and were double normalized to the expressions of the *ACTIN 2* and non-infected plants (Fig. 13). The transcript level of most of the *AtPDF* genes from Class-I and III was upregulated in the OE-*PDF1.2a* plants and few were significantly reduced in the *KO-pdf1.2a* plants. Signifying a positive interaction with the Class-I and III, *AtPDF* genes upon fungal infection. Most of the Class-II, *AtPDF* genes showed constitutive expression as Col-0 wild-type. However, *AtPDF2.2*, *2.3* and *2.5* expressions were upregulated in the *KO-pdf1.2a* plants and noticeably downregulated in the OE-*PDF1.2a* plants. Suggesting a negative interaction of *AtPDF1.2a* to these Class-II genes.

Transcript levels of the JA biosynthesis gene, *LOX3*, *AOC3*, and *OPR3* was only induced in the *KO-pdf1.2a* plants. Often, perturbation of a particular gene's function in a network may alter the expression of other genes within the same network, in order to reclaim the overall balance and function (Barabasi et al. 2004; Davidson et al. 2005). Similarly, ET biosynthesis genes, *EIN3* was not significantly altered in the OE-*PDF1.2a* plants while it was upregulated in the *KO-pdf1.2a* plants. *ETR2* was not significantly changed in the OE-*PDF1.2a* plants while it was downregulated in the *KO-pdf1.2a* plants. ET commonly acts

in cooperation with the JA pathway and activates the expression of defense-related genes (Ding et al. 2011; Rojo et al. 2003). Hence, the upregulation of *EIN3* was rather obvious in the *KO-pdf1.2a* plants where JA biosynthesis gene expressions were notably induced (Fig. 13B). SA defense pathways are generally known to work antagonistically to the JA pathway (Thomma et al. 1998). Consequently, to comprehend how the SA signaling, which governs local resistance and SAR, reacted to *V. longisporum* infection, the expression of *PR1*, *WRKY70*, and *WRKY53* was investigated responding to *V. longisporum* infection (Fig. 13B).

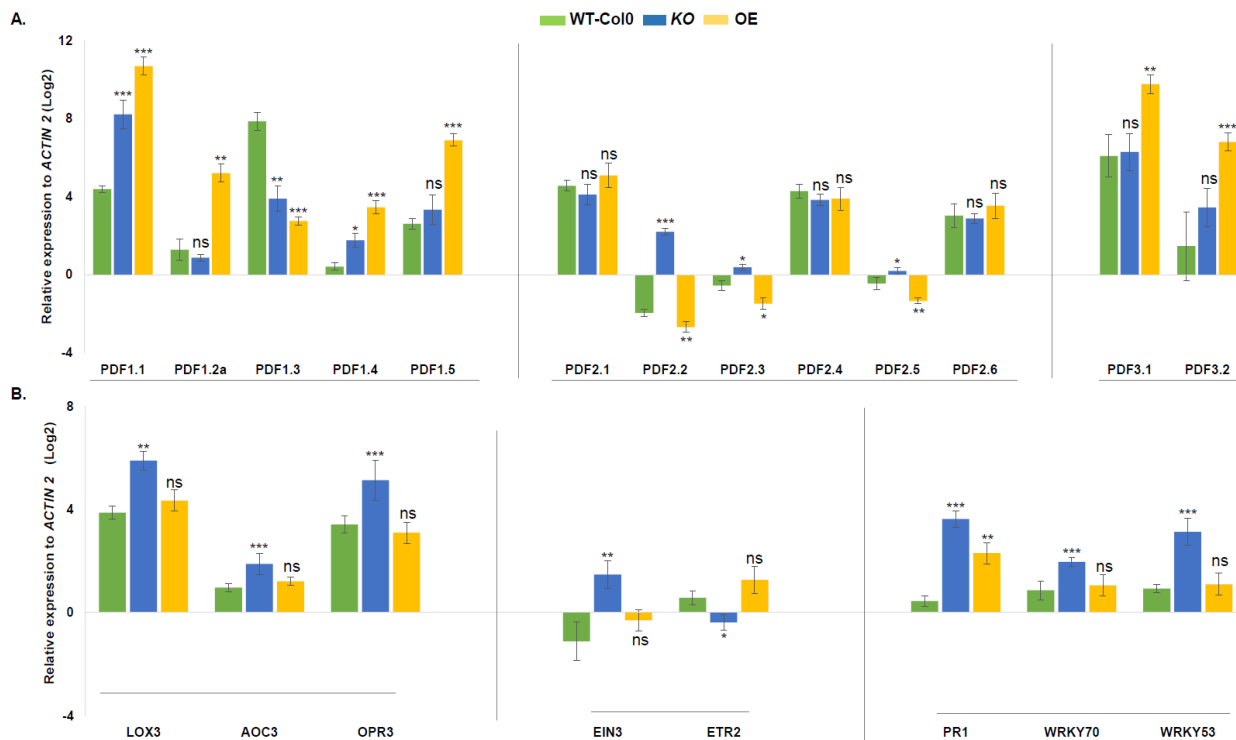


Figure 13 | Defense related gene expression in *AtPDF1.2a* transgenic plants upon *V. longisporum* infection. Relative expression levels of defense-related genes in the roots of *AtPDF1.2a* transgenic plants for *KO-PDF1.2a* (*KO*) and *OE-PDF1.2a* (*OE*) at 6 dpi of *V. longisporum*. **(A)** The *AtPDF1.1*, *AtPDF1.2*, *AtPDF1.3*, *AtPDF1.4*, *AtPDF1.5*, *AtPDF2.1*, *AtPDF2.2*, *AtPDF2.3*, *AtPDF2.4*, *AtPDF2.5*, *AtPDF2.6*, *AtPDF3.1* and *AtPDF3.2* represent fifteen Arabidopsis plant *AtPDF* genes from the three classes. **(B)** *LOX3*, *AOC3* and *OPR3* represent the JA synthesis-related gene; *ETR2* and *EIN3* are ethylene signaling pathway regulator genes and *PR1*, *WRKY70* and *WRKY53* are the major corresponding proteins involved in the SA-synthesis pathway. The gene transcript levels were normalized to the expression of *ACTIN 2* measured in the same samples and expressed logarithmically. Data are mean  $\pm$  s.d. ( $n = 3$  to  $4$ ). Asterisks indicate statistically significant difference at  $***P < 0.01$  and  $*p < 0.05$  using two-tailed Student's t-test.

Expression of SA responsive gene, *PR1* was significantly increased in both *KO-pdf1.2a* and *OE-PDF1.2a* plants. Nevertheless, the other two analyzed genes, *WRKY70* and *WRKY53* had similar expression intensity as the Col-0 wild-type.

Nevertheless, *WRKY70* and *WRKY53* were also found induced at 12 dpi only in *KO-pdf1.2a* plants (not shown). Based on the expression analysis in *KO-pdf1.2a* and OE-PDF1.2a plants, it was observed that *V. longisporum* infection essentially triggers all three pathways. In turn, JA, ET and SA pathways regulate the expression of *AtPDF1.2a*. And, in lack of basal expression of *AtPDF1.2a* in *KO-pdf1.2a* plants, a concentration-specific feedback loop, type of mechanism was observed which induced the expressions of JA, ET and SA responsive genes, which otherwise was not induced in OE-PDF1.2a plants.

The results of this study implicate that OE-PDF1.2a plants led to increased resistance to *V. longisporum* infection. Transcript analysis of *AtPDF1.2a* transgenic plants revealed that *V. longisporum* activates JA, ET, and SA pathways. Signaling through these pathways was demonstrated using the well-characterized marker genes *AtPDF1.2a*. It was previously suggested that the SA pathway protects against biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling enhances resistance to necrotrophic pathogens (Thomma et al. 1998). However, our study clearly shows that all three pathways were involved in defense against *V. longisporum*. Furthermore, similar activation of the three pathways has been demonstrated for many fungal pathogens. As well as, JA-dependent induced systemic resistance (ISR) pathway, which is triggered by nonpathogenic rhizobacteria has already proven to occur through the SA-dependent and the JA-dependent signal pathways with the involvement of the FLS2 flagellin receptor and the WRKY22 transcription factor and is characterized by an accumulation of transcripts of PR1 and *AtPDF1.2* proteins (Denoux et al. 2008). A question remains as to the relevance of synergistic signal interactions to resistance responses. Often-simultaneous activation of signaling pathways has no additive effects with resistance patterns to discrete pathogens being maintained (e.g. van Wees et al. 2000), although there are instances where both SA and JA signaling pathways are required (Su et al. 2018; Ellis et al. 2002a). It seems likely that variably employed synergistic/antagonistic mechanisms, which involve not only SA and JA but also, for example, ethylene (Penninckx et al. 1998; Tuominen et al. 2004), may represent positive and negative feedback loops allowing the tailoring of the plant response to particular stress (Maleck and Dietrich 1999).

OE-PDF1.2a plants did not upregulate the expression of JA, ET or SA responsive genes. Although, *KO-pdf1.2a* plants showed induced expression of all the three pathways.



Possibly, low basal expression of *AtPDF1.2a* in *KO-pdf1.2a* plants during ongoing infection work as a concentration-specific signal. In response to a gene knockout, plants or other organisms as well, may accumulate mutations in one or more genes modulating the affected pathway, thereby partially or fully rescuing the outcome (Teng et al. 2013; Chen et al. 2016). Hence, it indicates that *AtPDF1.2a* does not essentially interacts directly with any of these pathways and gets induced only as a result of activated JA, ET and SA pathway upon pathogen attack to mediate the defense response and signalling (Fig. 14B). Following our findings, we propose a working model for *AtPDF1.2a* interactions (Fig. 14). In absence of infection, *AtPDF1.2a* expression is independent of JA/ET pathway. However, SA pathway negatively regulates it (Fig. 14A). That is the strategy utilised by SA pathway to maintain hormone equilibrium in non-induced state (Mur et al. 2006). Upon *V. longisporum* infection, *AtPDF1.2a* expression is regulated by the JA, ET and SA pathways but only at low basal concentrations, *AtPDF1.2a* mediates the expression of JA, ET and SA responsive genes. In response of activated defense pathways and signalling, *AtPDF1.2a* positively regulates the expression of nearly all the *AtPDF* genes from Class-I and III and suppresses the expression of *AtPDF1.3, 2.2, 2.3 and 2.5*.

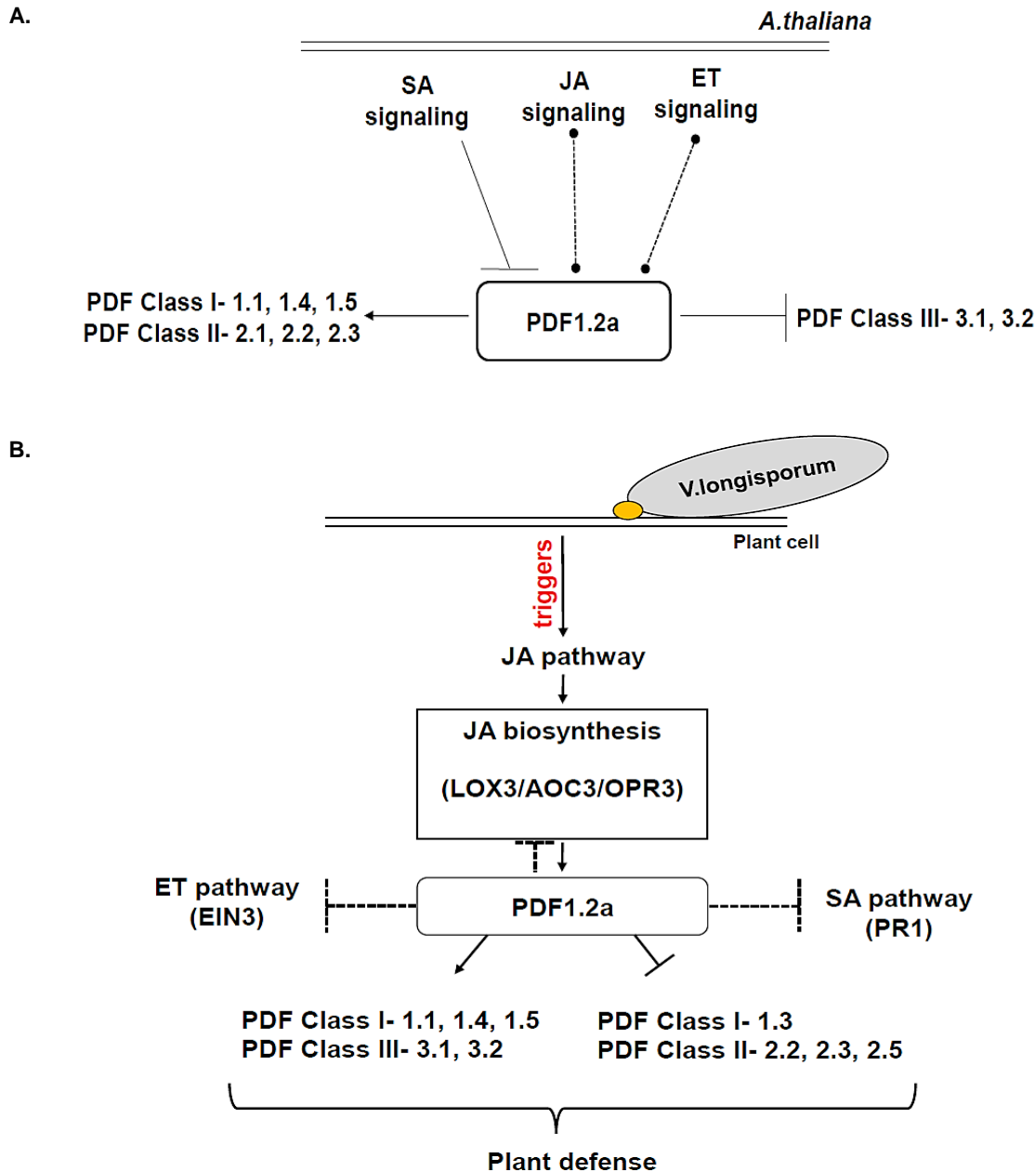


Figure 14 | Proposed working model of *AtPDF1.2a*.

(A) In the absence of infection, *AtPDF1.2a* positively interacts with some of the Class-I and II *AtPDF* gene family members and negatively to Class-III. Together these genes maintain the downstream JA mediated *AtPDF* signaling independent of JA biosynthesis. *AtPDF1.2a* doesn't hint a direct interaction with any of the pathways, JA, ET or SA. However, Arabidopsis mutant analysis showed that the SA pathway suppresses the expression of *AtPDF1.2a* and JA/ET had no impact on the expression of *AtPDF1.2a*. (B) Upon *V. longisporum* infection, *KO-pdf1.2a* plants caused an upregulated expression of JA, ET, and SA responsive genes. Suggesting a possible concentration-specific feedback loop while OE-*PDF1.2a* plants did not impact the expression of upstream JA, ET, or SA pathway responsive. Hence, evidently *AtPDF1.2a* is regulated by JA/ ET and SA pathway. In response to activated defense pathways, *AtPDF1.2a* positively interacts with many *AtPDF* genes from Class-I, and III and suppresses the expression of *AtPDF1.3*, 2.2 and 2.3, 2.5 to mediate the defense response.

In conclusion, *AtPDF1.2a* expression modulation revealed a possible antifungal activity of *AtPDF1.2a* towards *V. longisporum* and *S. sclerotiorum*. However, not against bacterial pathogens. A better understanding of each step will help elucidate its mode of action in detail. Moreover, in modern agriculture, biotic stress has become a great challenge, and many researches are actively working to develop resistant varieties using different approaches including the PDFs. So far, plant transgenic technology has shown remarkable success in plant disease-resistant program and will continue to improve plant health. However, plant pathologists have more focused on commonly known PDFs therefore, future studies are required to characterize or overexpress other PR proteins as well in different models and crop plants against different traits, which may be the breakthrough in disease development. Another future challenge is that increasing the rate of global climate change will possibly increase the emergence of virulent strains of phytopathogens with a broad host range. Hence, there is a need to functionally characterize as well as identify novel plant PDF genes/alleles to cope with such drastic challenges. In this regard, advances in “omics” approaches viz., genomics, transcriptomics, phenomics, proteomics, metabolomics, and ionomics will greatly help us to understand the detailed network of plant PDF genes as well as the interaction of PDFs with other proteins belonging to both plants and pathogens. These studies will provide us new genetic stocks of PDFs that can be effectively used to counter the disease epidemic. Therefore, PDFs can serve as potential candidates for engineering crop plants to improve resistance to multiple stresses.

## **6 Acknowledgments**

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## 7 Supporting Information

### Supplement Tables

Table S1 | Mutants used for genotyping to obtain homozygous knockout plants.

Line	Database name	Origin	ID	Features
<i>pdf1.2a</i>	SALK_063966.49.80.x	NASC	N563966	T-DNA insertion in the exon
<i>coi1-16</i>	coi1-16	NASC	N67817	EMS mutant
<i>acs1-7</i>	acs1-1acs2-1acs4-1acs5-2acs6-1acs7-1acs9-1	NASC	N16650	T-DNA insertion in the exon
<i>sid2</i>	SALK_088254	NASC	N65675	T-DNA insertion in the exon
<i>jar1</i>	SALK_059774C	NASC	N658259	T-DNA insertion in the exon
<i>ein2-1</i>	SALK_086500C	NASC	N654757	T-DNA insertion in the exon
<i>ein2-1; coi1-16</i>	ein2-1; coi1-16	NASC	N67818	EMS mutant

Table S2 | Primers used in this study.








Gene	Identifier	forward	reverse	[bp]	T [°C]
<i>ACTIN2</i>	AT5G09810	ACCTTGCTGGACGTGACCTTACTGAT	GTTGTCTCGTGGATTCCAGCAGCTT	298	58
<i>AtPDF1.2</i>	AT5G44420	CGCTGCTCTTGTCTCTTTGC	TCCATGTTTGGCTCCTCAA	154	57
<i>AtPDF1.2-OE</i>		ACATGGTCAGGGGTTTGC GGAAAC	ATGGAAGTAGTCTTACGTCGTACA	297	58
<i>AtPDF2.2</i>	AT2G0210	TCTCTTGCAATGAAGCTCTCTATG	ATGGGTAGATCAGCAATGTCTGGT	252	58
<i>AtPDF1.4</i>	AT1G19610	AGTCATGGCTTCTTCTTACACACT	TGAAGTAGCAGAAACATGCCAA	233	58
<i>AtPDF2.1</i>	AT2G02120	GATCGACGATTGAGTCATGC	CTCAGCCGTTCTTTTCTTGG	250	58
<i>AtPDF2.3</i>	AT2G02130	CACACACAAGTGTGCAACG	CGGAAACACACAAACCAATG	234	59
<i>AtPDF2.5</i>	AT5G63660	TGGAGAACAAGTTTTTCGCTGC	AAACTAGTTAGCAAAGGCCGGG	228	60
<i>PR1</i>	AT2G14610	TCAGGTTGTTGGAGAAAGTC	CATTAGTAAGGCTTCTCGTTCACA	126	57
<i>ETR2</i>	AT3G23150	CGTTGGAATTTACAGGTCGATGAG	CGTCTTCGAGTTACATCGTGGA	213	59
<i>EIN3</i>	AT3G20770	TGGTGGCCTCAACTTGGTTT	CGAGTTTCTGCTGGGACTT	459	60
<i>ACO3</i>	AT3G25780	GAATTGGGGCAAGAAGACCGAAA	TTGCCGAGTTTAAGAAGTCTGGA	111	62
<i>WRKY70</i>	AT3G56400	CCGCCGTTGAGGGATCT	GGACGAACCATGATGACGAT	479	58.8
<i>WRKY53</i>	AT4G23810	GCACGACTTAGAGAAGCTC	ACTCCTTGGGAATTTGGCG	450	56
<i>VI43 ITS</i>	OLG70/71	CAGCGAAACGCGATATGTAG	GGCTTGTAGGGGGTTAGA	261	58
<i>Pst DNA</i>	oprF	AACTGAAAAACACCTTGGGC	CCTGGGTTGTTGAAGTGTA	304	62
<i>Plant DNA</i>	AT4G2641	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC	81	56
<i>COI1</i>	AT2G39940	CCCCGAAAACATATGTCGGCT	AGGACGCATTCCCAAGTATGT	217	60
<i>ACS2</i>	AT1G01480	GGTGTGAGATAATCCCGGT	CCAAGTTCGTGAGTGTGTCC	181	58.8
<i>SID2</i>	AT1G74710	ATGAGATTCAGCCTCGCTGT	GGATCAAGGTCACGGAAGAA	178	60
<i>JAR1</i>	AT2G46370	ATCCGTTTCTGCTGATCGGG	GTGGAGAGGTAACAATCAGACCC	150	59.5
<i>EIN2</i>	AT5G03280	ACAAAGAGGTTCTGTCACCGT	TGACGGATCCAGTATCCACT	205	60

Accession Numbers starting with 'AT' were taken from Arabidopsis database (TAIR) and others were taken from NCBI database.

Table S3 | Primers used in mutant genotyping.

Name	Sequence (5' → 3')	Description
AtPDF1.2a_F	ACGTTGATCTTTGGCTTATTTCTTC	SALK_063966.49.80.x LP Primer for <i>AtPDF1.2a</i>
AtPDF1.2a_R	CATTGCCGGTGCGTCTGCATAT	SALK_063966.49.80.x RP Primer for <i>AtPDF1.2a</i>
COI1_R	ACCACAAATAAAGTTTCAGATGCCA	Flanking Primer for COI1 (Ellis et al. 2002)
COI1_R	CCCCGAAAATATGTCGGCT	Flanking Primer for COI1 (Ellis et al. 2002)
ACS2_F	GCAGTTTTGTGCGAAGATAGCG	T-DNA LP Primer for <i>acs2-1</i> (Tsuchisaka et al. 2009)
ACS2_R	ATCTGTACCTGATTCTCTGCAA	T-DNA RP Primer for <i>acs2-1</i> (Tsuchisaka et al. 2009)
SID2_F	TCTCCCGCAAGGTCTTTTT	SALK_088254 LP Primer for SID2
SID2_R	TCAGAGACGGCGGAGATTAG	SALK_088254 RP Primer for SID2
JAR1_F	ACGCTCATCAAGTCCAGAAACA	SALK_059774C LP Primer for JAR1
JAR1_R	GGAACAGGGTGACCAGTGAG	SALK_059774C RP Primer for JAR1
EIN2_F	ACAAAGAGGTTTCGTCACCGT	SALK_086500C LP Primer for EIN2
EIN2_R	TGACGGATCCCAGTATCCAAT	SALK_086500C RP Primer for EIN2

Table S4 | Protein Homologs present in Arabidopsis, where bit score for *AtPDF1.2b*, *1.2c*, *1.3* and *1.1* indicates high level of similarity with *AtPDF1.2a*.

Similar proteins in Arabidopsis thaliana:	Similar proteins anywhere in STRING:	Similar proteins in a taxon of choice:	
		Similarity (Bit Score)	Orthology (level)
LCR77- AT5G44420-PDF1.2a			
→ LCR- AT2G26020-PDF1.2b		157.9	(paralog)
→ LCR- AT5G44430-PDF1.2c		156.0	(paralog)
→ LCR- AT2G26010-PDF1.3		152.5	(paralog)
→ LCR67- AT1G75830-PDF1.1		144.4	(paralog)
→ LCR- AT1G55010-PDF1.5		75.5	-
→ LCR78- AT1G19610-PDF1.4		65.9	-

## 8 Reference (see page 146)

## Chapter IV: General discussion

*Verticillium longisporum* is becoming a global problem in oilseed rape production. Recently, the disease has been reported outside the continent of Europe in two important oilseed rape production areas, the UK in 2011 (Gladders et al. 2011) and Canada in 2015 (CFIA, 2015). To improve the management of the *V. longisporum* disease of oilseed rape and other crops, several approaches. One such approach includes host resistance, which is considered to be the most desirable control strategy. For oilseed rape resistance against *P. brassicae* for instance, dominant resistance genes have been identified and introduced into current *B. napus* germplasm, by which clubroot disease can efficiently be controlled in oilseed rape cultivation (Hwang et al. 2012; Řičařová et al 2017). However, no effective resistance against *V. longisporum* could be identified within the gene pool of *B. napus* or its progenitor species *B. rapa* and *B. oleracea* (Depotter et al. 2016). Quantitative genetic approaches have identified some QTLs for *V. longisporum* resistance, which partly overlaps with phenylpropanoid production, but their exact function or responsible genes remain unknown (Rygulla et al. 2008; Obermeier et al. 2013). Therefore, new strategies for improving the oilseed rape germplasm are required, especially with regard to resistance against *V. longisporum*. With that in mind, we characterized the role of plant defensin peptides (PDFs) in regulating plant-pathogen interactions and their potential for improving resistance as well. In addition, attempts have been made to identify the underlying molecular mechanisms. PDFs are small, cysteine-rich cationic antimicrobial peptides that possess biological activity towards a broad range of pathogenic organisms (Wang et al. 1999; Lacerda et al. 2014; Parisi et al. 2018). The expression of plant defensins can be induced in response to the pathogenic attack (Penninckx et al. 1996). From past few years PDFs have become interesting and important research targets, owing to their multifunctional but specific biological roles, especially their broad-spectrum antifungal activity.

### 1 Sequence analysis of plant defensins (PDFs) in Arabidopsis

By performing a TBLASTN search on the fully sequenced genome of Arabidopsis using the amino acid sequence of the previously identified Arabidopsis plant defensin *AtPDF1.2*

as a template, a total of thirteen putative plant defensin genes were identified (Penninckx et al. 1996; Epple et al. 1997; Thomma and Broekaert 1998). Based on phylogenetic analysis, the deduced putative protein sequences were classified into two families (Fig. 1). The first family contains seven peptides (*AtPDF1.1*, *1.2a*, *1.2b*, *1.2c*, *1.3*, *1.4* and *1.5*), five of which are highly similar (*AtPDF1.1* to *1.3*). Moreover, the predicted mature peptides of three of them are identical (*AtPDF1.2a*, *b*, and *c*). Remarkably, the genes encoding *AtPDF1.2a* and *AtPDF1.2c* are present in a tandem repeat on chromosome 5, while the gene encoding *AtPDF1.2b* is present on chromosome 2 in the tandem array with the gene encoding *AtPDF1.3* together (Thomma et al. 2002). Possibly, segmental duplication is the cause of this spatial organization. It was reported that 58% of the Arabidopsis genome shows large segmental duplications; moreover, that 17% of all Arabidopsis genes are arranged in tandem arrays (The Arabidopsis Genome Initiative 2000). The high homology between members within the *PDF* gene family suggests that duplication and subsequent diversification occurred evolutionarily quite recently. The putative protein sequences within the second family (*AtPDF2.1* to *2.6*) show more variation at the amino acid level (Fig. 1) (Thomma et al. 2002). This implies that duplication and the subsequent segregation of these members occurred before the diversification of the members of the first family. In addition, for the second family, some members are found in close proximity. *AtPDF2.1*, *2.3*, and *2.6* occur in a tandem array and *AtPDF2.2* is separated from this array by one gene. This separation is possibly caused by shuffling, a phenomenon that frequently happened in the Arabidopsis genome (The Arabidopsis Genome Initiative 2000). In addition to the thirteen putative plant defensin genes, two genes were identified that encode proteins containing only a plant defensin domain (Thomma et al. 2002). Gene *At4g30070* encodes a protein of 129 amino acids, of which the C-terminal domain displays the conserved cysteine pattern shared by all plant defensins in addition to a number of residues that are conserved among the Arabidopsis plant defensins (Fig. 1a). Gene *At5g38330* encodes a similar protein consisting of 122 amino acids, sharing 56% identical residues with the protein encoded by the gene *At4g30070*. Both proteins have a putative 26-amino-acid signal sequence. The amino-terminal domains of both proteins are rich in cysteine residues but do not share significant homology to any previously characterized protein (Fig. 1B). Possibly, these proteins are fusion proteins. Alternatively, these proteins could be precursors, as reported

for a tobacco defensin and thionins (Bohlmann and Apel 1991; Gu et al. 1992). The precursor of these proteins is characterized by the presence of a C-terminal acidic domain that has been proposed to neutralize the toxicity of the basic effector domain and thus mitigate toxicity during synthesis (Bohlmann and Apel 1991; Gu et al. 1992). However, in the case of the two Arabidopsis proteins, the basic defensin domain is at the C-terminus and the N-terminal domain has an excess of one basic residue. Taken together, *AtPDF* genes can be divided into three classes in Arabidopsis, namely Class-I (*AtPDF1.1-1.5*), Class-II (*AtPDF2.1-2.6*) and Class-III (*AtPDF3.1-3.2*) (Thomma et al. 2002).

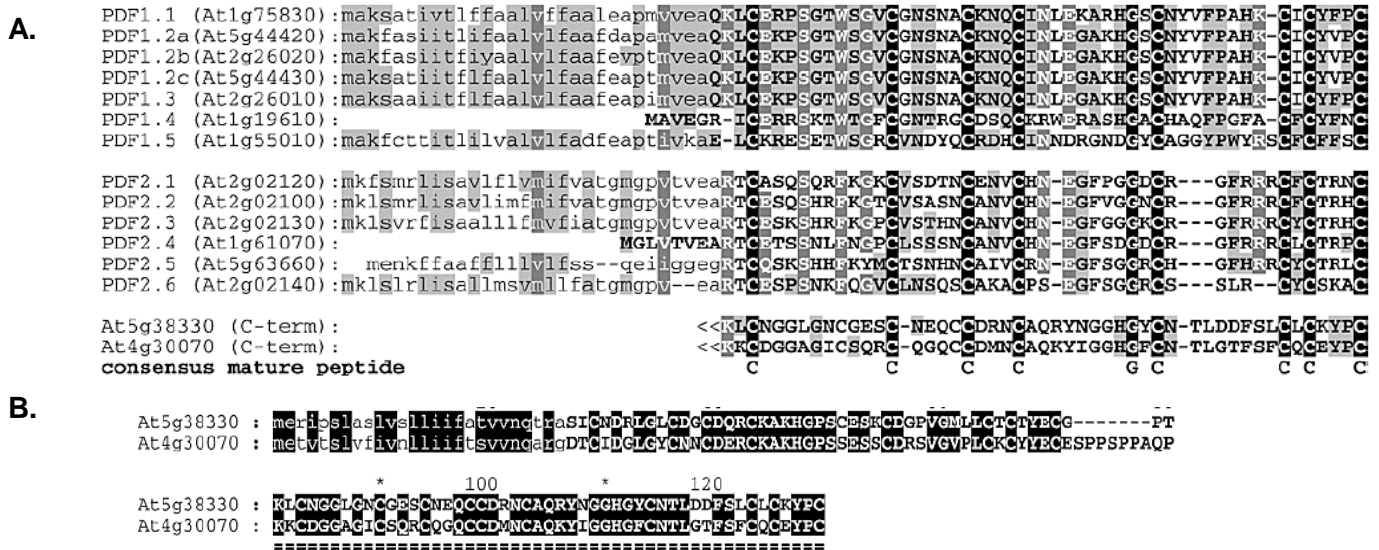


Figure 1 | Multiple sequence alignment of Arabidopsis *AtPDF* proteins.

(A) Alignment of putative Arabidopsis defensins (*AtPDFs*). Deduced amino acid sequences from putative defensin genes are aligned together with the carboxy-terminus of the putative fusion proteins encoded by Arabidopsis genes *At5g38330* and *At4g30070*. Predicted signal sequences are in lower case. Shading indicates the degree of conservation: 100%, 75%, and 40%. The consensus motif for Arabidopsis plant defensins is shown in the bottom line. (B) Alignment of the predicted proteins encoded by the genes *At5g38330* and *At4g30070*. Both proteins share a carboxy-terminal plant-defensin domain (double underlined). Predicted signal sequences are in lower case. Identical amino acids are shaded in black. (Thomma et al. 2002).

## 2 *V. longisporum* suppresses the expression of *AtPDF2.2* and induces the expression of *AtPDF1.2a* in the early phase of infection

Most plant defensins isolated to date exhibit antifungal activity against a broad range of fungi, including various plant pathogens. It was postulated that *PDFs* might play a role in the early stages of pathogenesis in the signaling process that informs plants about the attack from biotic stresses (Wu et al. 2014). To delve deeper into the role of *PDFs* in Plant-



*V. longisporum* interactions, Arabidopsis plants were infected and characterized. At 18 dpi, plants displayed extensive disease symptoms and stunted growth as expected. Transcript profiling on fifteen *AtPDF* genes at 6 dpi in Arabidopsis roots revealed that almost all the *AtPDF* genes from Class-I, II and III were significantly induced except for *AtPDF2.2*, *AtPDF2.3* and *AtPDF2.5*. Strikingly, the transcript levels of *AtPDF2.2* were too contrast and highly suppressed followed by *AtPDF2.5* and *AtPDF2.3*. It has been reported that infection with *A. brassicicola* downregulates the basal expression level of *AtPDF2.2* in the leaves along with the slightly affected expression of *AtPDF2.3*, along with the upregulation of *AtPDF1.2a* (Thomma and Broekaert. 1998, Penninckx et al. 1996). Additionally, a previously conducted transcriptomic study in our group revealed a few compatibility factors in *B. napus* - *V. longisporum* interactions. When one of these compatibility factors (CF) was knocked out in the transgenic Arabidopsis plants and subjected to *V. longisporum* infection, a highly elevated transcript level of *AtPDF2.2* in Arabidopsis mutants were detected (Pröbsting et al. 2020). This encouraged us to postulate that, *AtPDF2.2* plays a crucial role in plant-pathogen interaction. Interestingly, *AtPDF1.2a* expression was significantly upregulated by the *V. longisporum* infection. *AtPDF1.2a* is a well-known Arabidopsis plant defensin and has been demonstrated to get induced by necrotrophic pathogens such as *A. brassicicola* and *B. cinerea* (Penninckx et al. 1996; Manners et al. 1998). In addition, purified *AtPDF1.2* showed *in-vitro* antifungal activity (Penninckx et al. 1996; Sels et al. 2007; Terras et al. 1993), and several Arabidopsis mutants with altered *AtPDF1.2a* expression levels often show altered pathogen sensitivity. For instance, Arabidopsis mutants with diminished *AtPDF1.2a* expression showed a higher sensitivity against aggressive necrotrophic pathogens like *B. cinerea* as observed in the JA signaling mutant *coi1* and the ET signaling mutant *ein2* (Thomma et al. 1999) and *bos2*, *bos3* and *bos4* mutants (Veronese et al. 2004). Further analysis of the signaling pathways leading to pathogen-induced expression of *AtPDF1.2a* indicated the induction of plant hormones ethylene (ET) and methyl jasmonate (MeJA). Consistently, *AtPDF1.2a* transcripts fail to accumulate after inoculation with *A. brassicicola* in Arabidopsis mutants being involved in methyljasmonate (*coi1*) and ethylene signaling (*ein2*) (Penninckx et al. 1996; 1998). Therefore, *AtPDF1.2a* is now considered a general marker gene for MeJA/ JA and ET-mediated plant responses. On

the other hand, while *AtPDF1.2a* was not found to be induced by nematodes such as *Heterodera schachtii*, *AtPDF2.2* was considerably induced by this cyst nematode in syncytia (Szakasits et al. 2009; Siddique et al. 2011). The latter seems to be a specific response since the general defense response was repressed in syncytia (Siddique et al. 2011). These data suggest a specific regulation of defensins upon various biotic stresses. However, the role of PDFs in plant-*V. longisporum* interactions remain largely unresolved.

### **3 *In-silico* promoter analysis of *AtPDF2.2* and *AtPDF1.2* indicates complex regulatory mechanisms of *AtPDFs***

To comprehend the transcriptional regulation of *AtPDF2.2* and *AtPDF1.2a*, the promoter region was first analyzed *in-silico* for the presence of *cis*-acting elements involved in response to biotic or abiotic stress by using PlantCARE (Lescot et al. 2002). A motif search (Fig. 2A) revealed a JA response *cis*-element (CGTCA) in both of *AtPDF1.2a* (Penninckx et al. 1996) and *AtPDF2.2* promoters. While the majority of Class-I *AtPDF* genes contain the JA response motif, but only *AtPDF2.1*, 2.2, 2.3, and 2.6 from Class-II. Genevestigator ([www.genevestigator.com](http://www.genevestigator.com)) was used to look into gene expression specificity in relation to the respective motifs found (Fig. 2B). Among these *AtPDFs*, *AtPDF1.2c* and *AtPDF3.2* did not show a significant (above 2-fold) induction by MeJA treatment, while *AtPDF 2.5* and *AtPDF3.1* did show an induction. Several studies have reported the induced expressions of *AtPDF1.2a* upon fungal infection and wounding, hence, the presence of fungal elicitor responsive elements in this study were expected to be involved in the stress response. *AtPDF2.2* promoter contains single copies of the TC-rich motif and the fungal elicitor responsive element, respectively, while *AtPDF1.2a* only contains TC-rich repeats but not the fungal elicitor responsive elements. A motif for the SA response was also found in *AtPDF2.2* promoter almost in the middle of the fragment, indicating a possible interaction with SA. Contrastingly, *AtPDF1.2a* has neither the SA response elements in its promoter nor an induction upon SA treatment according to Genevestigator analysis. This is in line with the observation that in contrast to most pathogen-induced genes, *AtPDF1.2* is independent of the SA pathway (Manners et al. 1998).

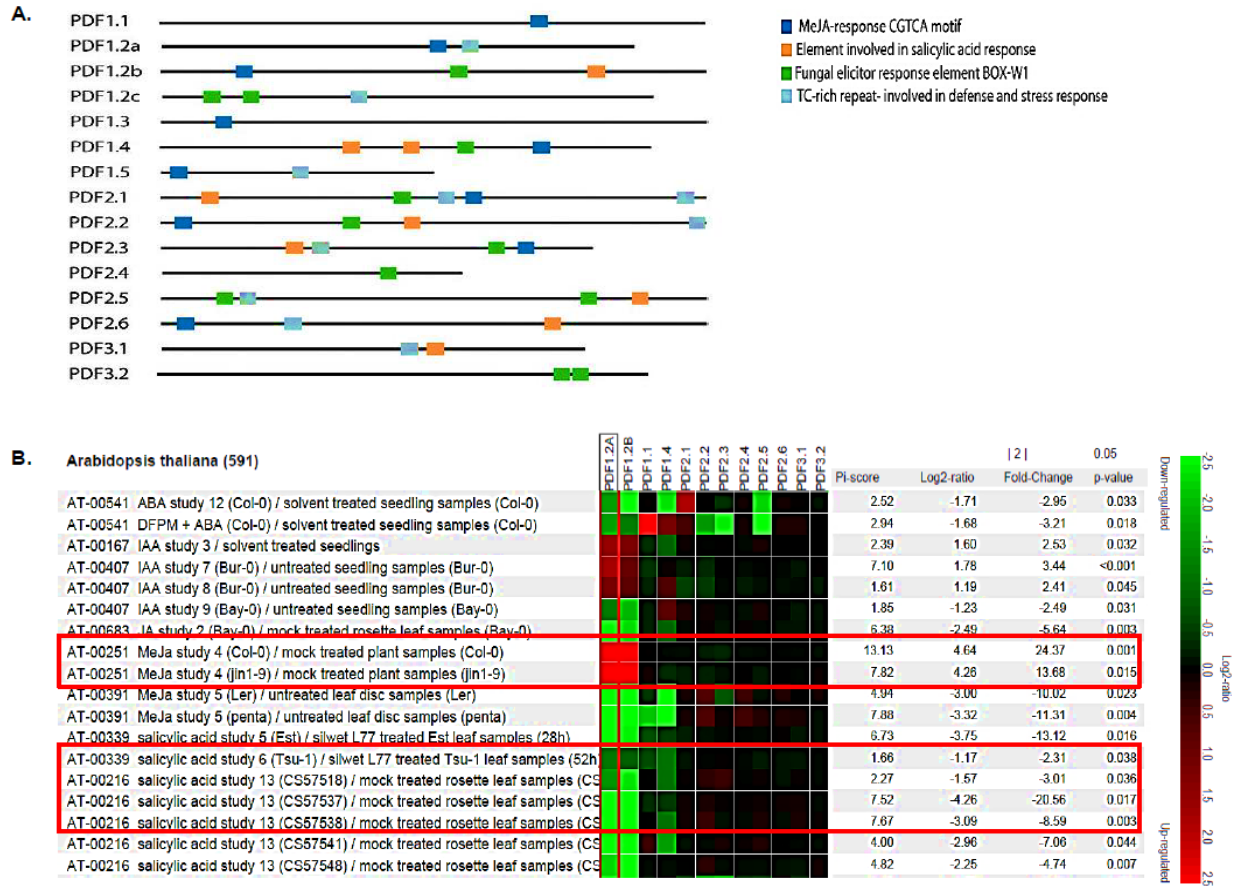


Figure 2 | *In-silico* analysis of *AtPDF2.2* and *AtPDF1.2a* promoter and hormone treatment.

(A) The solid black line represents the promoter DNA sequence; the coloured boxes represent the various motifs found with PlantCare (Lescot et al. 2002). (B) *In-silico* analysis showing expression of *AtPDF2.2* and *AtPDF1.2a* with other *AtPDF* genes using GENEVESTIGATOR for JA and SA treatments.

#### 4 Promoter analysis revealed a gradual suppression of *AtPDF2.2* from roots to leaves

In order to test the responsiveness of the *AtPDF2.2* promoter that contain the motif clusters, a 1519 bp upstream of the translation start codon was fused to the *uidA* (*GUS*) reporter gene and transferred to *Arabidopsis*. A *GUS*-histochemical and *GUS*-gene expression analysis was conducted in the resulting transgenic plants upon the fungal infection. While in the non-infected control plants, the *GUS* expression and *GUS* staining were detected in both roots and leaves at 6 dpi, the *GUS* staining/expression was nearly absent in the roots, suggesting a suppression by the fungus. Likewise, first at 9 dpi, a highly decreased *GUS* staining/expression was observed also in the leaves. Interestingly, the *GUS* staining/expression could be observed at the later infection stages. The data that transcript levels of the *GUS* gene varied similarly as the *GUS* staining patterns in response

to the fungal infection, strongly suggests the transcriptional regulation of *AtPDF2.2* by the *V. longisporum* infection directly or indirectly. Thus, it is reasonable to assume that the suppression of *AtPDF2.2* might be triggered by the fungus to initiate its infection process. In support of this, it has been demonstrated that *A. brassicicola* infection suppresses the expression of *AtPDF2.2* in Arabidopsis leaves (Thomma and Broekaert. 1998; Penninckx et al. 1996). While, it has been also reported that, *AtPDF1.2a* promoter can be activated by MeJA treatment and pathogen infection (Brown et al. 2003; Manners et al. 1998; Mitter et al. 1998). Examination of the 1183 bp region upstream of the transcription start site of the *AtPDF1.2a* revealed several putative motifs sharing homology to known cis-elements being involved in the jasmonate-mediated gene expression. Two of these motifs in the *AtPDF1.2a* promoter are the jasmonate-responsive element, TGACG (-392 to -396), reported in the *PDF1* of barley (*Hordeum vulgare*, Rouster et al. 1997), and a 9-bp sequence, AAATGTTGT (-410 to -419), similar to the jasmonate-responsive region of the promoter of the soybean (*Glycine max*) gene *VspB* (Mason et al. 1993). Moreover, GCC-box and its surrounding bases can interact with several members of the ERF family of transcription factors, resulting in transcriptional activation of *AtPDF1.2a* (Brown et al. 2003). The response of *AtPDF1.2a* to jasmonic acid and ethylene is mediated through the transcription factor ORA59 that binds to GCC boxes in the *AtPDF1.2a* promoter (Pre et al. 2008). In addition MeJA induction, *AtPDF1.2a* was found to be induced during non-host interaction of Arabidopsis plants with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Siddique et al. 2011).

## **5 Plant defensins are functionally involved in plant growth and development**

Gathering evidence suggests that plant defensins and defensin-like peptides not only play a role in defense response but also that they are involved in regulating plant growth and development. Plant defensins are widely distributed in various tissues across the plant. At least one defensin gene is expressed in each plant tissue and some tissues show expression of two or more defensins. Plant defensins have been identified in leaves, tubers, flowers, pods, seeds, germinating seeds, seedlings and also localized in other peripheral sites like xylem, stomata, stomata cells and parenchyma cells, where they are expressed either constitutively or upon pathogenic infection, by mechanical wounding and other stress responses (Broekaert et al. 1997). The tissue-specific localization and

expression patterns of these peptides unfold the critical roles they play in the defense and development of plants (Carvalho and Gomes 2011). Comparative expression analysis of *AtPDF1.2a* and *AtPDF2.2* along with other members of *AtPDF* showed an expression of *AtPDF2.2* across all developmental stages, indicating their vital roles in growth and development, while *AtPDF1.2a* expression was limited to young and developed rosette stage of plant growth (Fig. 3A). Within Class-II genes, *AtPDF2.3* shows a similar expression pattern as *AtPDF2.2*, followed by *AtPDF2.1*. In contrast to Class-II, Class-I exhibited very low expression levels across the all investigated stages and was mostly restricted to the young plant only (Fig. 3A). In plant tissues, *AtPDF* genes exhibit differential expression patterns across various organs, in which *AtPDF2.2* was found in almost all organ tissue except for the primary root and root endodermis. It is to be noted that our promoter analysis demonstrated a strong GUS staining in the primary roots of transgenic plants carrying the Prom::*AtPDF2.2*::GUS construct. In addition, *AtPDF2.2* has been reported to be constitutively present in the Arabidopsis roots (Szakasits et al. 2009; Siddique et al. 2011). A possible function of plant defensins on root development was reported and controversially discussed (Allen et al. 2008). It is observed that the antifungal PDF, MsDef1 from alfalfa can block mammalian L-type calcium channels (Spelbrink et al. 2004) and therefore the growth of plant root hair (Schiefelbein et al. 1992) to restrict the calcium influx. Intriguingly, the application of other plant defensins such as MtDef2 or RsAFP2 without effect on calcium channels was also able to result in a similar inhibitory effect on root hairs (Vijayan et al. 2008), and overexpression of *MsDef1* in Arabidopsis did not alter root morphology (Allen et al. 2008). *AtPDF2.2* and *AtPDF2.1* showed a higher expression level in the endosperm, whereas *AtPDF1.2a* was found neither in the embryo nor in the endosperm. But, their expression was found in rosette and leaves at a comparable level (Fig. 3B). It is believable that the varied expression levels in different tissues probably reflect their distinct but specific roles, even though their functional redundancy still exists.

Increasing data demonstrates that plant defensin-like peptides have evolved specific functions during plant reproduction. A number of PDFs and defensin-like peptides were reported to be expressed specifically in the cells of the female and male gametophyte of Arabidopsis (Punwani et al. 2007; Jones-Rhoades et al. 2007; Steffen et al. 2007; Wuest et al. 2010), *Zea mays* (Cordts et al. 2001; Amien et al. 2010) and *Torenia*

*fournieri* (Okuda et al. 2009). In Brassicaceae, for example, they are involved in the self-incompatibility system, developed by plants to prevent self-fertilization (Higashiyama, 2010; Marshall et al. 2011). *AtPDF2.2* also displayed its expression in reproductive organs (Fig.1A). Thus, ubiquitous expression of *AtPDF2.2* along with *AtPDF2.3* but not *AtPDF1.2a* support their possible involvement during plant growth and developmental responses (Figure 3B).

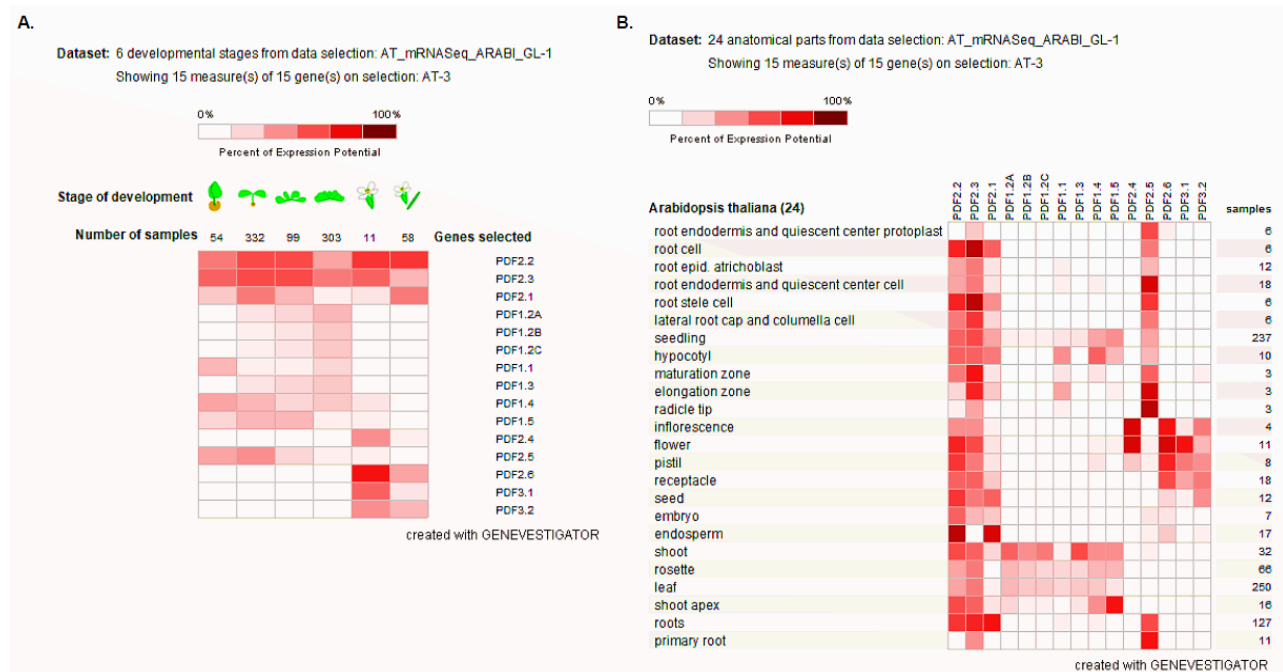


Figure 3 | *In-silico* expression analysis of AtPDF gene family.

(A) GENEVESTIGATOR microarray expression analysis of *AtPDF2.2* and *AtPDF1.2a* with other AtPDF gene family members during different developmental stages of plant growth. (B) *In-silico* microarray analysis showing tissue-specific expression of *AtPDF2.2* and *AtPDF1.2a* with other AtPDF genes using GENEVESTIGATOR.

## 6 Knockdown and overexpression of *AtPDF2.2* and *AtPDF1.2a* lead to altered phenotype in transgenic plants

In this study, knockdown, and overexpression of *AtPDF2.2* and *AtPDF1.2a* respectively resulted in altered phenotypes. We observed that knockdown in *KD-pdf2.2* plants displayed weak phenotype, including a reduction in primary root length, smaller rosette area, early bolting, and below-average seed generation. Similarly, the *KO-pdf1.2a* mutant displayed a reduction in primary root length. Whereas, the overexpression, OE-PDF2.2 plants exhibited superior phenotype with increased growth of primary root length and rosette size. Likewise, OE-PDF1.2a plants exhibited increased primary root length but

smaller rosette size. A study on overexpression of plant defensin gene *DEF2* in tomato showed that growth was initially retarded. The transgenic plant leaves were smaller and growth was more upright (Stotz et al. 2009). Although the role of plant defensins in plant development and growth is still poorly understood, we believe that varying expression levels of *AtPDF2.2* and *AtPDF1.2a* in the tissue/organs as revealed by Genevestigator analysis affect the plant development and growth. Generally, tissue-specific gene expression is associated with specific physiological and developmental functions (Zhang et al. 2012). *AtPDF1.2a* has been reported to be involved in growth and developmental processes (Schmid et al. 2005) as well as in plant architecture (Epple et al. 1997). A study on Arabidopsis *AtPDFs* reported the presence of *AtPDF1.2a* expression only in a rosette in the absence of any pathogen infection (Epple et al. 1997). Besides, *AtPDFs* are JA responsive genes and the JA pathway is well documented to be functional in plant growth and reproduction process. For instance, mutants impaired in JA biosynthesis and perception genes display insufficient filament elongation, non-viable pollen, male sterility, and delayed anther dehiscence (Mandaokar et al. 2006; Wasternack and Hause 2013). That fact that knockdown and overexpression of *AtPDF2.2* resulted in clearly contrasting effects on the phenotype of transgenic plants, evidence for its crucial role in regulating plant growth and development. To understand the underlying mechanism further study is needed.

## **7 Digital transcript profiles of *AtPDF2.2* and *AtPDF1.2a* under biotic and abiotic stresses**

Expression analysis by GENEVESTIGATOR depicted a certain correlation in expression between *AtPDF2.2* and *AtPDF1.2a* (Fig. 4) in response to biotic and abiotic stresses, respectively. The expression levels of *AtPDF2.2* and *AtPDF1.2a* varied upon exposure to several abiotic stresses like cold, heat, photoperiod, ozone, and hormone pathway mutants. In response to cold, ABA, heat, and ozone treatments, both *AtPDF2.2* and *AtPDF1.2a* showed about 1-1.5-fold expressional upregulation (Fig. 4), while, contrastingly, in response to photoperiod shift from the short day (SD) to darkness, *AtPDF2.2* was upregulated while *AtPDF1.2a* downregulated, respectively. From this microarray data and literature, it can be enumerated that the JA pathway might modulate these *AtPDFs* to cope up with the changing day lengths ratios (Cerrudo et al.



2012; de Wit et al. 2013; Cagnola et al. 2008). Following this, the *AtPDF2.2* expression should be decreased along with the increase in darkness as it happened for *AtPDF1.2a*. It is reasonable to speculate that the upregulated expression of *AtPDF2.2* is possibly independent of the JA pathway.

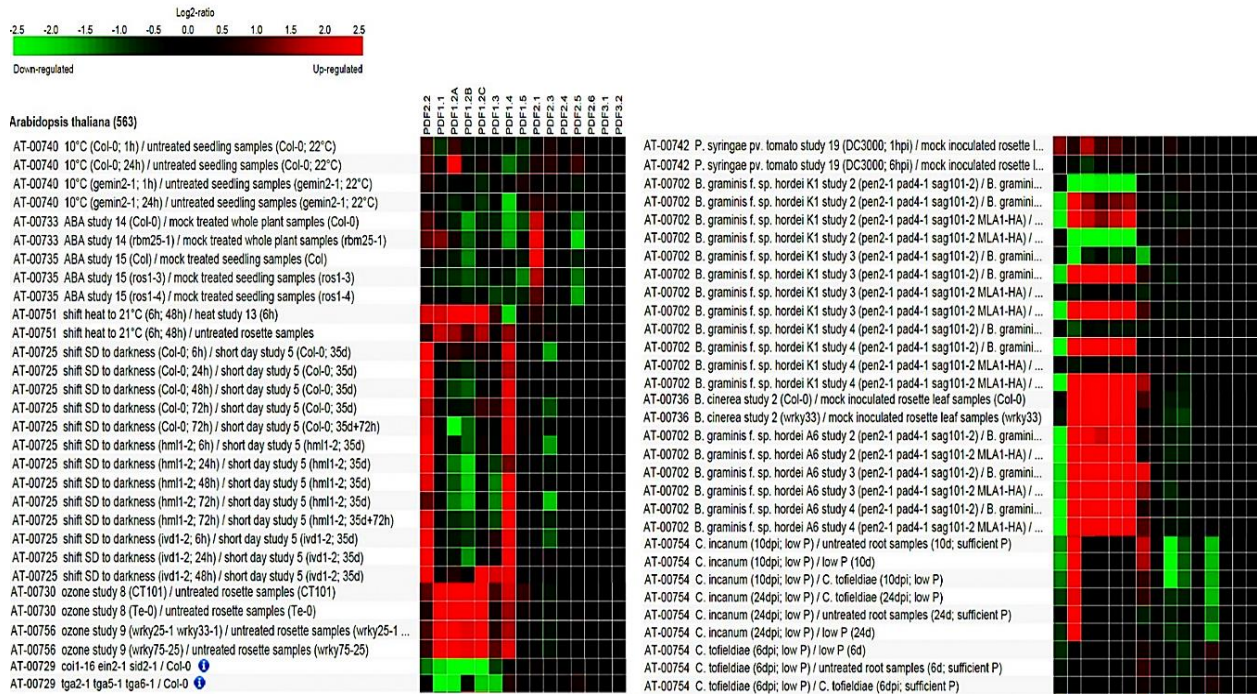


Figure 4 | *In-silico* microarray expression analysis of *AtPDFs*.

All fifteen *AtPDF* genes under various abiotic and biotic stress conditions were used to generate heatmap using GENEVESTIGATOR. Colour bar represents fold change in gene expression, red color represents the highest level of expression and green signifies the lowest level of expression. Expressions for *AtPDF2.2* and *AtPDF1.2a* are shown in first and third rows respectively, starting from left to right.

Similar to the abiotic stress, *AtPDF2.2* along with *AtPDF2.1* and *AtPDF2.3* showed retarded expression patterns under fungal infestations, while *AtPDF1.2a* along with other Class-I, *AtPDF* genes were upregulated in response to *Blumeria graminis*, *Colletotrichum incanum*, and *Colletotrichum tofieldiae*, respectively. We believe that the suppression of the gene expression is indicative that *AtPDF2.2* is targeted by the fungal pathogens directly or indirectly, while the activated JA pathway (Penninckx et al. 1996) stimulates *AtPDF1.2a*. It is notable that challenging with *Pseudomonas syringae* pv. tomato DC3000 just *AtPDF2.2* and *AtPDF1.2a*, induced the expressions, but not other *AtPDFs*. This strongly supports a pathogen-specific suppression of *AtPDF2.2* in the plant-fungus interactions only.



## 8 Knockdown/out of *AtPDF2.2* and *AtPDF1.2a* prompt the differential expressions of *AtPDF* genes and JA, SA or ET-associated genes in transgenic plants

To shed some light on underlying molecular mechanisms, the levels of *AtPDFs*, SA, JA, and ET associated gene expression was compared in non-infected *KD-pdf2.2*, OE-*PDF2.2*, and *KO-pdf1.2a*, OE-35S-*PDF1.2a* plants. The expressions of *AtPDF1.1*, *1.2*, *1.5*, *2.1*, *2.3*, *2.4*, *2.6* and *3.1* were downregulated in the *KD-pdf2.2*. While a significant reduction of *AtPDF1.1*, *1.4*, *1.5*, *2.1*, *2.2*, *2.3*, *3.1*, and *3.2* were observed in the *KO-pdf1.2a* plants. OE-*PDF2.2*-expressing plants exhibited the upregulation of all *AtPDFs* except for *AtPDF3.2*. Whereas, OE-*PDF1.2a*- expressing plants showed an increase in the expression of only Class-II, including *AtPDF2.1*, *2.2*, and *2.3*, respectively. Interestingly, expressions of *AtPDF3.1* and *3.2* were found to be downregulated in the OE-*PDF1.2a*-expressing plants. It is remarkable to discover that the overexpression of *AtPDF2.2* influences the expression levels of almost all the *AtPDF* genes, while *AtPDF1.2a*-overexpression specifically activates the expression of just three *AtPDF* genes from Class II. Comparing the expression patterns between the Class- I and II, it is obvious that there is a co-regulation, as knockdown or overexpression of *AtPDF2.2* or *AtPDF1.2a*, respectively altered the expression patterns of each other and further Class-I and II members as well. Therefore, it can be concluded that, these *AtPDF* genes can regulate with each other, probably independent of JA mediated signaling.

To comprehend the interference of *AtPDF2.2* and *AtPDF1.2a* with JA signaling, three JA-biosynthesis genes, *LOX3*, *AOC3*, and *OPR3* were analyzed for their expression in the transgenic plants. Expression levels of all three genes were not significantly altered in both the OE-*PDF2.2* and OE-*PDF1.2a* plants as compared to the Col-0 wild-type plants, suggesting that overexpression of JA responsive *AtPDF* genes doesn't seem to have an impact on the JA pathway. It has been reported that JA biosynthesis can work independently from changes in the downstream gene expression (Wasternack 2007; Browse, 2009a, b). In support of this, no detectable changes were observed for the three genes (*LOX3*, *AOC3*, and *OPR3*) in the *KO-pdf1.2a* plants. Arbitrarily, transcript levels of *AOC3* and *OPR3*, but not *LOX3* were raised in the *KD-pdf2.2* plants. It is known that *AOC3* and *OPR3* functions downstream to *LOX3* a gene for JA biosynthesis

(Zimmerman and Feng 1978; Wasternack 2007; Browse 2009a; b; Schaller and Stintzi 2009). Many studies also demonstrated that *AOC3* and *OPR3* can be induced exclusively or together with other JA biosynthesis upon pathogen attack. Often, pathogens try to alter the expression of downstream responsive genes to bypass the defense response (Chehab et al. 2011; Chehab et al. 2012; Chini et al. 2018). As we observed that *V. longisporum* suppresses the expression of *AtPDF2.2*, it could be expected that knockdown of *AtPDF2.2* may stimulate similar response inducing gene expression of JA biosynthesis. The expression change of *AtPDF1.2a* does not influence the JA pathway in any manner.

SA-associated genes, *PR1*, *WRKY70* and *WRKY53* were differentially regulated in both the OE-PDF2.2 and OE-PDF1.2a plants. OE-PDF2.2 plants had an extensive reduction in expression levels of the SA associated genes. Since, it is proven that under high JA signaling, few NAC and other transcription factors act as a SA repressor (Zheng et al. 2012; Bu et al. 2008). Generally, this antagonism is achieved by changes in the expression of pathway-associated downstream responsive genes (Mur et al. 2013; Leon-Reyes et al. 2010; Ning Li et al. 2019). Therefore, it can be presumed that *AtPDF2.2* mediates robust downstream JA signaling and conceivably antagonizes to SA signaling. However, in both the OE-PDF1.2a and *KO-pdf1.2a* plants, we found that expression of SA-associated genes was equally repressed as in the Col-0 wild-type plants. It might be explained that in the absence of infection or ongoing disease, SA pathway marker genes are not affected by the *AtPDF1.2a* expression. In accordance, SA responsive genes were remarkably induced in the *KD-pdf2.2* plants. Despite the proven antagonism between SA- and JA-dependent pathways, transcript analysis identified a high number of genes co-induced or co-repressed by the two hormones, pointing to a certain degree of overlap between the two pathways (Glazebrook et al. 2003; Schenk et al. 2000).

Finally, expression levels of ET-associated genes, *EIN3*, a positive and *ETR2*, a negative regulator of ethylene biosynthesis was analyzed. The transcription of *EIN3* was highly suppressed but substantially induced for *ETR2* in the OE-PDF2.2 however not in the OE-PDF1.2a plants. Nevertheless, *EIN3* was noticeably induced while *ETR2* was diminished in the *KD-pdf2.2* plants, indicating an active role of *AtPDF2.2* in ethylene signaling. It is

believed that ET commonly acts in cooperation with JA pathway and activates the expression of defense-related genes (Ding et al. 2011; O'Donnell et al.1996; Penninckx et al.1998; Rojo et al. 2003; Xu et al.1994). Hence, the upregulation of *EIN3* was rather obvious in the *KD-pdf2.2* plants where the expression of JA biosynthesis genes was induced. In the *pdf1.2* plants, the ET-responsive genes were not significant. Considering the synergistic regulation of JA and ET biosynthesis, JAZ proteins repress the JA biosynthesis which results in deactivated downstream ERF branch, being responsible for the ET response (Zhu et al. 2011). Thus, this crucial mechanism governing the JA/ET synergistic interactions could explain the downregulation of the ET pathway in the OE-PDF2.2 plants and upregulation in the *KD-pdf2.2* plants. However, an alike basal expression of ET-responsive genes in OE-PDF1.2a and *KO-pdf1.2a* plants as Col-0 wild-type signifies that *AtPDF1.2a* does not affect the defense pathway directly.

### **9 Translational regulation of *AtPDF2.2* and *AtPDF1.2a***

For the investigation of an inherent mode of interaction between defense pathways and the *AtPDFs*, the expression levels of *AtPDF1.2a* and *AtPDF2.2* genes were determined in different Arabidopsis genotypes (Wt-Col-0, *jar1*, *acs1-7*, *ein2*, *coi1-16* and *ein2-1*; *coi1-16* and *sid2*) without fungal infection by RT-qPCR. Both the *AtPDF* genes were differentially regulated via defense pathways. The *AtPDF1.2a* expression was not impacted in the JA and ET mutants (*jar1*, *acs1-7*, *ein2*, *coi1-16* and *ein2-1*; *coi1-16*), which suggests that the basal expression of *AtPDF1.2a* is independent of JA and ET pathways. Ellis and Turner (2001) have shown that the expression of *AtPDF1.2* was constitutive in the Arabidopsis ET mutant (*cev1*) and on the other hand, activation of the SA pathway suppresses a large set of JA-responsive genes, including the JA marker genes, like *AtPDF1.2a* (Van Wees et al. 1999, Van der Does et al. 2013). Our data with the SA mutant (*sid2*) revealed an increased expression of *AtPDF1.2a*. There are many examples of cross talk between SA and JA/ET signaling pathways. Many SA mutants like (*cpr5*, *cpr6*, *acd2*, *dnd1*, *dnd2*, and *ssi1*) display constitutively high expression of *AtPDF1.2a* (Glazebrook 2001; Spoel et al. 2003), suggesting either that the block in JA signaling relieves the suppression of SA signaling or that the activation of SA signaling blocks JA signaling. The former explanation is supported by the observation that the activation of *AtPDF1.2* expression is also blocked in *nahG mpk4* double mutants

(Petersen et al. 2000; Gupta et al. 2000). Moreover, many studies have identified antagonistic interactions between the SA and JA/ET mediated signaling pathways (Feys and Parker 2000; Kunkel and Brooks 2002). Therefore, it is reasonable to believe that SA pathway regulates directly or indirectly some key regulatory pathway genes of JA signaling to alter the expression of *AtPDF1.2a*. Interestingly, a completely different expression patterns of *AtPDF2.2* were observed in the mutant plants of JA and ET pathway (*jar1*, *acs1-7*, *ein2*, *coi1-16* and *ein2-1; coi1-16*) showing a significantly depleted expression of *AtPDF2.2*. However, in *sid2* mutant plants, the expression levels of *AtPDF2.2* was elevated. This observation further fortifies that the SA pathway most likely suppresses the JA responsive gene expression even in the absence of the fungal infection. Similarly, Gupta et al. (2000) demonstrated that in comparison to the wild-type plant, mutants impaired in SA accumulation exhibited the increase in JA-dependent gene expression. Following this, it can be concluded that *AtPDF2.2* is a vital member of the JA pathway signaling.

### **10 Overexpression of *AtPDF2.2* and *AtPDF1.2a* enhances plant resistance to *V. longisporum* infection in transgenic plants**

To comprehend the involvement of *AtPDF2.2* and *AtPDF1.2a* in the plant-fungus interaction, the knockdown and overexpression plants were subjected to the *V. longisporum* infection. The *V. longisporum* infection affected the primary root length of OE-PDF2.2, *KD-pdf2.2*, OE-PDF1.2a, and *KO-PDF1.2a* seedlings differentially. Root length was measured consecutively at three-time points from 3 to 12 dpi. Fungi were visibly growing alongside the Col-0 wild-type primary roots while *KD-pdf2.2* and *KO-pdf1.2a* roots did not grow and stagnated after infection and were covered by fungi at 12 dpi. To contrast, OE-PDF2.2 and OE-PDF1.2a roots were growing in length and devoid of fungi showing resistance to the fungal infection. It has been observed that Arabidopsis wild-type plants express the leaf-specific defensin *AtPDF1.2* upon pathogen challenge (Penninckx et al. 1996; Thomma et al. 1998; 1999). Hence, our data support that *AtPDF2.2* and *AtPDF1.2a* are functional to restrict fungal infection, conferring plant resistance to the fungal infection. As revealed by the infection assays at 18 dpi mutant plants of both *KD-pdf2.2* and *KO-pdf1.2* were more susceptible with pronounced stunted growth as compared to the Col-0 wild-type plants. The disease symptoms were further

intensified and caused the early senescence, followed by the death of *KD-pdf2.2* plants as well as extremely reduced growth of *KO-pdf1.2* plants at 24 dpi. On the contrary, overexpression (OE-PDF2.2 and OE-PDF1.2a) plants demonstrated significant resistance and fewer disease symptoms at 24 dpi in comparison to the Col-0 wild-type plants. Though overexpression of plant defensins in several plant species had been reported to increase the plant resistance against various fungal pathogens (Tab. 1), underlying mechanisms remain largely unsolved. It is discussed that PDFs containing eight cysteine residues interact with various types of receptors, present in the fungal plasma membrane (PM) and/or in the cell wall (CW), where they target fungal sphingolipids or related glucosylceramides (Aerts et al. 2008; Lay et al. 2005; Wilmes et al. 2011). These sphingolipids colocalize in fungal membranes with ergosterol in lipid-rafts rich in membrane proteins with high-affinity binding sites for the defensins. Different antifungal defensins bind in different manners giving rise to different effects, including membrane disruption, inactivation of calcium channels, activation of signaling cascades, induction of ROS production, and induction of apoptosis (Aerts et al. 2008; Wilmes et al. 2011). In the light of these evidences, the heightened resistance in the OE-PDF2.2 or OE-PDF1.2a and the enhanced susceptibility to *V. longisporum* infection in the *KD-pdf2.2* or *KO-pdf1.2* plants, respectively strongly suggest that *AtPDF2.2* and *AtPDF1.2a* play a crucial role in defense response in *A. thaliana*-*V. longisporum* interactions. Therefore, subsequent research on the underlying mechanisms as well as their auxiliary effects on other pathogens is of great interest.

Table 1 | Overview of transgenic plants overexpressing a plant defensins and their resulting phenotype.

Species of origin	Defensin name	Transformed plant	Phenotype (increased resistance/tolerance to the indicated stressor)	Reference
<i>Arabidopsis halleri</i>	AhPDF1.1	<i>Arabidopsis</i>	Zn	Oomen et al. 2011
<i>Arabidopsis</i>	AtPDF1.1	<i>Arabidopsis</i>	<i>Cercospora beticola</i>	De Coninck et al. 2010
<i>Brassica campestris</i>	BsD1	<i>Nicotiana tabacum</i>	<i>Phytophthora parasitica</i>	Park et al. 2002
<i>Brassica juncea</i>	BjD	<i>Arachis hypogaea</i>	<i>Pheoisariopsis personata</i> <i>Cercospora arachidicola</i>	Swathi et al. 2008
		<i>Nicotiana tabacum</i>	<i>Fusarium moniliforme</i> <i>Phytophthora parasitica</i> pv. <i>nicotianae</i>	
<i>Brassica rapa</i>	BrD1	<i>Oryza sativa</i>	<i>Nilaparvata lugens</i>	Choi et al. 2009

Species of origin	Defensin name	Transformed plant	Phenotype (increased resistance/tolerance to the indicated stressor)	Reference
<i>Dahlia merckii</i>	DmAMP1	<i>Carica papaya</i>	<i>Phytophthora palmivora</i>	Zhu et al. 2007
		<i>Oryza sativa</i>	<i>Magnaporthe oryzae Rhizoctonia solani</i>	Jha et al. 2009
		<i>Solanum melongena</i>	<i>Botrytis cinerea Verticillium albo-atrum</i>	Turrini et al. 2004a
<i>Medicago sativa</i>	MsDEF1/alfAFP	<i>Solanum lycopersicum</i>	<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i>	Abdallah et al. 2010
		<i>Solanum tuberosum</i>	<i>Verticillium dahliae</i>	Gao et al. 2000
<i>Nicotiana megalosiphon</i>	NmDef02	<i>Solanum tuberosum</i>	<i>Phytophthora infestans Alternaria solani</i>	Portieles et al. 2010
		<i>Nicotiana tabacum</i>	<i>Phytophthora parasitica</i> var. <i>nicotianae</i> <i>Peronospora hyoscyami</i> f.sp. <i>tabacina</i>	
<i>Orychophragmus violaceus</i>	Ovd	<i>Brassica napus</i>	<i>Sclerotinia sclerotiorum</i>	Wu et al. 2009
<i>Pisum sativum</i>	DRR230	<i>Brassica napus</i>	<i>Leptosphaeria maculans</i>	Wang et al. 1999
<i>Raphanus sativus</i>	RsAFP2	<i>Nicotiana tabacum</i>	<i>Alternaria longipes</i>	Terras et al. 1995
		<i>Oryza sativa</i>	<i>Magnaporthe oryzae Rhizoctonia solani</i>	Jha and Chattoo 2010
		<i>Solanum lycopersicum</i>	<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i> <i>Botrytis cinerea</i>	Kostov et al. 2009
		<i>Triticum aestivum</i>	<i>Fusarium graminearum Rhizoctonia cerealis</i>	Li et al. 2011
<i>Solanum lycopersicum</i>	DEF2	<i>Solanum lycopersicum</i>	<i>Botrytis cinerea</i> reduced seed setting pollen viability growth changes	Stotz et al. 2009b
<i>Wasabia japonica</i>	WT1/WjAMP1	<i>Colocynthis citrullus</i>	<i>Alternaria solani Fusarium oxysporum</i>	Ntui et al. 2010
		<i>Oryza sativa</i>	<i>Magnaporthe grisea</i>	Kanzaki et al. 2002
		<i>Phalaenopsis orchid</i>	<i>Erwinia carotovora</i>	Sjahril et al. 2006
		<i>Solanum tuberosum</i>	<i>Botrytis cinerea</i>	Khan et al. 2006
<i>Zea mays</i>	ZmDEF1	<i>Nicotiana tabacum</i>	<i>Phytophthora parasitica</i>	Wang et al. 2011

## **11 *AtPDF2.2* overexpression in transgenic *Arabidopsis* plant reduces susceptibility to *P. syringae* pv. tomato DC3000 but *AtPDF1.2a* does not**

In order to investigate the possible antibacterial activity of *AtPDFs*, *Pseudomonas syringae* pv. tomato DC3000, a Gram-negative bacterial pathogen causing bacterial speck disease of tomato (*Solanum lycopersicum*) and *Arabidopsis* (Worley et al. 2012), was examined. *P. syringae* is considered a hemibiotrophic plant pathogen, whose natural infection cycle begins with entering the plant through surface wounds and natural openings, such as stomata (Worley et al. 2012). Aggressive endophytic growth within the host plant eventually leads to disease. The *P. syringae* interaction with *Arabidopsis* is mediated by the interplay of both SA and JA (Brooks et al. 2005). So far, mostly plant PDF proteins are known to exhibit defense mainly against fungal pathogens. *AtPDF2.2* and *AtPDF1.2a* transgenic *Arabidopsis* plants along with Col-0 wild-type was challenged with *Pst* DC3000. OE-PDF2.2 plants were more tolerant while *KD-pdf2.2* plants were more susceptible as compared with the Col-0 wild-type plants. MeJA treatment has been shown to be effective against *P. syringae* in *Arabidopsis* and tomato (Pieterse et al. 1998; Thaler et al. 2002; Van Wees et al. 1999). The expression of *AtPDF2.2* in the Col-0 wild-type plants were induced at 1 and 3 dpi. Thus, induction in the expression of *AtPDF2.2* suggests a possible pathogenesis-related response to *P. syringae*. Notably, bacterial DNA quantified was least in the OE-PDF2.2 plants followed by Col-0 wild-type and *KD-pdf2.2* plants respectively. A plausible explanation of this enhanced resistance could be the overexpression of *AtPDF2.2*. There are various compelling evidence demonstrating the role of *PDFs* in basal resistance. The *PDFs* inhibit the growth of the gram-positive and gram-negative bacteria, but their antibacterial activity is less pronounced than their ability to inhibit the growth of phytopathogenic fungi. The antibacterial activity has been described for the *So-D* defensins from spinach, the *StPTH1* defensin from the *Solanum tuberosum* potato (Segura et al. 1998), *Dm-AMP1* from dahlia, *Ct-AMPI* from the *Clitoria ternatea*, *Ah-AMP1* from the *Aesculus hippocastanum* (Osborn et al. 1995) *PsDef1* from a pine (Khairutdinov et al. 2017) and *VaD1* from the *Vigna angularis* azuki bean (Chen et al. 2005). Antibacterial activity has also been demonstrated for different defensin-like peptides from legumes, but these peptides inhibit bacterial growth in concentrations >50  $\mu$ M (Wong et al. 2006; Wong and Ng 2005; Wong and Ng 2006; Srikanth et al. 2016). The Cp-thionin-2 from the *Vigna*

*unguiculata* and fabatin-2 from the *Vicia faba* broad beans have been shown to exhibit the antibacterial effect synergistically with the HBD3 human  $\beta$ -defensin. It is assumed that this combination of peptides can be used for fighting against bacteria that cause food spoilage and food poisoning (Kraszewska et al. 2016). Altogether, these examples and our data point towards a feasible role of *AtPDF2.2* in resistance against pathogens with diverse lifestyles, an important finding of this study.

On the other hand, both OE-PDF1.2a, and KO-PDF1.2a transgenic plants presented almost similar bacterial disease progression. Bacterial biomass quantified was also in the same range as the Col-0 wild-type plants. Significantly, induced expression of *AtPDF1.2a* was however detected at 3 dpi. It is well known that *P. syringae* interaction with Arabidopsis is mediated by the interplay of both SA and JA (Brooks et al. 2005). *P. syringae* produces the nonhost-specific phytotoxin coronatine (COR) (Worley et al. 2012). COR is a structural mimic of the active form of jasmonate, jasmonoyl- L - isoleucine (JA-Ile) (Zheng et al. 2012) This COR stimulates JA-signaling and consequent suppression of SA-dependent defense through antagonistic crosstalk (Geng et al. 2012; Geng et al. 2014; Kloeck et al. 2001; Brooks et al. 2005). Henceforth, COR, through its ability to mimic active JA-conjugates could similarly alter gene expression independent of targeting COI. This might can explain why we observed an induced expression of *AtPDF1.2a*. However, the induction of *AtPDF1.2a* did not result in disease resistance most likely because of the inhibitory effects of JA on SA signaling, benefiting the bacterial pathogen to advance its infection course. Plant defensins reported with resistance against Gram-positive and Gram-negative bacteria were summarized in Tab. 2.

Table 2 | Plant defensins presenting recognized antibacterial activity.

Defensin	Source	Bacteria		Reference
		Gram-negative	Gram-positive	
Cowpea-thionin II (Cp-thionin II)	<i>Vigna unguiculata</i>	<i>Escherichia coli</i> , <i>Pseudomonas syringae</i>	<i>Staphylococcus aureus</i>	Franco et al. 2006
Pseudothionin (PTH1)	<i>Solanum tuberosum</i>	<i>Pseudomonas syringae</i> pv. <i>Tabaci</i>	<i>Clavibacter michiganensis</i>	Kovalskaya et al. 2009, Segura et al. 1999
<i>Spinacia oleracea</i> Defensins	<i>Spinacia oleracea</i>	<i>Ralstonia solanacearum</i>	<i>Clavibacter michiganensis</i>	Segura et al. 1998



1 to 7 (So-D1 to So-D7)				
Fabatins	<i>Vicia faba</i>	<i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>	<i>Enterococcus hirae</i>	Zhang et al. 1997
<i>Tulipa</i> antimicrobial peptides (Tu-AMP 1 and Tu-AMP 2)	<i>Tulipa gesneriana</i>	<i>Erwinia carotovora</i> , <i>Agrobacterium radiobacter</i> , <i>Agrobacterium rhizogenes</i>	<i>Clavibacter michiganensis</i> , <i>Curtobacterium flaccumfaciens</i>	Fujimura et al. 2004
Sweet potato defensin 1 (SPD1)	<i>Ipomoea batatas</i>		<i>Staphylococcus aureus</i>	Huang et al. 2008
<i>Triticum aestivum</i> defensin 1 (Tad1)	<i>Triticum aestivum</i>	<i>Pseudomonas cichorii</i>		Koike et al. 2002
White cloud bean peptide	<i>Phaseolus vulgaris</i>	<i>Mycobacterium phlei</i>		Wong et al. 2006
			<i>Bacillus megaterium</i> , <i>Bacillus subtilis</i>	

## 12 Knockout/down of *AtPDF2.2* and *AtPDF1.2a* results in altered resistance to *S. sclerotiorum* in transgenic plants

Sclerotinia stem rot caused by *S. sclerotiorum* is one of the most important diseases of oilseed rape (*Brassica napus* L.) (Lane et al. 2019). The fungus severely reduces the yield and quality of some important crops, worldwide, including oilseed rape (Kirkegaard et al. 2006). Upon *S. sclerotiorum* infection, the expression of *AtPDF2.2* was suppressed while *AtPDF1.2a* being induced in the Col-0 wild-type plants support their involvement in plant-fungus interactions. We believe that as *AtPDF2.2* works downstream the JA signaling, its downregulation adversely impairs plant resistance. The induction of *AtPDF1.2a* might be an effect of plant defense response. Some research suggests that the induced expression of *AtPDF1.2a* comes from stimulation of all three SA, JA and ET pathways together (Guo and Stotz 2007). Subsequently, infection assays on *AtPDF2.2* and *AtPDF1.2a* transgenic plants showed an enhanced susceptibility in *KD-pdf2.2* while *KO-pdf1.2a* plants were similarly infected as the Col-0 wild-type. Contrastingly, both the OE-PDF2.2 and OE-PDF1.2a plants showed improved resistance as compared with the Col-0 wild-type plants. Various studies had shown that the knockout or overexpression of JA pathway genes is responsible for the heightened susceptibility or resistance, respectively against *S. sclerotiorum* infection (Anderson et al. 2004;

Donaldson et al. 2001; Kesarwani et al. 2007). For instance, overexpression of PDF from *Orychophragmus* in *Brassica napus* reduced damage to plants caused by *Sclerotinia sclerotiorum* (Wu et al. 2009; Zarinpanjeh et al. 2016). PDFs proved to be involved in the innate immunity responses to different fungal pathogens, including *F. oxysporum*, *Alternaria alternata*, *Botrytis cinerea*, *Verticillium dahlia* (Gaspar et al. 2014; Graham et al. 2008; Ahmed et al. 2012). Transgenic over-expression of antimicrobial peptides or proteins in crop plants is the most common and a promising strategy to combat bacterial and fungal pathogens. Many antimicrobial peptides have shown broad-spectrum activities against different pathogens and plant defensins, like their animal counterparts can have multiple functions in plant defense response and plant development.

### **13 AtPDF2.2 and AtPDF1.2a transgenic plants exhibit the modulated expression of AtPDFs and JA, SA or ET responsive genes**

Finally, to examine the effect of *V. longisporum* infection on the underlying defense mechanisms, expression patterns of SA, JA, and ET associated marker genes were comparatively analyzed in the roots of 6 dpi knockdown (*KD-pdf2.2* and *KO-pdf1.2a*) and overexpression (OE-PDF2.2 and OE-PDF1.2a) plants. We observed that the transcript levels of Class-I and II *AtPDF* genes were downregulated in the *KD-pdf2.2* plants but significantly induced in the OE-PDF2.2 plants. Marginal repression was detected among the Class-II *AtPDF* genes in the OE-PDF2.2 plants, infected by the fungus, as compared with non-infected plants. The transcript levels in the both Class-III *AtPDF* genes were induced in the *KD-pdf2.2* plants, and *AtPDF3.1* was upregulated in the OE-35S-PDF2.2 plants. Although these two genes are probably not directly involved in the defense responses (Thomma et al. 2002), but *AtPDF3.1* was induced in the OE-PDF2.2 plants. We conclude that *AtPDF2.2* positively interacts with *AtPDF3.1* but not with *AtPDF3.2*. In contrast to *AtPDF2.2*, *AtPDF1.2a* transgenic plants presented a rather complex gene expression patterns. Transcript levels of the mostly all Class-I and III *AtPDF* genes in the OE-PDF1.2a plants were elevated except for *AtPDF1.3* while none of Class-II genes. This might suggest a positive regulation between Class-I and III *AtPDF* genes with *AtPDF1.2a*. Since *AtPDF1.2a* shows functional redundancy with *AtPDF1.2b* and *AtPDF1.2c* as they encode the same mature peptide. High homology between the other members of this Class-I is obvious (Thomma et al. 2002). This can be explained that genetic robustness

may arise from redundant genes, whereby the mutation of one gene may be compensated by another with overlapping functions and expression patterns (Tautz et al. 1992; Wang et al. 1996; Santamaria et al. 2007). Moreover, Class-III *AtPDF* genes could be precursors for these peptides. Class-II *AtPDF* genes including *AtPDF2.2*, *2.3* and *2.5* were found to be induced in the *KO-pdf1.2a* plants though they were not significantly affected in the OE-PDF1.2a plants. It is to notice that *AtPDF1.3*, *AtPDF2.3* and *AtPDF2.5* were suppressed in the transgenic *AtPDF1.2a* plants. Strikingly, these genes (*AtPDF1.3*, *AtPDF2.3*, and *AtPDF2.5*) which are induced or suppressed in the OE-PDF1.2a or *KO-pdf1.2a* plants respectively were all highly upregulated in the OE-PDF2.2 and depressed in the *KD-pdf2.2* plants. It is reasonable to speculate a negative regulation of *AtPDF2.2* by *AtPDF1.2a* including three other *AtPDF* genes, in contrast to regulation of *AtPDF1.2a* by *AtPDF2.2*. Understanding the underlying mechanisms remains a great challenge.

It is widely accepted that the JA signaling mediates resistance to necrotrophic and hemibiotrophic pathogens (Li and Yen 2008). Thus, the impact of *V. longisporum* infection on JA biosynthesis in transgenic *AtPDFs* plants could provide valuable clues on *AtPDFs* working mechanisms. Transcript levels of the JA biosynthesis gene, *LOX3*, *AOC3*, and *OPR3* was measured relatively lower in the *KD-pdf2.2* and higher in the OE-PDF2.2 plants. Additionally, even the non-infected JA mutants (*jar1* and *coi1*) showed a noticeable decrease in the expression of *AtPDF2.2*. Hence, a substantial fluctuation in the expressions of JA biosynthesis genes in the *AtPDF2.2* transgenic plants indicates a direct interaction of *AtPDF2.2* with the upstream JA biosynthesis genes. For *AtPDF1.2a*, these JA biosynthesis genes were upregulated in the *KO-pdf1.2a* plants, not in the OE-PDF1.2a as compared with Col-0 wild-type plants. It seems that there is no direct interaction with the JA pathway, explaining why the expression of *AtPDF1.2a* was not altered in the non-infected JA mutants (*jar1* and *coi1*). As *KO-pdf1.2a* plants showed the upregulation of JA biosynthesis genes along with the induced expressions of *AtPDF2.2*, *2.1*, and *2.3*, it might imply an indirect targeting by *V. longisporum*, since the fungus negatively affects the expression of *AtPDF2.2*, this in turn, triggers the JA biosynthesis consequently. During the co-evolution of plants and microbes, pathogens have evolved certain mechanisms to

suppress defense responses by meddling with key pathway regulators, thereby forcing plants to evolve bypass mechanisms (Zhang et al. 2017; McDowell and Dangl 2000). This explanation can also clarify why JA biosynthesis genes were induced in the non-infected *KD-pdf2.2* plants. Reasonably, suppression of *AtPDF2.2* triggers the JA biosynthesis in plants. These arguments strengthen our observation of suppression of *AtPDF2.2* and activation of JA biosynthesis occurred in plants, simultaneously. On the other hand, *AtPDF1.2a* was detected negatively to be interacting with *AtPDF2.2* along with *AtPDF2.1* and *2.3* genes. Nonetheless, *KO-pdf1.2a* plants gave hints on

how *AtPDF1.2a* could interference with *AtPDF2.2* in the initial phase of infection. Since both the mutant plants, *KD-PDF2.2* and *KO-PDF1.2a* were highly vulnerable while overexpression; OE-PDF2.2 and OE-PDF1.2a plants were noticeably resistance to the fungal infection, so, it can be deduced that there is an intricate early reprogramming of gene expression by pathogen attack. Following this, a two-step model of the mechanism of action for the *PDFs* in plant-fungus interactions was proposed, in which the first step is the interaction of the defensins with sphingolipids, GlcCer or M(IP)<sub>2</sub>Con fungal membranes, and in the next step the defensin inserts itself into the membrane causing permeabilization provoking an event of membrane destabilization (Thevissen, François et al. 2003; Thevissen, Ferket et al. 2003).

SA defense pathways are generally known to work antagonistically to JA pathway (Thomma et al. 1998). To comprehend how the SA signaling reacts to the *V. longisporum* infection, transcript levels of *PR1*, *WRKY70*, and *WRKY53* were investigated. OE-PDF2.2 plants showed highly elevated expressions of SA associated genes and OE-PDF1.2a plants had induced expression only for *PR1* as compared with Col-0 wild-type. It has been shown that inoculation of Arabidopsis with the hemibiotrophic fungal pathogen, *Alternaria brassicicola* resulted in a strong systemic response leading to the sustained induction of genes such as *PR1* and *PDF1.2* (Penninckx et al. 1996), which are markers for the salicylate and jasmonate defense-signaling pathways, respectively (Ryals et al 1996; Penninckx et al 1996; Manners et al. 1998). This aligns with our observation, in which the overexpression of *AtPDF1.2a* resulted in induced expression of *PR1* while the overexpression of *AtPDF2.2* elevated the expression of all analyzed SA responsive genes. As anticipated, in the *KD-pdf2.2* plants, SA responsive gene

expressions were significantly reduced. However, the expressions of SA-genes were induced in the *KO-pdf1.2a* plants. The stimulation of SA responsive genes in the *KO-pdf1.2a* plants could be a consequence of JA responsiveness upon lower levels of *AtPDF1.2a* (Thomma et al. 1998; 1999). Emerging evidences also suggest that some of the well-known hemibiotrophic pathogens can induce different defense signaling pathways due to their overlapping biotrophic and necrotrophic lifestyles. Zhu et al. (2012) demonstrated that *PR1* protein exhibited antifungal activity. In addition, JA dependent defense responses can contribute to resistance against pathogens with a hemibiotrophic lifestyle, possibly by acting in concert with other defense signaling pathways (Broekaert et al. 2006; Browse 2009; Meng and Zhang 2013). Besides, a recent study further claims that the early activation of the JA pathway requires SA through SA receptors, instead of the conventional JA pathway (Liu et al. 2016). Considering that both SA and JA pathways were activated in the OE-PDF2.2 and deactivated in the *KD-pdf2.2* plants, we conclude that *AtPDF2.2* is regulated by both pathways in response to the *V. longisporum* infection. A lower expression of *AtPDF1.2a* conversely stimulates intricate signaling to defense pathways. However, the overexpression data clarifies that *AtPDF1.2a* is not directly involved in the regulation of the SA pathway. Nevertheless, there is a possibility of dose-dependent interactions. At lower concentrations, JA and SA can stimulate with each other to bring the synergistic effect (Mur et al. 2006). Since it is well established that *AtPDF1.2a* is directly activated by the JA pathway, it is obvious in our study that the changes in its transcriptional abundance when the JA pathway is modulated.

Consistently, ET biosynthesis genes, *EIN3* and *ETR2* gene expressions were unaffected in the *KD-pdf2.2* while *EIN3* was upregulated and *ETR2* was downregulated in the OE-35S-PDF2.2 plants. ET commonly acts in cooperation with the JA pathway and activates the expression of defense-related genes (Ding et al. 2011; O'Donnell et al.1996; Penninckx et al.1998; Rojo et al. 2003; Xu et al.1994). Hence, the change in the expression of *EIN3* was not obvious in the *KD-pdf2.2*, in which the expression of JA biosynthesis genes was highly reduced. Moreover, in absence of JA biosynthesis, ET-response cannot be initiated. Whereas the expression of ET biosynthesis genes was not significantly altered in the OE-35S-PDF1.2a plants, contrariwise, *EIN3* was upregulated and *ETR2* was downregulated considerably in the *KO-pdf1.2a* plants, respectively.

Similarly, when JA biosynthesis genes were markedly induced, then we observed the induction of *EIN3* in the both of OE-35S-PDF2.2 and *KO-pdf1.2a* plants. The possible involvement of ethylene and jasmonate signaling pathways in the pathogen-induced systemic activation of *AtPDF1.2* has been reported by the study of various Arabidopsis mutants (Penninckx et al. 1996; Manners et al. 1998).

The results of this study evidence that the *V. longisporum* is able to depress *AtPDF2.2* and activates the JA pathway at the early stage in infected plants. Coincidentally, we demonstrate that *AtPDF2.2* simultaneously interacts with SA and ET pathways upon fungal attack. The expression of *AtPDF1.2a* is induced as a result of the active JA pathway, though it could be responsible for the expression of several *AtPDF* genes during the early infection. Moreover, our data support that the SA pathway predominantly protects plants against biotrophic and hemibiotrophic pathogens, whereas JA and ET signaling enhances resistance to necrotrophic pathogens. However, our study clearly shows that all three pathways are involved in plant defense against *V. longisporum*.

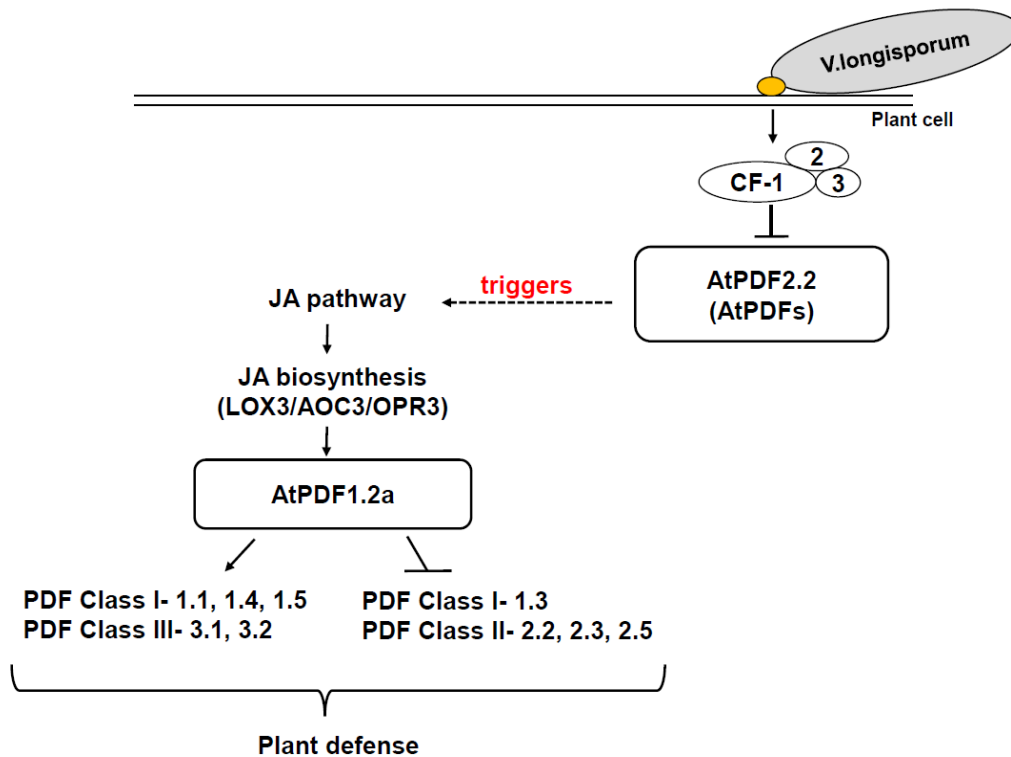


Figure 5 | Proposed functional model.

*V. longisporum* mediated suppression of *AtPDF2.2* expressions probably triggers the JA pathway. Activated JA pathway starts downstream signaling and induces *AtPDF1.2a* expression to start the plant defense.

In the light of our findings, we propose a working model for *AtPDF2.2* and *AtPDF1.2a* in plant-fungus interactions. As shown in Figure 5, *AtPDF2.2* expression is targeted by *V. longisporum*, utilizing putative compatibility factors genes, which in turn affect the few additional *AtPDF* genes. This suppression triggers the JA biosynthesis, which subsequently induces the *AtPDF1.2a* expression. *AtPDF1.2a* regulates positively with Class-III, and I genes but negatively regulates *AtPDF2.2* and some other Class-II genes to initiate the defense and signaling reprogramming.

#### **14 Concluding remarks**

Our data demonstrate 1) that suppression of *AtPDF2.2* is required by *V. longisporum* to establish its infection, 2) that *AtPDF2.2* probably prompts the JA biosynthesis along with the suppression in a direct or indirect manner. However, *AtPDF1.2a* is responsible for effective downstream JA signaling by potentiating the expression of *AtPDF* genes to initiate the defense response and 3) overexpression of the *AtPDF1a* and *AtPDF2.2* in transgenic plants confers resistance against two fungi, *V. longisporum* and *S. sclerotiorum*, and in addition, *AtPDF2.2* was able to improve plant resistance to the *P. syringae* infection as well. Furthermore, our data suggest that these PDF genes might be valuable candidates for genetic engineering or molecular breeding for a broad range of disease resistance against necrotrophic fungal or bacterial pathogens in plants. A great challenge remains to understand the model of action and dissect underlying mechanisms.

In plants, defensins have been used in the development of transgenic plants resistant to fungal attacks (Dias and Franco 2015; Portieles et al. 2010). These transgenic plants present resistance and no detrimental agronomic features under field conditions and they provide a useful alternative for the current techniques for pathogen control based on chemical treatment (Gao et al. 2000; Dias and Franco 2015). Moreover, tobacco plants expressing the *Tephrosia villosa* defensin (*TvD1*) gene present concomitant activity against fungi and insects (Vijayan et al. 2013) These findings show that defensins applied to improve host defenses against microbial pathogens have a high prospect of success.

More than sequence and structural similarity, some defensins containing a CS $\alpha$  $\beta$  motif seem to act on bacterial pathogens in a similar way (Dias and Franco 2015). This similarity is clear in the common mechanism of action observed in some fungal, insect and mussel

defensins, which is based on the inhibition of bacterial cell-wall biosynthesis, mediated by binding to Lipid II. However, the antibacterial activity observed in some plant defensin mechanisms is still a challenge. More than this, the determinants of the variable specificity observed in these molecules still needs to be established. Genetic engineering as a promising method for the effective control of plant diseases has the advantage of incorporating genes, which produce resistance proteins from any species to any crop (der Biezen 2001). Among the antifungal proteins, pathogenesis-related (PR) proteins such as defensins are well known to provide resistance to fungal infection in various plants (Thomma et al. 2002). However, the use of defensins for biotechnological purposes is still a challenging area, due to the difficulties of producing these peptides on a large scale. The purification of these peptides from their natural sources in general results in a low final concentration (Valore and Ganz 1997). Additionally, their chemical synthesis and their expression in heterologous systems faces misfolding problems intrinsic to cysteine-rich peptides, since these peptides require specific environments and sometimes propeptide regions to assist in the correct protein folding and disulfide bond linkage (Valore and Ganz 1997). Large-scale production is an important challenge that needs to be met to allow for widespread agricultural uses, decreasing costs, and the production time of these defensins. For this purpose, advanced detailed researches are needed to explore the mode of action of PDFs and dissect the molecular mechanisms underlying as well.

### **15 Reference (see page 146)**



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## Summary

The hemibiotrophic fungal pathogen *Verticillium longisporum* infects oilseed rape (*Brassica napus*), posing a real threat to its cultivation. According to Eurostat (2019), *Brassica napus* was 2017 the major oilseed cash crop in Germany, cultivated on 1.3 mio. ha. Intensive cultivation with insufficient crop rotation and tillage operations raises severe problems, e.g. diseases caused by soil-borne pathogens. Although increased understanding of plant-*Verticillium* interactions has been achieved relying on the model plant *Arabidopsis*, further research is necessary to meet current agricultural demands for advanced strategies in resistance breeding. Conventional approaches have their limitations and are predominantly based on the availability of a broad genetic spectrum and the selection of suitable genotypes to improve for example resistance to abiotic stresses or pathogens. To achieve this goal also in crops with a narrow genetic gene pool such as oilseed rape, new strategies are required. An example of this is the expression of plant defensins (PDFs), which are known to possess antifungal activity. In our lab, we identified host compatibility factors (CFs), loss of which resulted in elevated ethylene response and *AtPDF2.2* gene expression. Overexpression of PDFs in both model and crop plants has shown to enhance and provide long-lasting disease resistance. In this thesis, two chapters are presented aiming at the characterization and functional analysis of *AtPDFs* with emphasis on the role of antifungal activity in regulating *Arabidopsis*-*Verticillium* interactions.

Chapter II describes that *AtPDF2.2* promoter-driven GUS expression highlighted the initial suppression of this gene at 6 dpi in the *Arabidopsis* roots, followed by leaves at 9 dpi. Transcript analysis further confirmed the strong downregulation of *AtPDF2.2* expression along with two closely related *Arabidopsis* genes of PDF Class-II. Transgenic lines for *AtPDF2.2* overexpression (*OE-PDF2.2*) and knockdown (*KD-pdf2.2*) demonstrated also an up-regulation of *AtPDF2.3* and *AtPDF2.5* in the overexpression line and corresponding suppression in the knockdown line. Another important observation in *KD-pdf2.2* plants was an elevated jasmonic acid (JA) response with concomitant upregulation of ethylene (ET) and salicylic acid (SA) responsive genes, an effect also observed during infection with other hemibiotrophic fungi. Therefore, based on available data it can be elucidated that, in order to bypass the defense response and establish the infection, *V.*

*longisporum* during the initial phase of infection, preferably utilizes the compatibility factors (CFs) to suppress the expression of *AtPDF2.2*. The suppression of *AtPDF2.2* further affects the expression of two other closely related *AtPDF* genes, *AtPDF2.3* and *2.5*, helping the fungus to establish a successful infection process. However, the suppression of these three PDFs, in turn, triggers the JA response, which subsequently activates ET and SA signaling to mediate the defense response. Lastly, to study the antifungal activity of *AtPDF2.2* the *OE-PDF2.2* line was analyzed and displayed enhanced resistance to two fungal pathogens (*V. longisporum* and *Sclerotinia sclerotiorum*) as well as one bacterial pathogen (*Pseudomonas syringae*). Contrastingly, *KD-pdf2.2* led to highly susceptible plants to both fungal and bacterial pathogens.

Hence, namely, three important conclusions can be drawn based on this study; firstly, *AtPDF2.2* is targeted by *V. longisporum* to establish the infection. Secondly, *AtPDF2.2* probably prompts the JA response upon suppression. And, lastly, it displays an apparent antifungal activity against *V. longisporum* infection and could provide disease resistance against the necrotrophic fungal pathogen *S. sclerotiorum* and the biotrophic bacterium *P. syringae* as well. The exact mechanism of how *AtPDF2.2* influences these effects remains to be determined. A better understanding of each step will help elucidate its mode of action in detail.

Chapter III reports that the fungus *Verticillium longisporum* suppresses plant defensin *AtPDF2.2* to achieve its colonization on its host *Arabidopsis*. *Verticillium longisporum* at 6 dpi induced the expression of *AtPDF1.2a* in *Arabidopsis* wild-type Col-0 plants. Transcript study in the *AtPDF1.2a* overexpression (*OE-PDF1.2a*) and knockout (*KO-pdf1.2a*) plants revealed that, *AtPDF1.2a* expression positively correlates with Class I and III *AtPDF* genes, but negatively to *AtPDF2.2*, *2.3*, and *2.5*. Moreover, *OE-PDF1.2a* plants did not upregulate the expression of JA, ET or SA marker genes but *KO-pdf1.2a* plants showed induced expression of all the three pathways responsive genes. Possibly, low expression of *AtPDF1.2a* in *KO-pdf1.2a* plants during the ongoing infection works as a concentration-specific signal. Since *OE-PDF1.2a* plants did not show significant changes in the expression of upstream JA, ET and SA responsive genes it might rather be that upon *V. longisporum* attack *AtPDF1.2a* positively co-regulates

several *AtPDF* genes from Class-I and III, while suppressing the expression of *AtPDF2.2*, *2.3* and *2.5* to mediate the plant defense response.

*AtPDF1.2* has been shown to have antifungal activity but its precise role in plant defense remains unclear. To study the antifungal activity of *AtPDF1.2a*, overexpression and *Arabidopsis mutant* plants were utilized, which displayed improved resistance to both fungal pathogens but not to the biotrophic bacterial pathogen. In conclusion, *AtPDF1.2a* expression appears to only an antifungal effect on *V. longisporum* and *S. sclerotiorum*. By a detailed characterization of the biological functions of PDFs, we will gain a deeper insight into their modes-of-action. This will help to better understand the plant-pathogen interaction and provide an excellent basis for new breeding approaches.

Taken together, this thesis demonstrates that plant PDFs play a vital role in the *Arabidopsis-Verticillium* interaction. Both, *AtPDF1.2a* and *PDF2.2* show antifungal activity against *V. longisporum* and *S. sclerotiorum* infections, but *AtPDF2.2* was also able to enhance resistance to *P. syringae*. Importantly, *AtPDF2.2* appears to be targeted by *V. longisporum* to successfully infect the plant. PDFs have already been successfully used in the development of transgenic plants resistant to fungal attacks. Hence, these *AtPDF* genes with their broad activity against biotrophic, hemibiotrophic, and necrotrophic pathogens are ideal candidates to breed resistant plants.

So far, transgenic plants have been successfully applied in plant disease resistance programs. Future studies are required to characterize or overexpress PDFs or other PR proteins as well in different models and crop plants against different pathogens, which may lead to a breakthrough in disease development. Another future challenge is that global climate change will possibly increase the emergence of virulent strains of phytopathogens with broad host ranges. Hence, there is a need to functionally characterize as well as identify novel plant defensin genes/alleles to cope with such drastic challenges. In this regard, advances in “omics” approaches *viz.*, genomics, transcriptomics, phenomics, proteomics, or metabolomics will greatly help us to understand the detailed network of plant defense reactions, including the interaction of plant defensins with other proteins belonging to both plants and pathogen. These studies will provide us new insight to develop genetic stocks of plant defensins that can be



effectively used in engineered crop plants to counter the epidemic diseases or multiple stresses.

## Zusammenfassung

Das hemi-biotrophe pilzliche Pathogen *Verticillium longisporum* infiziert Raps (*Brassica napus*) und stellt eine reale Bedrohung für dessen Kultivierung dar. Nach Eurostat (2019) war Raps 2017 die wichtigste Ölf Frucht in Deutschland mit 1,3 Mio. ha Anbaufläche. Die intensive Kultivierung mit zu geringer Fruchtfolge und fehlender Bodenbearbeitung verstärkt das Problem durch Anreicherung bodenbürtiger Pathogene. Obwohl es durch Untersuchungen an der Modellpflanze Arabidopsis bereits Fortschritte im Verständnis der Pflanzen-Verticillium Interaktion gibt muss weiterhin geforscht werden, um die aktuelle landwirtschaftliche Nachfrage hinsichtlich fortschrittlicher Strategien in der Resistenzzüchtung zu bedienen. Konventionelle Ansätze sind limitiert und basieren in erster Linie auf der Verfügbarkeit eines breiten Genpools, um geeignete Genotypen für die Züchtung zu identifizieren um so eine verbesserte Resistenz, z.B. gegen abiotischen Stress oder Pathogene zu erzielen. Um dieses Ziel auch in Kulturpflanzen mit sehr engem Genpool wie beim Raps zu erreichen, müssen neue Strategien entwickelt werden. Ein Beispiel stellt die Expression pflanzlicher Defensine (PDFs), die für ihre anti-fungale Wirkung bekannt sind. In unserem Labor wurden im Vorfeld Kompatibilitätsfaktoren (KF) in Arabidopsis identifiziert, deren Verlust zu einer gesteigerten Ethylen-Antwort und Expression von *AtPDF2.2* führte. Es gibt bereits zahlreiche Beispiele, wie die Überexpression solcher PDFs in Modell- und Kulturpflanzen zu einer verbesserten und anhaltenden Resistenz führte. In dieser Arbeit wird in zwei Kapiteln gezeigt, wie die Charakterisierung und Funktionale Analyse der Rolle antifungaler AtPDFs zur Regulierung der Arabidopsis-Verticillium Interaktion beitragen kann.

Kapitel I beschreibt, dass durch GUS Expression unter dem *AtPDF2.2* Promoter konnte gezeigt werden, dass dieses Gen 6 Tage nach Infektion (dpi) in Arabidopsis Wurzeln unterdrückt wurde und später auch in Blättern (9 dpi). Diese wurde durch eine Transkript-Analyse bestätigt, welche eine starke Unterdrückung der *AtPDF2.2* Expression und zweier weiterer Arabidopsis-Gene der PDF Klasse II zeigte. Transgene Linien zur *AtPDF2.2* Überexpression (*OE-PDF2.2*) und zum Knock-Down (*KD-pdf2.2*) zeigten auch eine erhöhte Expression von *AtPDF2.3* und *AtPDF2.5* in der Überexpressionslinie, während eine entsprechende Unterdrückung in der Knock-Down Linie zu verzeichnen war. Eine weitere wichtige Beobachtung in der *KD-pdf2.2* Linie war eine verstärkte

Jasmonsäure (JA) Antwort, begleitet von einer Hochregulation Ethylen (ET) und Salizylsäure (SA)-abhängiger Gene, ein Effekt der auch in anderen Infektionen mit hemibiotrophen Pathogenen beobachtet wurde. Daher kann auf Basis der verfügbaren Daten angenommen werden, dass *V. longisporum* während der frühen Phase der Infektion Kompatibilitätsfaktoren des Wirts nutzt, um *AtPDF2.2* zu unterdrücken, um die pflanzliche Abwehr zu umgehen. Damit wird auch die Expression der nahe verwandten Gene *AtPDF2.3* und *2.5* unterdrückt, was es dem Pilz ermöglicht einen erfolgreichen Infektionsprozess zu etablieren. Allerdings geht die Unterdrückung dieser drei PDFs mit einer aktivierten JA-Antwort einher und im Nachhinein auch der ET und SA Signalwege um die Abwehrreaktion herbeizuführen. Um die antifungale Wirkung von *AtPDF2.2* zu untersuchen wurde die *OE-PDF2.2* Linie analysiert, welche eine erhöhte Resistenz gegenüber zwei pilzlichen (*V. longisporum* und *Sclerotinia sclerotiorum*) und einem bakteriellen Pathogen (*Pseudomonas syringae*) zeigte. Im Gegensatz dazu zeigte die *KD-pdf2.2* Linie eine höhere Anfälligkeit gegenüber diesen pilzlichen und bakteriellen Pathogenen. Damit lassen sich drei wichtige Schlussfolgerungen auf Grundlage dieser Studie ziehen: erstens wird *AtPDF2.2* gezielt durch *V. longisporum* unterdrückt, um die Infektion zu ermöglichen. Zweitens löst die Unterdrückung von *AtPDF2.2* eine JA-Antwort aus. Und drittens hat *PDF2.2* eine antifungale Wirkung gegen *V. longisporum* und vermittelt ebenfalls eine Resistenz gegen den nekrotrophen Pilz *S. sclerotiorum* und das bakterielle biotrophe Pathogen *P. syringae*. Der genaue Mechanismus wie *AtPDF2.2* diese Effekte beeinflusst muss allerdings noch genauer untersucht werden. Die einzelnen Schritte zu verstehen wird es dann ermöglichen ein genaueres Modell der *Verticillium* Infektion zu erstellen.

Kapitel II zeigt, dass *Verticillium longisporum* induziert im Arabidopsis Wildtyp Col-0 die Expression von *AtPDF1.2a* zum Zeitpunkt 6 dpi. Transkript-Studien mit *AtPDF1.2a* Überexpressions (*OE-PDF1.2a*) und Knock-Out (*KO-pdf1.2a*) Linien zeigten, dass *AtPDF1.2a* expression positiv mit anderen PDFs der Klasse I und III korreliert, aber negativ mit *AtPDF2.2*, *2.3* und *2.5*. Die Überexpression von *AtPDF1.2a* hatte keinen Einfluss auf die Expression von JA, ET und SA Marker-Genen, während in *KO-pdf1.2a* Pflanzen eine erhöhte Expression solcher Gene verzeichnet wurde. Möglicherweise fungiert eine schwache Expression von *AtPDF1a* in *KO-pdf1.2a* Pflanzen während der Infektion als konzentrations-spezifisches Signal. Weil aber in *OE-PDF1.2a* Pflanzen keine

signifikante Änderung der Expression von JA, ET und SA regulierten Genen verzeichnet werden konnte, ist es wahrscheinlicher, dass unter *V. longisporum* Befall *AtPDF1.2a* mehrere *AtPDF* Gene der Klasse I und III positiv koreguliert, während es die Expression von *AtPDF2.2*, 2.3 und 2.5 unterdrückt, um so die pflanzliche Abwehrreaktion zu vermitteln.

Für *AtPDF1.2a* wurde bereits antifungale Aktivität nachgewiesen, aber seine genaue Rolle in der pflanzlichen Abwehr ist noch ungeklärt. Daher wurden OE-*PDF1.2a* Linien genutzt, welche eine erhöhte Resistenz gegenüber beiden pilzlichen, nicht aber dem biotrophen bakteriellen Pathogen zeigten. *AtPDF1.2a* Expression scheint also nur eine antifungale Wirkung auf *V. longisporum* und *Sclerotinia sclerotiorum* zu haben. Durch eine genauere Charakterisierung der biologischen Funktion der PDFs werden wir ein tieferes Verständnis ihrer Wirkweise erlangen. Dies wird helfen die Pflanze-Pathogen Interaktion besser zu verstehen und dieses Wissen in für neuartige Zuchtansätze zu nutzen. Zusammenfassend zeigt diese Arbeit wie wichtig pflanzliche PDFs in der Arabidopsis-*Verticillium* Interaktion sind. Beide, *AtPDF1.2a* und *AtPDF2.2* zeigen antifungale Wirkung gegen *V. longisporum* und *S. sclerotiorum*, aber *AtPDF2.2* erhöhte auch die Toleranz gegenüber *P. syringae*. Wichtig ist hervorzuheben, dass *V. longisporum* gezielt *AtPDF2.2* zu manipulieren scheint, um so die Pflanzen erfolgreich infizieren zu können. PDFs wurden bereits in der Entwicklung transgener Pflanzen mit Resistenz gegen pilzliche Pathogene erfolgreich eingesetzt. Daher bieten sich diese *AtPDF* Gene mit ihrer breiten Wirkung gegen biotrophe, hemi-biotrophe und nekrotrophe Erreger als geeignete Kandidaten an, um resistenterere Pflanzen zu züchten.

## Material and Methods

This chapter includes detailed protocols for the main procedures used in this study. Solutions and reagents used in the stated protocols are mentioned within each procedure. Whereas the organisms, vectors, software, machines and chemicals consumed are listed in form of Tables at the end.

### 1 gDNA isolation

Total genomic DNA was isolated by CTAB (cetyltrimethylammonium bromide) buffer (Rogers and Bendich 1985) (Tab. 1) supplemented with  $\beta$ -Mercaptoethanol.

- Tissues were sampled in a 2ml eppi and directly grounded by a cold plastic stick grinder using liquid nitrogen.
- 1 ml prewarmed 2x CTAB (premixed with 3  $\mu$ l  $\beta$ -Mercaptoethanol per tube) was added to 100 mg plant material and incubated at 65°C for 30 min.
- 500  $\mu$ l Chloroform / Isoamylalcohol (24:1) was added to each sample and incubated on an overhead shaker at RT for 5 min.
- Centrifugation was performed for 15 min. at 14.000 rpm at RT.
- The upper phase (~750  $\mu$ l) was transferred into a new 1.5 ml tube, an equal amount of isopropanol was added, and tubes were incubated at 4°C for 30 min.
- Centrifugation was performed for 15 min. at 14.000 rpm at 4°C to obtain gDNA pellet.
- Pellets were subsequently washed with 1 ml of DNA washing solution I, 500  $\mu$ l DNA washing solution II (Table II), resuspended in 50-200  $\mu$ l TE-Buffer and samples were stored at 4°C.
- 1:10 diluted samples (2  $\mu$ l of the sample in the dilution) were run in a 1% agarose gel at 90V for 30 min. To determine the concentration of the isolated DNA,  $\lambda$ DNA with the varying concentration of (10ng, 20ng, 30 ng) was also loaded on the same gel. Image Lab software (Bio-Rad Laboratories GmbH) was used for gel visualization.

**Table 1:** Solutions used in gDNA isolation

Solution	Composition
2x CTAB	Tris/HCL (pH 7.5) 200 mM EDTA (pH 8.0) 20 mM NaCl 1.4 M
Washing Solution I	CTAB 2% (w/v) 76% EtOH (v/v) 0.2 M NaAc
Washing Solution II	76% EtOH (v/v) 10 mM NH <sub>4</sub> Ac
TE-Buffer	10 mM Tris/HCL (pH 8.0)

## 2 Plasmid isolation by alkaline lysis

Plasmid isolation from bacterial cells is performed excessively in the cloning experiments, alkaline lysis protocol was used for this purpose (modified from Birnboim and Doly (1979)). All the solutions used in this protocol are stated in Tab. 2. Bacterial strains used for plasmid isolation are usually grown in the appropriate sterile liquid culture overnight before performing the protocol.

- 2 ml of bacterial cultures were transferred to the 2ml eppis, samples were centrifuged for 5 min. at 14.000 rpm at RT; the supernatant was discarded.
- Bacterial pellets were resuspended in 200 µl Buffer I which was premixed with 1 µl RNaseA Sigma-Aldrich, article nr: R500-250MG) (10 mg / ml) and 1 µl Lysozym (Carl Roth GmbH + Co. KG, article nr: 8259.1) (10 mg / ml) per tube, mixed thoroughly on a vortex mixer and incubated for 5 min at RT.
- 250 µl of lysis solution (Buffer II) was added and mixed carefully by gently inverting 2-3 times and kept on ice for more than 5 min.
- 250 µl of chilled Buffer III was added, mixed by inverting and incubated on ice for 10 min.
- Tubes were centrifuged for 15 min. at 14.000 rpm, 4°C and the supernatant (~600 µl) was transferred into a new 1.5 ml tube, an equal amount of cold isopropanol was added. Solution was mixed by invert mixing and tubes were placed at 4°C for 30 min for precipitation of the plasmid DNA.
- Tubes were centrifuged for 20 min. at 14.000 rpm at 4°C to obtain the pellet of plasmid DNA.

- Each pellet was washed with 1 ml 70% ethanol for 5 min., re-suspended in 50-100  $\mu$ l TE-Buffer and samples were stored at 4°C for short term storage and for long term storage at -20°C.
- 1:10 diluted samples (2  $\mu$ l of the sample in the dilution) were run in a 1% agarose gel at 90V for 30 min. To determine the concentration of the isolated plasmid DNA,  $\lambda$ -DNA with the varying concentration of (10ng, 20ng, 30 ng) was also loaded on the same gel. Image Lab software (Bio-Rad Laboratories GmbH) was used for gel visualization.

**Table 2:** Solutions used in plasmid DNA isolation

Solution	Composition
Plasmid isolation Buffer I	25mM Tris/HCL (pH 8.0) 10mM EDTA pH 8.0 (adjust with HCL)
Plasmid isolation Buffer II	200mM NaOH 1% SDS (w/v)
Plasmid isolation Buffer III	3M Potassium acetate pH 5.5 (adjust with glacial acetic acid)

### 3 Vector preparation via gateway cloning for *AtPDF2.2*, *AtPDF1.2* and *Prom::AtPDF2.2::GUS* overexpressing, knockdown and GUS

The overexpressing (OE), (KD) and GUS constructs of *AtPDFs* (*AtPDF2.2* and *AtPDF1.2*) were prepared by GATEWAY cloning using the Gateway™ (Thermo Fisher Scientific) Invitrogen cloning kit. The media consumed in this protocol are listed in Tab. 3.

- Bacterial strain (*DB3.1*) containing the vectors used in gateway cloning (pDONR, pGWB series vectors, all listed in Table 11) were first isolated from the overnight stock cultures by alkaline lysis that is already been described above
- Arabidopsis gDNA was used to amplify the desired inserts for cloning (all the primers are listed under the separate chapter's supplementary material list) using Phusion DNA polymerase (Thermo Fisher Scientific).
- In a 1.5 ml microcentrifuge tube, BP recombination reaction was performed. An *attB*-flanked DNA fragment and an *attP*-containing donor vector (pDONR201) were used to generate an entry clone. 1-10  $\mu$ l *attB*-PCR product of 40-100 fmol concentration was mixed with 2  $\mu$ l of the pDONR™ 201 vector (supercoiled, 150 ng/ $\mu$ l). BP Clonase™ enzyme was mixed briefly then 4  $\mu$ l was added to the tube and mixed well by vortexing briefly twice. TE buffer (pH: 8.0) was used to increase the volume upto 16  $\mu$ l.
- Reaction was incubated at 25°C for 1 hour.

- 2 µl of 2 µg/µl Proteinase K solution was added to the mix and incubated at 37°C for 10 minutes to terminate the reaction.
- Competent *E. coli DH5α* were transformed by heat-shock method and selected for kanamycin resistant entry clones.
- Alkaline lysis method was used to isolate the plasmids from the positive clones which were further confirmed by PCR using vector specific primers
- In a 1.5 ml microcentrifuge tube, LR recombination reaction was performed. 1-10 µl of the entry clone having 100-300ng concentration was mixed with 2 µl of the desired destination vectors. LR Clonase™ enzyme was mixed briefly then 4 µl was added to the tube and mixed well by vortexing briefly twice. TE buffer (pH: 8.0) was used to increase the volume up to 16 µl.
- Reaction was incubated at 25°C for 1 hour.
- 2 µl of 2 µg/µl Proteinase K solution was added to the mix and incubated at 37°C for 10 minutes to terminate the reaction.
- Competent *E. coli DH5α* were transformed by heat-shock method and select for kanamycin and spectinomycin resistant destination clones.
- Alkaline lysis method was used to isolate the plasmids from the positive clones which were further confirmed by PCR using vector specific primers.
- The destination vector containing the desired fragment was then used to transform *A. tumefaciens* (GV3101).
- The positive clones were used to transform Arabidopsis (Col-0) plants by floral dip, according to Clough and Bent (1998).



**Table 3:** Media used in cloning experiments

Media	Composition
LB medium (for <i>E.coli</i> )	Tryptone/Peptone 10 g/l Yeast Extract 5 g/l NaCl 10 g/l pH 7 Bacto Agar 15 g/l (for solid medium) Kanamycin 50 mg/l (pAM194 and pDONR201) Spectinomycin 100mg/l (various pGWB destination vectors)
2YT medium (for <i>A. tumefaciens</i> )	Tryptone/Peptone 16 g/l Yeast Extract 10 g/l NaCl 5 g/l pH 7 (adjust with NaOH) Bacto Agar 15 g/l (for solid medium) Kanamycin 50 mg/l (pAM194) Rifampicin 100 mg/l ( <i>A. tumefaciens</i> GV3101) Gentamycin 10 mg/l (Ti-Plasmid GV3101, pGWB plasmids)

#### 4 Isolation of total RNA

Total RNA was isolated using TRIzol®.

- Tissue were sampled in a 2ml eppi and directly grounded by a cold plastic stick grinder using liquid nitrogen. In 2 ml tubes 1 ml TRIzol® reagent was added to 100 mg plant material, vortexed and incubated for at RT 5 min.
- 200µl Chloroform was added to each sample, vortexed and incubated on ice for 3 min.
- Tubes were centrifuged for 15 min. at 14.000 rpm at 4°C.
- The upper phase (~500 µl) was transferred into a new 1.5 ml tube, an equal amount of chilled isopropanol was added, and tubes were incubated at -20°C over-night.
- Tubes were centrifuged for 20 min at 14.000 rpm at 4°C to obtain RNA pellet.
- Each pellet was washed twice with 1 ml 75% (DEPC)-ethanol for 3-5 min., resuspended in 20-100 µl (DEPC)-ddH<sub>2</sub>O and samples were stored at -20°C.

RNA quality was checked by running the 1:10 dilution of the isolated RNA sample in a 1.3% MOPS-buffer agarose gel at 110V for 30 min. RNA concentrations were determined using the NanoVue Plus Spectrophotometer (GE Healthcare Life Science).

#### 5 Expression analysis of AtPDFs transgenic lines

Expression of *AtPDF2.2* and *AtPDF1.2a* transgenic plants were analysed PCR amplification. Gene-specific primer pairs were utilised for it.

## 6 Reverse transcription (cDNA synthesis)

The reverse transcription was performed with 1000 ng total RNA and 1 µl primer mixture (10 µM each), according to the manufacturer's instructions (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific). Gene transcripts were reverse transcribed using oligo-dT primers. The cDNA quality was checked by PCR amplification of the *ACTIN2* transcript and ran on 0.8% agarose gel electrophoresis.

## 7 Realtime-qPCR

SYBR-Green from (Maxima SYBR Green Kit, Thermo Fisher Scientific) (Tab. 4) was employed for the RT-qPCR experiments according to the manufacturer's instructions. Specific annealing temperatures and extension time was used depending on the primer pairs. Dilutions of the cDNA were used for the qPCR experiments. 1 µl of 1:10 cDNA dilutions for the *AtPDF* gene transcripts were used. For amplification of the genes, genes specific primers were used. In order to determine the primer efficiency, standard curve was generated by cDNA dilutions (1:10; 1:100; 1:1000). The relative transcript levels were calculated by Pfaffl (2001) method. Relative expression values are transformed to  $\log_2$  and the induction between 2 groups was measured. For the normalization of the gene expression, housekeeping gene *ACTIN-2* (AT3G18780) was employed.

**Table 4:** Kits used in cDNA synthesis and qPCR

Kits	Article number	Company
Maxima SYBR Green/ROX qPCR Master Mix	K0222	Thermo Fisher Scientific
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Fisher Scientific

## 8 Cultivation of *V. longisporum* for infection purpose

All the procedures were conducted under sterile conditions. Media used in the whole infection assay is listed in Tab. 5.

- Stock tubes of *V. longisporum* conidia were thawed and centrifuged at 6000g at room temperature for 8 min.; supernatant was discarded, and conidia were resuspended in 300 µl CDB.
- 150 µl conidia solution was spread on one PDA plate and subsequently incubated in complete darkness at RT for 14 days.

- Eight slices were cut from the PDA agar covered with the fungus growth and two slices were used to inoculate 200 ml CDB in one sterile flask. Liquid cultures were incubated on a rotary shaker in complete darkness at 120 rpm and RT for 3 days.
- In order to increase the concentration of the conidia stock, 200ml of inoculated CDB liquid cultures were filtered through sterile gaze (200 µm), centrifuged (6000g; RT; 8 min.) and resuspended in one-fourth of the original volume, sterile glycerol was also added at a 22% concentration of the final volume. These stocks were stored at -80°C and used for infection when needed.
- On the day of infection *Verticillium* stocks were thawed, centrifuged (6000g; RT; 8 min.) and resuspended in either sterile CDB or tap water for in vitro infections or for in vivo infections respectively.
- The concentration of conidia was determined by a Fuchs-Rosenthal chamber counting where 1:10 diluted conidia was applied, and the conidia were counted in 5 small squares of 4 big squares, respectively. The concentration was calculated by the following formula:

$$\frac{\text{Mean of conidia per small square} \times \text{dilution factor}}{0.00125 \text{ mm}^3 \text{ per small square}} \times 1000 = \text{conidia/ml}$$

- Concentrations were adjusted to 1-5 x10<sup>6</sup> conidia / ml either by sterile CDB or tap water depending on the infection assay.

### **9 *V. longisporum* in-vivo infection**

In order to perform an infection on soil, *A. thaliana* seeds were surface sterilized and seeds were grown in petri dishes containing ½ MS medium for 14 days in short day conditions (8h light) at 22°C. After that plants were removed from dishes, either root dipped for 15 min. in *V. longisporum* conidia (2x 10<sup>6</sup> / ml) and subsequently transferred to pots containing sand and soil (1:1), or directly transferred to pots and subsequently drenched with 5 ml of conidia solution (5x 10<sup>6</sup> / ml). Plants were regularly watered, and disease progression was monitored for up to a month.

## 10 *V. longisporum in-vitro* infection

For the qPCR experiments or for expression analysis study using the infected roots of either *A. thaliana* or *B. napus*, infection was carried out in a controlled environment (in-vitro infection on ½ MS plate). Sterilized seeds for *B. napus* was pre-grown in a plastic container for 7-10 days containing about 60ml of sterile ½ MS media and sterilized *A. thaliana* seed were grown in a petri dish containing ½ MS medium for 14 days in short day conditions (8h light) at 22°C. For the vertical root growth of *A. thaliana* and *B. napus* seedlings, the plantlets were transferred to a square plate (120 mm), arranged vertically at the same height and *A. thaliana* plantlet were grown like this for 7 more days however *B. napus* roots were inoculated directly. The roots were inoculated by using a sterile paint brush dipped in *V. longisporum* conidia ( $2 \times 10^6$  / ml). The inoculated root area was covered by aluminum foil as fungus is known to propagate in darkness and plates were continuously grown in a vertical position in short day (8h light) at 22°C. Root tissues were cut and sampled separately at 6 dpi.

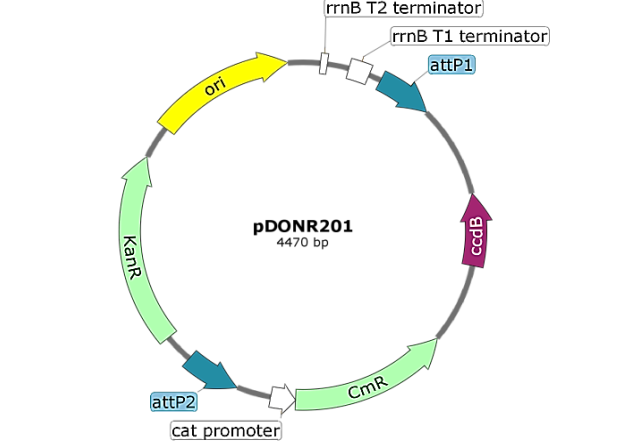
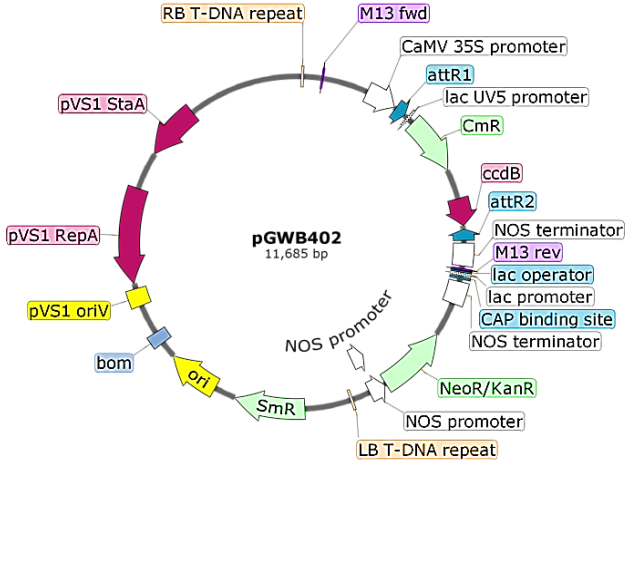
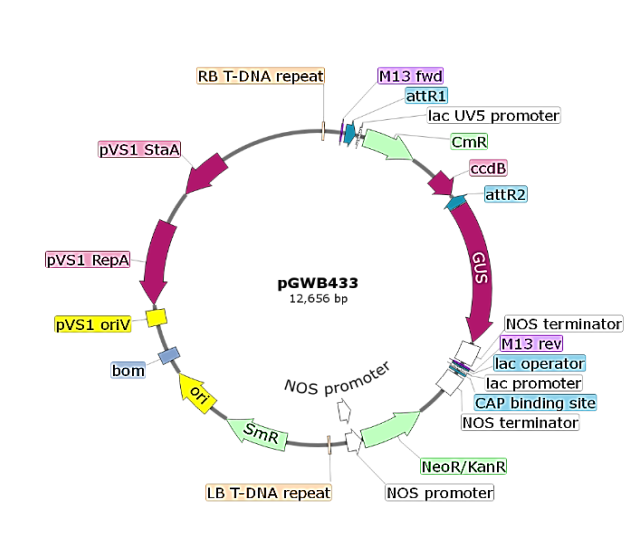
**Table 5:** Media used in the infection assay

Solution	Composition
PDA	1x PDB pH 5,6 Agar-Agar 15 g/l
Czapek Dox	1x CDB
½ MS medium	0,5x MS salts incl. Vitamins and MES Sucrose 5 g/l pH 5,8 (adjust with KOH) Daishin Agar 8 g/l

**Table 6:** Organisms used in this study

Organism	Genotype / Line / Isolate	Source
<i>Escherichia coli</i>	DH5α/DB3.1	lab stock
<i>Agrobacterium tumefaciens</i>	GV3101	lab stock
<i>Verticillium longisporum</i>	VI43	Dr.E.Diederichsen (FU Berlin, Germany)
<i>A. thaliana</i>	Col-0	Lehle Seeds (Round Rock, USA)
	OE-PDF2.2 #1, #7, #8, #12 (in Col-0)	self-provided
	<i>KD-pdf2.2</i> #1, #4, #7, #8 (in Col-0)	self-provided
	Prom:: <i>AtPDF2.2</i> ::GUS #13, #21, #22 (in Col-0)	self-provided
	OE-PDF1.2a #1, #2, #3, #7 (in Col-0)	self-provided
	<i>KD-pdf1.2a</i> #3, #4, #6 (in Col 0) (SALK_063966.49.80.x)	Nottingham Arabidopsis Stock Centre
	<i>coi1-16</i> (in Col 0) (N67817)	Nottingham Arabidopsis Stock Centre
	<i>acs1-7</i> (in Col 0) (N16650)	Nottingham Arabidopsis Stock Centre
<i>sid2</i> (in Col 0) (SALK_088254)	Nottingham Arabidopsis Stock Centre	
<i>jar1</i> (in Col 0) (SALK_059774C)	Nottingham Arabidopsis Stock Centre	
<i>ein2-1</i> (in Col 0) (SALK_086500C)	Nottingham Arabidopsis Stock Centre	
<i>ein2-1; coi1-16</i> (in Col 0) (N67818)	Nottingham Arabidopsis Stock Centre	

**Table 7:** Vectors used in this study.

Vector name and map	Features
 <p><b>pDONR201</b> 4470 bp</p>	<p><b>Use in the present work:</b> Common entry plasmid (BP clones) in all the cloning experiment performed by gateway cloning.</p> <p><b>Features:</b></p> <ul style="list-style-type: none"> <li>– attP1-P2 sequences (BP recombination)</li> <li>– ccdB: toxin</li> <li>– CmR: Chloramphenicol-resistance gene</li> <li>– KanR: Kanamycin-resistance gene</li> <li>– ori: origin of replication</li> <li>– rrnB T1 and T2 terminators</li> </ul>
 <p><b>pGWB402</b> 11,685 bp</p>	<p><b>Use in the present work:</b> Binary vector used in Cloning of all the genes fused with the constitutive 35s promoter by gateway cloning (LR).</p> <p><b>Features:</b></p> <ul style="list-style-type: none"> <li>– attR1-R2 sequences (LR recombination)</li> <li>– ccdB: toxin</li> <li>– CmR: Chloramphenicol-resistance gene</li> <li>– CaMV 35S and lac promoters</li> <li>– pUC/M13 forward and reverse primer binding sites</li> <li>– KanR: Kanamycin-resistance gene</li> <li>– SmR: Spectinomycin-resistance gene</li> <li>– pVS1 and ori: origins of replication</li> <li>– RB and LB T-DNA repeats, pVS1 Rep A and pVS1 StaA (Plant transformation)</li> </ul>
 <p><b>pGWB433</b> 12,656 bp</p>	<p><b>Use in the present work:</b> Binary vector used in Cloning of all the promoters fused with the GUS reporter gene.</p> <p><b>Features:</b></p> <ul style="list-style-type: none"> <li>– attR1-R2 sequences (LR recombination)</li> <li>– ccdB: toxin</li> <li>– CmR: Chloramphenicol-resistance gene</li> <li>– Lac UV 5 promoter</li> <li>– GUS reporter gene</li> <li>– pUC/M13 forward and reverse primer binding sites</li> <li>– KanR: Kanamycin-resistance gene</li> <li>– SmR: Spectinomycin-resistance gene</li> <li>– pVS1 and ori: origins of replication</li> <li>– RB and LB T-DNA repeats, pVS1 Rep A and pVS1 StaA (Plant transformation)</li> </ul>

**Table 8:** Softwares used in this study

Software / Database	Source / Company
AxioVision rel. 4.8	Carl Zeiss Microscopy GmbH
CFX Maestro™ Software for CFX Real-Time PCR Instruments	Bio-Rad Laboratories GmbH
Clustal Omega	<a href="https://www.ebi.ac.uk/Tools/msa/clustalo/">https://www.ebi.ac.uk/Tools/msa/clustalo/</a>
Image J Software	<a href="https://fiji.sc/">https://fiji.sc/</a>
Image Lab™ Software	Bio-Rad Laboratories GmbH
MEGA6	<a href="https://www.megasoftware.net/">https://www.megasoftware.net/</a>
Microsoft® Office	Microsoft Corporation
NCBI - BLASTn / Primer-BLAST / CDSEARCH	<a href="https://blast.ncbi.nlm.nih.gov/Blast.cgi">https://blast.ncbi.nlm.nih.gov/Blast.cgi</a>
NCBI - Open Reading Frame Finder	<a href="https://www.ncbi.nlm.nih.gov/orffinder/">https://www.ncbi.nlm.nih.gov/orffinder/</a>
PlantCARE	<a href="http://bioinformatics.psb.ugent.be/webtools/plantcare/html/">http://bioinformatics.psb.ugent.be/webtools/plantcare/html/</a>
SnapGene software	<a href="https://www.snapgene.com/">https://www.snapgene.com/</a>
SIGNAL T-DNA Verification Primer Design	<a href="http://signal.salk.edu/tdnaprimers.2.html">http://signal.salk.edu/tdnaprimers.2.html</a>
The Arabidopsis Information Resource (TAIR)	<a href="http://www.Arabidopsis.org/">http://www.Arabidopsis.org/</a>
Unipro UGENE: a unified bioinformatics toolkit	<a href="http://ugene.net/">http://ugene.net/</a>

**Table 9:** Machines and devices used in this study.

Machine	Model	Company
Autocalve	VX-75	Systec GmbH
Camera	D3000	Nikon GmbH
Centrifuge	5417 R	Eppendorf AG
Centrifuge	Heraeus Multifuge X3R	Thermo Fisher Scientific
Climate chamber	VB0714	Vötsch Industrietechnik GmbH
Electroporation Systems	Gene Pulser Xcell™	Bio-Rad Laboratories GmbH
ELISA reader	Model 680	Bio-Rad Laboratories GmbH
Freezer -80°C	HERAfreeze Basic	Thermo Fisher Scientific
Gel documentation	Gel Doc™ XR+	Bio-Rad Laboratories GmbH
Gel electrophoresis, chamber	Wide Mini-Sub® Cell GT Bio-Rad Laboratories GmbH	Bio-Rad Laboratories GmbH
Incubator	Excellent UFE 400-800	Memmert GmbH + Co. KG
Incubator, shaking	CERTOMAT® IS	Sartorius Stedim Biotech GmbH
Magnetic stirrer	Combimag REO	IKA® Works, Inc.
Microscope	Stereo Discovery.V20	Carl Zeiss Microscopy GmbH
Microscope	TCS SP1	Leica Biosystems Nussloch GmbH
Microwave	hNN-E235M	Panasonic
PCR thermocycler	PCR Biometra TOne 96	Analytik Jena AG
pH meter	inoLab pH 720	WTW GmbH
Pipettes	2,5 µl, 20 µl, 200 µl, 1000 Reserch/Research plus	Eppendorf AG
Realtime PCR System	CFX96 Touch™ Real-Time PCR Detection System	Bio-Rad Laboratories GmbH
Scale	ABJ	KERN & SOHN GmbH
Shaker	Vibramax 100	Heidolph Instruments GmbH & Co.KG
Shaker, overhead	Reax 2	Heidolph Instruments GmbH & Co.KG
Spectrophotometer	NanoVue Plus	GE Healthcare Life Science
Thermomixer	TSC ThermoShaker	Analytik Jena AG
Vortex	MS 2	IKA®-Werke GmbH & CO. KG
Water bath	Immersion Circulators Model 1112A	VWR International GmbH
Water bath, shaking	1083	GFL Gesellschaft für Labortechnik mbH
Workbench, sterile	HERAsafe™ KS 12	Thermo Fisher Scientific

**Table 10:** Chemicals used in this study

Chemical	Article number	Company
Agarose	840004	Biozym Scientific GmbH
Agar-Agar	5210.4	Carl Roth GmbH + Co. KG
Ammonium acetate	7869.2	Carl Roth GmbH + Co. KG
Ampicillin	K029.4	Carl Roth GmbH + Co. KG
Bacto-Agar	214010	OTTO NORDWALD GmbH
Boric acid	6943.1	Carl Roth GmbH + Co. KG
Chloroform	3313.2	Carl Roth GmbH + Co. KG
CTAB (cetyltrimethylammonium bromide)	9161.2	Carl Roth GmbH + Co. KG
Czapek Dox Broth	C1714.1000	Duchefa Biochemie B.V
D(+)-Sucrose	4321.2	Carl Roth GmbH + Co. KG
Daishin Agar	D1004.1000	Duchefa Biochemie B.V
DEPC (diethyl pyrocarbonate)	K028.1	Carl Roth GmbH + Co. KG
DMSO (dimethyl sulfoxide)	4720.2	Carl Roth GmbH + Co. KG
DNA Gel Loading Dye (6X)	R0611	Thermo Fisher Scientific
DNA oligonucleotides		Eurofins Genomics
dNTP Mix	R0181	Thermo Fisher Scientific
EDTA (ethylenediaminetetraacetic acid)	8043.2	Carl Roth GmbH + Co. KG
Ethanol	1.00983.2511	Diagonal GmbH & Co. KG
Ethidiumbromide	2218,2	Carl Roth GmbH + Co. KG
GeneRuler DNA Ladder 1kb	SM0311	Thermo Fisher Scientific
GeneRuler DNA Ladder 100bp	SM0242	Thermo Fisher Scientific
Gentamycin	M3121.0001	Genaxxon bioscience GmbH
Glycerol	4043.1	Carl Roth GmbH + Co. KG
HCl hydrochloric acid	P074.2	Carl Roth GmbH + Co. KG
HPLC water	A511.3	Carl Roth GmbH + Co. KG
Isoamylalcohol	8930.1	Carl Roth GmbH + Co. KG
Isopropanol	20842.330DB	VWR International GmbH
Kanamycin	T832.3	Carl Roth GmbH + Co. KG
Methanol	4627.5	Carl Roth GmbH + Co. KG
MgCl <sub>2</sub> magnesium chloride	kk36.2	Carl Roth GmbH + Co. KG
MgSO <sub>4</sub> magnesium sulfate	P027.3	Carl Roth GmbH + Co. KG
MOPS (3-(N- morpholino)propane sulfonic acid)	6979.3	Carl Roth GmbH + Co. KG
MS basal salts incl. Vitamins and MES	M0255.0050	Duchefa Biochemie B.V
PDB Potato-dextrose broth	CP74.2	Carl Roth GmbH + Co. KG
Phenol	0038.3	Carl Roth GmbH + Co. KG
Potassium acetate	T874.1	Carl Roth GmbH + Co. KG
KOH potassium hydroxide	6751.3	Carl Roth GmbH + Co. KG
Na <sub>2</sub> HPO <sub>4</sub> (disodium phosphate)	X987.2	Carl Roth GmbH + Co. KG
NaH <sub>2</sub> PO <sub>4</sub> (monosodium phosphate)	K300.2	Carl Roth GmbH + Co. KG
NaCl sodium chloride	3957.2	Carl Roth GmbH + Co. KG
NaOH sodium hydroxide	6771.2	Carl Roth GmbH + Co. KG
Rifampicin	R0146.0005	Duchefa Biochemie B.V
Sodium acetate	6773.1	Carl Roth GmbH + Co. KG
Sodium hypochloride	9062.4	Carl Roth GmbH + Co. KG
SDS (sodium dodecyl sulfate)	4360.2	Carl Roth GmbH + Co. KG
TEMED (tetramethylethylenediamine)	2367.3	Carl Roth GmbH + Co. KG
TRIS (tris(hydroxymethyl)aminomethane)	5429.3	Carl Roth GmbH + Co. KG
TRIS/HCL (tris(hydroxymethyl)aminomethane)/hydrochloric acid	9090.3	Carl Roth GmbH + Co. KG
TritonX-100	3051.3	Carl Roth GmbH + Co. KG
Tryptone/Peptone	8952.3	Carl Roth GmbH + Co. KG
TRIzol®	15596018	Thermo Fisher Scientific
Tween20	9127.1	Carl Roth GmbH + Co. KG
X-gal	2315.4	Carl Roth GmbH + Co. KG
X-glcA	X1405.1000	Duchefa Biochemie B.V
Yeast extract	A1552.1000	Diagonal GmbH & Co. KG



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