Study of the interaction between the model plant *Arabidopsis thaliana* and the pathogenic bacteria *Pseudomonas syringae*

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Résumé

Dans la nature les plantes sont soumises à de nombreuses attaques de l'environnement tel que stress biotiques (Insectes, microorganismes pathogènes…) et abiotiques (Sècheresse, froid…). Cependant les plantes disposent de mécanismes de défenses naturelles complexes. En agriculture, malgré les efforts des sélectionneurs pour obtenir des variétés de plantes cultivées résistantes, les pertes de récoltes dues aux agents pathogènes restent très importantes. Les agriculteurs protègent leurs cultures à l'aide de produits phytosanitaires non sans soulever de nombreux problèmes sanitaires et environnementaux. Certaines molécules, appelées éliciteurs permettent de stimuler les défenses des plantes tandis que les agents primant tel que le BABA (acide β-aminobutyrique) permettent aux plantes de se préparer à mieux se protégèrent en cas d'attaques. La caractérisation des mécanismes de défenses des plantes ainsi que des mécanismes de virulence des pathogènes représente un enjeu important afin d'aborder la protection des plantes sous un autre angle.

Le but de ce travail de thèse a été d'aborder la recherche en phytopathologie moléculaire et les interactions plantes/pathogènes. Dans un premier temps, des outils de biologie moléculaire basés sur les réactions de polymérase en chaine en temps réel ont été développés afin de mesurer chez la plante modèle *Arabidopsis thaliana* la croissance de plusieurs microorganismes pathogènes ainsi que de mesurer les réponses de défense des plantes. Dans un deuxième temps ces méthodes ont été mises à contribution afin de disséquer les interactions de signalisation hormonale s'établissant lors de l'infection d'Arabidopsis par la bactérie pathogène *Pseudomonas syringae*. Les résultats obtenus permettent de mieux comprendre comment les bactéries, par le biais de leurs mécanismes de virulence, réduisent la mise en place des défenses naturelles des plantes par un jeu complexe de signalisation hormonale. Cette stratégie de virulence bactérienne peut être contournée en préparant les plantes à mieux se défendre avec l'utilisation d'agents primant tel que le BABA qui permet de rétablir les défenses naturelles des plantes en cas d'attaques.

CONTENTS

Chapter 1

General introduction

General introduction

In their natural habitat as well as under culture conditions, plants are continuously exposed to biotic and abiotic stresses. Biotic stresses consist of numerous pathogens including bacteria, fungi, oomycetes, viruses, herbivorous insects and nematodes that have evolved specific parasitic relationships with the host plants according to their lifestyle. Biotrophic pathogens absorb nutrients from living host plants while necrotrophic pathogens kill the host cells in order to feed on the content (Glazebrook 2005). Hemibiotrophic pathogens use both lifestyles according to the infection stage. In addition plants are also exposed to varying environmental conditions such as cold, heat, drought and salt stresses (Mittler 2006; Tardieu and Tuberosa 2010). These threats can lead to serious damage in crop cultures, thus affecting yields (Strange and Scott 2005; Mittler 2006; Oerke 2006).

In nature, plant-pathogen interactions are often incompatible due to non-host resistance (Nurnberger and Lipka 2005; Jones and Dangl 2006). Non-host resistance results first from the host-specific constitutive physical defenses (cuticle, wax, pecto-cellulosic wall) and the range of preexisting antimicrobial compounds. In addition, inducible defenses are regulated by the innate immunity, characterized by the ability of plants to recognize host- or non-host pathogens and to respond by inducing a set of sophisticated defenses against the invader (Jones and Dangl 2006). These inducible defenses include re-enforcement of the cell wall achieved by callose and lignin synthesis and the production of antimicrobial secondary metabolites such as phytoalexins. In addition, plants produce a broad range of inducible peptides and pathogenesis-related proteins (PR-proteins) displaying various antimicrobial activities such as chitinases and glucanases (Van Loon and Van Strien 1999; van Loon *et al.* 2006b). Induction and regulation of plant defenses are controlled in part by a complex

network of interconnected endogenous hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) (Pieterse *et al.* 2009).

Although plant breeding has been able to select successfully some resistance traits, selection pressure often leads to adaptation of pathogens, some of which have evolved mechanisms to overcome plant defenses. Therefore, important yield losses occur in crops in part due to the increased susceptibility of monocultures to pathogenic microorganisms and attack by insects (Strange and Scott 2005; Oerke 2006). In order to reduce losses, large amounts of synthetic chemicals are almost systematically used to protect cultures. This leads to the rising problems of environmental pollution due to the toxicity of these products.

A major challenge of plant pathology has been to understand the mechanisms governing plant immunity against microbial pathogens. Over the past decades, knowledge about signals produced and exchanged during plant-pathogen interactions has rapidly progressed (Dangl and Jones 2001; Jones and Dangl 2006; Nishimura and Dangl 2010). The emergence of the biotechnologies has enabled to improve plant immunity (Gust *et al.* 2010). In addition to the plant immune system, induced disease resistance in the whole plant can be triggered by a primary localized infection (Ryals *et al.* 1996; Sticher *et al.* 1997). This has set the basis to study and develop unconventional protection strategies. For instance, the mechanism of priming can promote efficient disease resistance (Conrath *et al.* 2002; Prime-A-Plant Group 2006; Goellner and Conrath 2008). These novel approaches could revolutionize agriculture and offer exciting perspectives to exploit the plants' ability to express induced resistance (Beckers and Conrath 2007; Walters and Fountaine 2009; Walters 2010)

The plant immune system against microbial pathogens

Plant resistance against pathogens is governed by two distinct perception mechanisms (Jones and Dangl 2006). Both can be additive to mediate quantitatively the establishment of defense responses. Conversely, successful infections depend on the aptitude of pathogens to bypass the plant immune system and the triggered defenses. The ultimate outcome of the plant-pathogen battle depends on the opponents' abilities defined by their genetic features (Figure 1). Plants possess transmembrane pattern recognition receptors (PRRs) that recognize microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Jones and Dangl 2006; Nürnberger and Kemmerling 2009). These PAMPs such as flagellin, cold shock proteins, elongation factor Tu (EF-Tu), glycoproteins, chitin and lipopolysaccharides, and the related PRRs are well documented (Gomez-Gomez and Boller 2002; Schwessinger and Zipfel 2008; Nürnberger and Kemmerling 2009). The sensing of these molecules by plants results in the basal resistance called PAMP-triggered immunity (PTI). PTI is characterized by the rapid induction of defense responses such as transient activation of mitogen-activated protein kinase (MAPKs) and oxidative burst, callose deposition, and expression of a broad range of defense-related genes (Jones and Dangl 2006; Boller and He 2009; Nürnberger and Kemmerling 2009). In response, virulent pathogens secrete protein effectors that antagonize PTI (Mudgett 2005; Abramovitch *et al.* 2006; Grant *et al.* 2006; de Wit *et al.* 2009; Ellis *et al.* 2009; Guo *et al.* 2009; Stergiopoulos and de Wit 2009). This results in compatible interactions or effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Gohre and Robatzek 2008; Boller and He 2009). In turn, these effectors may be recognized directly or indirectly by specific plant resistance (*R*) genes, most coding for NB-LRR proteins (NUCLEOTIDE BINDING and LEUCINE RICH REAPEAT domains) (Dangl and Jones 2001). During gene for gene resistance (Flor 1971), the avirulent effector (Avr) secreted by a given pathogen is recognized directly or indirectly by the product of a specific *R* gene which triggers the effector-triggered

immunity (ETI) in addition to PTI (Chisholm *et al.* 2006; Jones and Dangl 2006). ETI is a faster and enhanced activation of PTI (Tao *et al.* 2003; Thilmony *et al.* 2006; Truman *et al.* 2006) and can be associated with a hypersensitive response (HR) characterized by the programmed cell death (PCD) surrounding the infection site (Dangl *et al.* 1996; Greenberg and Yao 2004). In addition, pathogens can acquire additional effectors that can suppress ETI, thus triggering ETS again (Tsiamis *et al.* 2000; Abramovitch, *et al.* 2006). Likewise, natural selection and co-evolution of plants with their pathogens can results into the acquisition of new NB-LRR receptors through multiple genetic processes such as *R*-genes duplication and polymorphism that can restore ETI (Van der Hoorn *et al.* 2002; Thrall and Burdon 2003; Bakker *et al.* 2006).

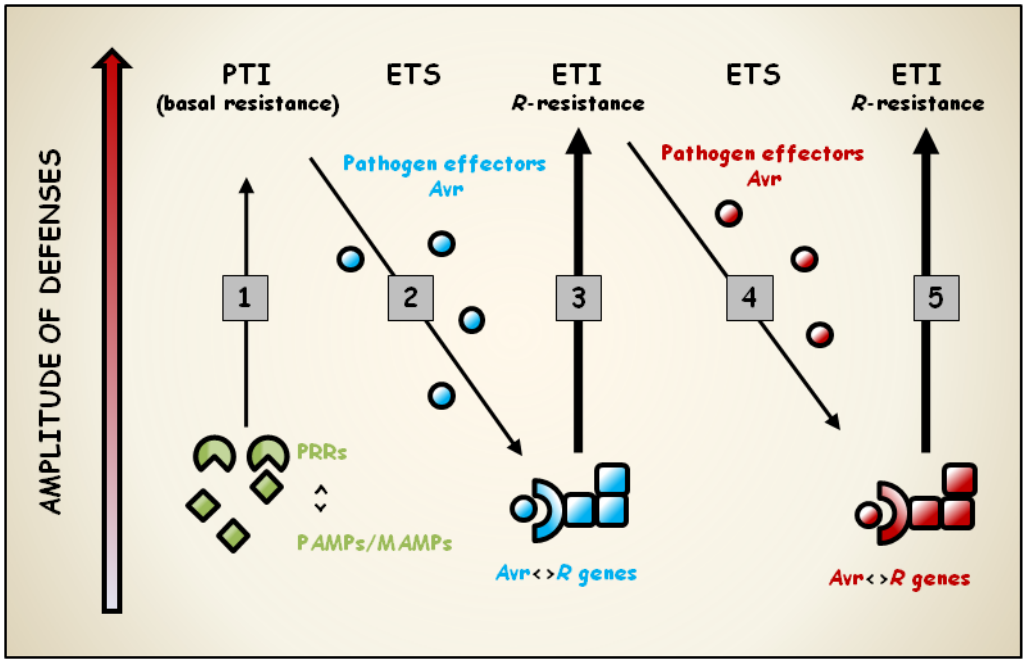

Figure 1. The plant immune system and the sequences leading to resistance or infection.

In phase 1 plants recognize microbial/pathogen-associated molecular patterns (MAMPs/PAMPs) through PRRs and induce PAMP-triggered immunity (PTI). In phase 2, pathogens can secrete effectors that repress PTI resulting in effector-triggered susceptibility (ETS). In phase 3, avirulent effectors (Avr) are recognized by the associated resistance (*R*) genes resulting in effector-triggered immunity (ETI), an enhanced version of PTI. In phase 4, pathogens can acquire further effectors that suppress ETI. In phase 5, plants can obtain new *R*-genes, resulting in ETI again. Adapted from Jones and Dangl, 2006.

The network of hormones regulating inducible plant defenses

Plant growth and development are regulated by the phytohormones auxins (AUX), cytokinins, gibberellins, ET and ABA. As well, activation of plant defenses resulting from PTI and ETI are controlled by a complex network of endogenous signaling hormones (Figure 2) (Bari and Jones 2009; Pieterse*, et al.* 2009). The most significant are SA (Loake and Grant 2007; Vlot *et al.* 2009), jasmonates (JAs) (Pozo *et al.* 2004), ET (van Loon *et al.* 2006a) and ABA (Mauch-Mani and Mauch 2005; Asselbergh *et al.* 2008; Ton *et al.* 2009). In addition, the role of AUX (Navarro *et al.* 2006; Chen *et al.* 2007; Wang *et al.* 2007; Zhang *et al.* 2007), gibberellins (Navarro *et al.* 2008), cytokinins (Siemens *et al.* 2006; Walters and McRoberts 2006) and brassinosteroids (Krishna 2003; Nakashita *et al.* 2003; Shan *et al.* 2008) has recently emerged in the regulation of the plant defense network (Bari and Jones 2009; Pieterse*, et al.* 2009).

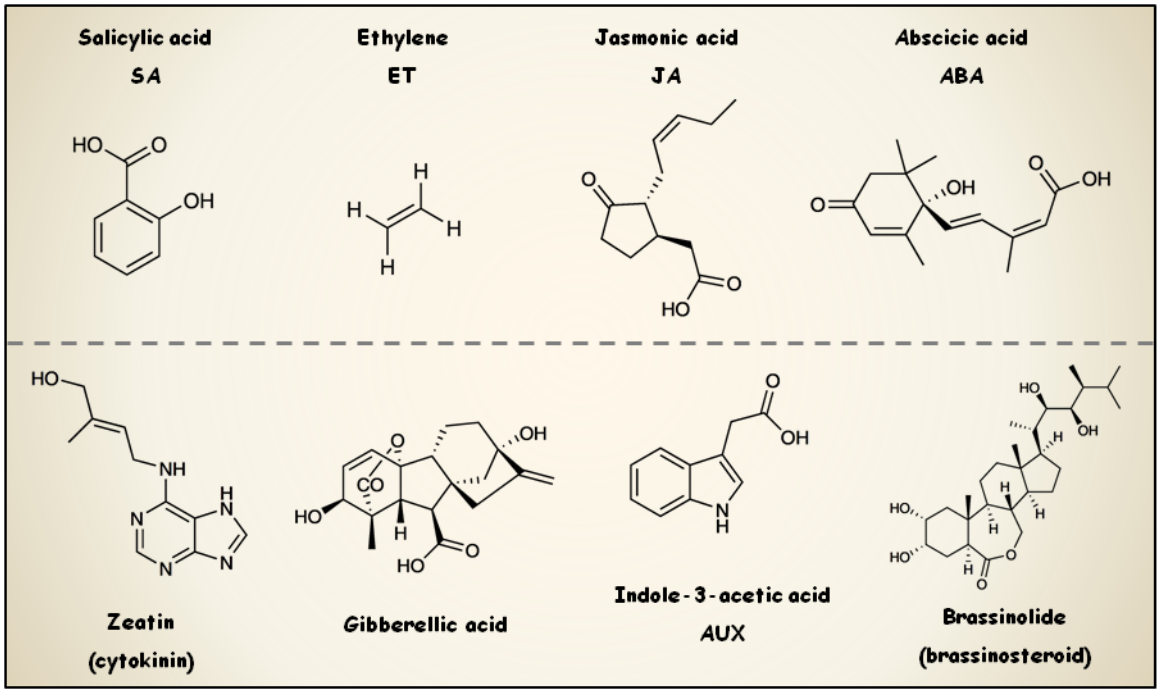

Figure 2. The major (top) and emergent (bottom) phytohormones regulating the plant defense network.

During plant-pathogen interactions, the amount and specificity of the expressed defenses result from the "signal signature" induced by the invader (De Vos *et al.* 2005). Expression of a given set of specific defenses results from the fine-tuned regulation between the cross-communication of phytohormones which includes synergistic and antagonistic effects (Kunkel and Brooks 2002; Robert-Seilaniantz *et al.* 2007; Koornneef and Pieterse 2008; Bari and Jones 2009; Pieterse, *et al.* 2009) (The Figure 3 summarizes the cross-communications between the different plant defense hormones).

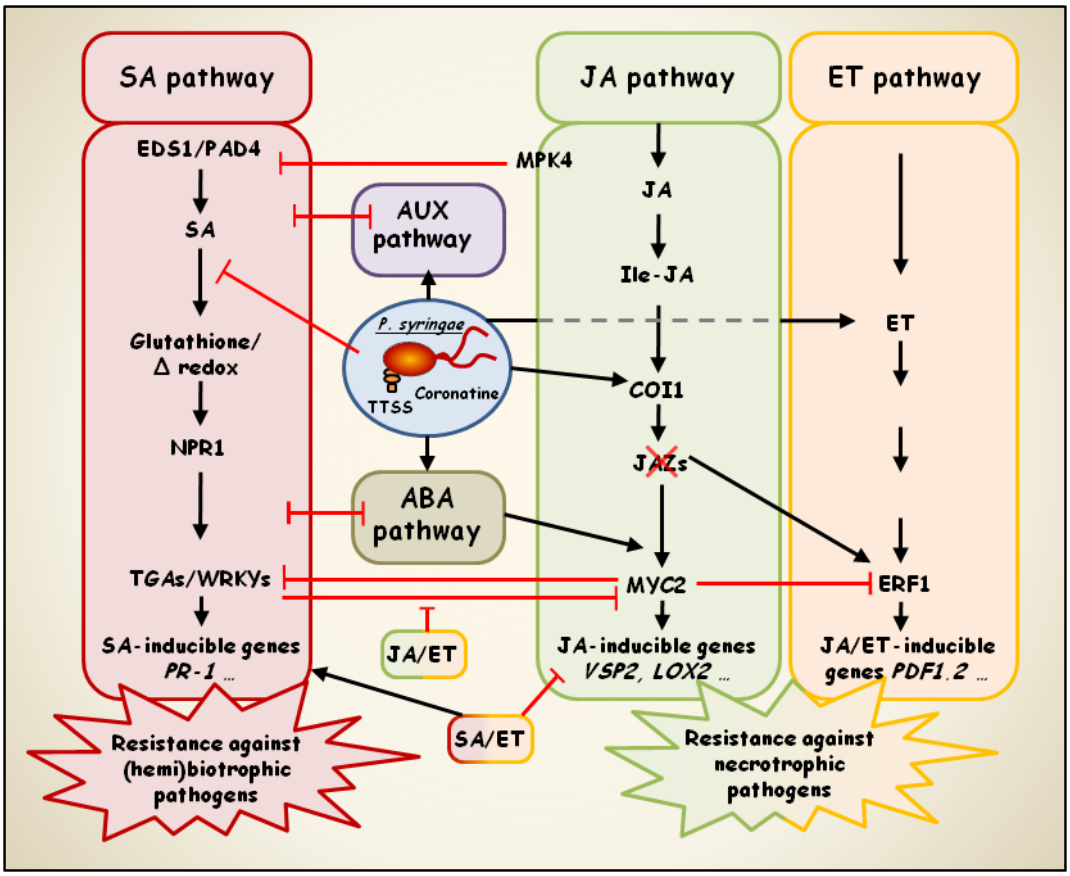

Figure 3. Networking between the principal plant defense hormones.

The signalization cascades of the major plant defense pathways including salicylic acid (SA), jasmonic acid (JA), ethylene (ET) are indicated. The Cross-communications between these signaling pathways include synergistic (in black) and antagonistic (in red) effects leading to protection against different pathogens. In addition, the hemibiotrophic bacteria *Pseudomonas syringae* can manipulate plant hormone pathways to suppress host defenses through secretion of the phytotoxin coronatine and effectors delivered by the type three secretion system (TTSS). Adapted from Pieterse *et al*., 2009.

The SA pathway is known to be the key player that mediates resistance against biotrophic pathogens while the JA/ET pathways regulate resistance against necrotrophic pathogens, insects and wounding (Kunkel and Brooks 2002; Howe 2004; Glazebrook 2005; von Dahl and Baldwin 2007). The antagonism between SA and JA is well documented (Kunkel and Brooks 2002; Glazebrook *et al.* 2003; Rojo *et al.* 2003; Bostock 2005; Beckers and Spoel 2006; Koornneef and Pieterse 2008; Pieterse*, et al.* 2009). The SA-mediated inhibition of the JA pathway requires the modulation of the cellular redox state through increased level of glutathione (Mou *et al.* 2003; Ndamukong *et al.* 2007; Koornneef *et al.* 2008) that regulates the function of the signaling transducer NPR1 (NON-EXPRESSOR OF PR GENES) (Spoel *et al.* 2003; Dong 2004; Pieterse and Van Loon 2004) and the downstream regulation of transcription factors such as WRKY70 (Li *et al.* 2004). Conversely, the JA-mediated inhibition of the SA pathway operates through the MPK4 kinase (Petersen *et al.* 2000; Brodersen *et al.* 2006) and the transcription factor MYC2/JIN1 (Nickstadt *et al.* 2004; Laurie-Berry *et al.* 2006). The outcome of SA/JA interaction is time- and concentration- specific and involves synergistic effects (Mur *et al.* 2006; Koornneef*, et al.* 2008). Synergistic and antagonistic effects between the ET and SA signaling pathways (Lawton *et al.* 1994; Glazebrook*, et al.* 2003; De Vos *et al.* 2006) and between the ET and JA signaling pathways (Penninckx *et al.* 1998; Lorenzo *et al.* 2003; Pre *et al.* 2008) have been reported but the second is more documented. JA and ET can both induce the expression of the transcription factor *ERF1* (*ETHYLENE RSPONSE FACTOR*) (Lorenzo*, et al.* 2003; Pre*, et al.* 2008) that mediates the expression of a set of JA/ET-dependent genes such as *PDF1.2* (Penninckx*, et al.* 1998). In addition, ET has recently been shown to be an important regulator of the SA/JA antagonism. ET can render the antagonistic effect of SA on JA-dependent responses NPR1 independent (Leon-Reyes *et al.* 2009). On the other side, activation of the JA/ET pathway through ERF1 renders the plant insensitive to future SA-mediated suppression of JA-

dependent defenses (Leon-Reyes *et al.* 2010). Alternatively, JA alone induces *MYC2* that represses *ERF1* and positively regulates the expression of a different set of JA-responsive genes such as *VSP2* and *LOX2* (Lorenzo *et al.* 2004; Lorenzo and Solano 2005). Although ABA is associated with plant responses against abiotic stresses, its role as a major regulator of plant defense expression against pathogens has become obvious (Mauch-Mani and Mauch 2005; Asselbergh*, et al.* 2008; Ton*, et al.* 2009). Interestingly, several studies have reported the importance of ABA to regulate both JA biosynthesis and expression of *MYC2* while also repressing *ERF1* (Anderson *et al.* 2004; Adie *et al.* 2007). The mutual antagonism between SA and ABA is increasingly documented but the regulation of this cross-talk is not totally understood (Mohr and Cahill 2007; Yasuda *et al.* 2008; Zabala *et al.* 2009; Jiang *et al.* 2010; Kusajima *et al.* 2010; Mosher *et al.* 2010). Beyond the SA-JA-ET-ABA network, several evidence support that the growth hormone AUX is involved in the regulation of plant defenses (Navarro*, et al.* 2006; Chen*, et al.* 2007), and can be antagonized by the SA pathway (Wang*, et al.* 2007; Zhang*, et al.* 2007). Interestingly, AUX positively regulates the JA pathway during flower maturation and represses it in seedlings (Nagpal *et al.* 2005; Liu and Wang 2006). In addition, other hormones involved in plant growth regulation such as gibberellins (Navarro*, et al.* 2008), cytokinins (Siemens*, et al.* 2006; Walters and McRoberts 2006) and brassinosteroids (Krishna 2003; Nakashita*, et al.* 2003; Shan*, et al.* 2008) have been shown to modulate plant disease resistance, but their connection with the major defense hormones SA, JA, ET and ABA remains to be settled.

The activation and fine-tuning of plant defenses are thus regulated by an extensive network of cross-communicating phytohormones (Figure 3). This complex network has recently been reviewed and will certainly be extended (Bari and Jones 2009; Pieterse*, et al.* 2009). Expression of plants defenses implies an ecological fitness cost (Heil and Baldwin 2002; Walters and Heil 2007; Bolton 2009). Thus, it is presumed that this complex network

regulates the fine-tuning of plant defenses from a state of alertness in stress-free conditions into an activated state during disease pressure. This fine regulation would avoid excessive energy loss for the benefit of plant growth (Pieterse*, et al.* 2009). On the other side, virulent pathogens have evolved complex mechanisms such as the use of toxins and/or effectors to manipulate the cross-communication of plant defense pathways. This can result in the suppression of plant defenses and in the manipulation of host cellular functions to optimize growth conditions (Jones and Dangl 2006; Lopez *et al.* 2008; Pieterse*, et al.* 2009). Thus, the outcome of plant-microbe interactions is determined by the complex hormonal interplay between plant defense pathways on the one side and the virulence factors of pathogens on the other side.

Arabidopsis/*Pseudomonas syringae* interaction

Significant advances in the comprehension of hormonal cross-communications (Figure 3) stem from studies of the compatible interaction between Arabidopsis and the hemibiotrophic bacteria *Pseudomonas syringae* (Nomura *et al.* 2005; Kim *et al.* 2008). PTI and the subsequent SA-dependent and independent defenses are activated in Arabidopsis upon infection with *P. syringae* (Tsuda *et al.* 2008). On the bacterial side, about 36 protein effectors (Block *et al.* 2008; Cunnac *et al.* 2009; Lindeberg *et al.* 2009) are secreted into the host cell by a remarkable system conserved in Gram-negative bacteria called the type three secretion system (TTSS) (Jin *et al.* 2003; McCann and Guttman 2008). These effectors suppress both SA-independent and SA-mediated basal defenses (DebRoy *et al.* 2004; Nomura*, et al.* 2005; Kim*, et al.* 2008). Furthermore, *P. syringae* pathovars secrete the phytotoxin coronatine (COR) involved in bacterial virulence (Mittal and Davis 1995; Kloek *et al.* 2001). COR has been shown to target JA, ET, AUX and ABA pathways known to antagonize the SA-mediated

defenses (Uppalapati *et al.* 2005; Thilmony*, et al.* 2006). COR also suppresses PTI (Li *et al.* 2005) and induces the re-opening of stomata that have been closed upon recognition of the bacterial flagellin and lipopolysaccharides by Arabidopsis (Melotto *et al.* 2006). Both COR and TTSS effectors display common and distinct virulence effects in Arabidopsis such as targeting the JA pathway, thereby repressing the SA-inducible defenses (Zhao *et al.* 2003; He *et al.* 2004; Uppalapati *et al.* 2007). More recently, an amino acid conjugated form of JA, jasmonoyl-isoleucine (Ile-JA), was identified as the signal molecule of the JA pathway. Ile-JA binds to the F-box protein CORONATINE-INSENSITIVE 1 (COI1) and subsequently promotes the ubiquitin-dependent degradation of JA ZIM domain (JAZ) proteins (Chini *et al.* 2007; Thines *et al.* 2007). These JAZ proteins repress the activity of MYC2/JIN1 known to stimulate the expression of a subset of JA-dependent genes (Lorenzo*, et al.* 2004). COR mimics Ile-JA and has a higher affinity to the COI1 receptor (Katsir *et al.* 2008; Melotto *et al.* 2008). Thus, during *P. syringae* infections, TTSS effectors and COR activate the COI1-dependent degradation of JAZ proteins enabling the expression of *MYC2/JIN1,* known to suppress SA-inducible responses and promote full susceptibility (Nickstadt*, et al.* 2004; Laurie-Berry*, et al.* 2006). Some TTSS effectors of *P. syringae* also interfere with the AUX (Navarro*, et al.* 2006; Chen*, et al.* 2007; Zhang*, et al.* 2007) and the ABA pathways (de Torres-Zabala *et al.* 2007; Zabala*, et al.* 2009), both promoting the repression of SA-inducible defenses.

Although recognized by the immune system of Arabidopsis, *P. syringae* deploy an arsenal of virulence effectors such as COR and TTSS effectors. These effectors interfere with the hormonal network of plant defenses, thereby leading to successful infection through the suppression of basal defenses including the SA pathway.

Induced disease resistance

The plant immune system cannot prevent successful infection of genetically susceptible plants by virulent pathogens. Nevertheless, plants protection can be enhanced against an extensive range of biotrophic and necrotrophic pathogens through a mechanism called induced disease resistance (Hammerschmidt 1999). This well described phenomenon is generally defined by the systemic expression of resistance induced by a local pre-treatment with necrotizing pathogens (systemic acquired resistance, SAR) (Sticher, *et al.* 1997; Durrant and Dong 2004) or with beneficial soil-borne microorganisms such as plant growth-promoting rizhobacteria (PGPRs) and mycorrhizal or endophytic fungi (Waller *et al.* 2005; Bakker *et al.* 2007; Pozo and Azcon-Aguilar 2007; Van Wees *et al.* 2008) (induced systemic resistance, ISR) (Figure 4).

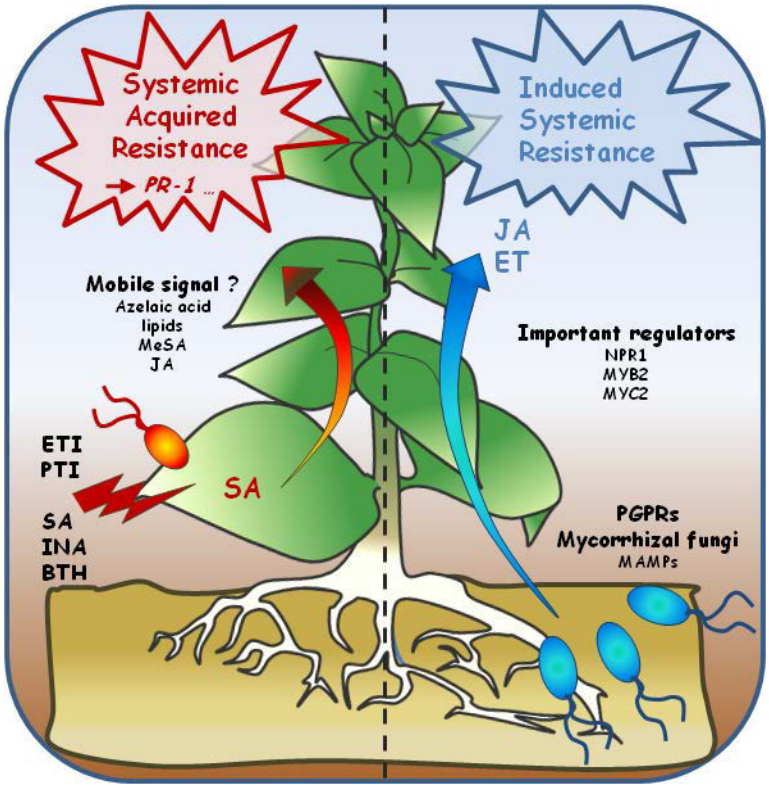

Figure 4. Model for the systemically induced immune responses.

SAR is activated locally by virulent (PTI) and avirulent (ETI) pathogens or chemicals. Systemic resistance is dependent of the SA pathway and is activated upon the transport of a mobile signal. ISR is induced by roots-colonizing beneficial microorganisms and activates defenses that are dependent of both the ET and JA pathways. Adapted from Pieterse et al., 2009.

SA, which accumulates in local and distant tissues upon SAR induction, is essential for establishment of systemic resistance and the expression of defenses such as *PR-1* (Gaffney *et al.* 1993; Delaney *et al.* 1994; Dong 2001). However, grafting experiments demonstrated that SA is not the mobile signal transmitted from the local site of SAR induction to distal tissues where it induces systemic resistance (Vernooij *et al.* 1994). One of the most exciting challenges over the past decades has been to identify this long-distance mobile signal (Vlot *et al.* 2008a; Parker 2009; Shah 2009). Several candidates have been proposed such as lipid molecules (Maldonado *et al.* 2002; Nandi *et al.* 2004), JA (Truman *et al.* 2007), metyl-SA (MeSA) (Park *et al.* 2007; Vlot*, et al.* 2008a; Vlot *et al.* 2008b; Liu *et al.* 2010) and recently azelaic acid (Jung *et al.* 2009). However, it remains controversial which of these molecules is the active mobile signal of SAR (Vlot*, et al.* 2008a; Parker 2009). Furthermore, it was recently demonstrated that MeSA and JA are not essential for SAR establishment (Attaran *et al.* 2009). In addition, SAR has recently been shown to be induced by virulent or avirulent microorganisms, triggering PTI and ETI, respectively (Mishina and Zeier 2007; Tsuda*, et al.* 2008). Furthermore, induced resistance can be mimicked through the application of natural or synthetic molecules (Ryals*, et al.* 1996; Sticher*, et al.* 1997; Kuc 2001). Treatment with SAR inducers such as SA, 2,6-dichloroisonicotinic acid (INA) (Kessmann *et al.* 1994), benzo(1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH or ASM) (Lawton*, et al.* 1994; Friedrich *et al.* 1996; Görlach *et al.* 1996) provide a phenotypic similar protection as classical SAR itself (Ryals*, et al.* 1996; Sticher*, et al.* 1997).

Although SAR and ISR induce a similar phenotypical systemic resistance, SAR protects mainly against biotrophic pathogens that are sensitive to SA while ISR protects essentially against pathogens and insects that are, with a few exceptions, sensitive to JA- and ET-dependent defenses (Ton *et al.* 2002; Van Oosten *et al.* 2008). In accordance with that,

rhizobacteria- (Van Wees*, et al.* 2008) and mycorrhizal fungi-mediated ISR (Stein *et al.* 2008; Segarra *et al.* 2009) are SA-independent but require ET and JA signaling pathways. More intriguingly ISR also requires a functional NPR1 protein as SAR does (Van Wees*, et al.* 2008). The ET- and JA-dependent transcription factors MYB2 (Van der Ent *et al.* 2008; Segarra*, et al.* 2009) and MYC2 (Pozo *et al.* 2008) have recently been identified as important regulators of ISR. In addition, recent studies reported the essential role of MAMPs from the beneficial soil-borne microorganisms to activate ISR (Bakker*, et al.* 2007; Van Wees*, et al.* 2008).

Priming

Induced disease resistance is generally associated with direct expression of plant defenses resulting in energy costs and reduced fitness (van Hulten *et al.* 2006; Walters and Heil 2007; Hammerschmidt 2008). In addition, the mechanism of priming is characterized by the ability of plants to "recall" a previous infection, roots colonization or chemical treatments (Figure 5) (Conrath*, et al.* 2002; Prime-A-Plant Group 2006; Goellner and Conrath 2008). A primed plant does not express substantial defenses, but potentiates a faster and stronger reaction to a given stress situation ultimately leading to an efficient protection. According to the first priming stimulus, plants are prepared to potentiate specific plant defenses dependent of the SA, JA/ET or ABA pathway, that are activated upon the second stimulus induced by the different biotic or abiotic stresses. The distinctive advantage of priming compared with direct plant defense inducers comes from the tradeoff between efficient protection and the low energy cost for defenses under both enemy-free conditions and disease pressure (van Hulten*, et al.* 2006; Walters and Heil 2007; Hammerschmidt 2008; Walters *et al.* 2008; Walters and Fountaine 2009).

The priming phenomenon was first described in plants treated with low concentrations of SAR inducers (Kauss *et al.* 1992; Kauss *et al.* 1993; Kauss and Jeblick 1995; Mur *et al.* 1996; Katz *et al.* 1998; Thulke and Conrath 1998; Kohler *et al.* 2002). In addition, ISR promoted by selected strains of PGPRs (Pieterse *et al.* 1996; van Loon *et al.* 1998; Bakker*, et al.* 2007) and mycorrhizal fungi (Pozo and Azcon-Aguilar 2007; Stein*, et al.* 2008) is not associated with direct expression of plant defenses but rather prepares plants to boost their defenses upon pathogen infection or insect attack.

In addition, several chemicals and minerals are referred to as priming agents such as thiamine (Vitamin B1) (Ahn *et al.* 2007), Phytogard® (Pajot *et al.* 2001), Brotomax, Pyraclostrobin, Oryzemate, Metalaxyl, $Cu(OH)_2$ and Fosethyl Aluminum (Prime-A-Plant Group 2006; Goellner and Conrath 2008).

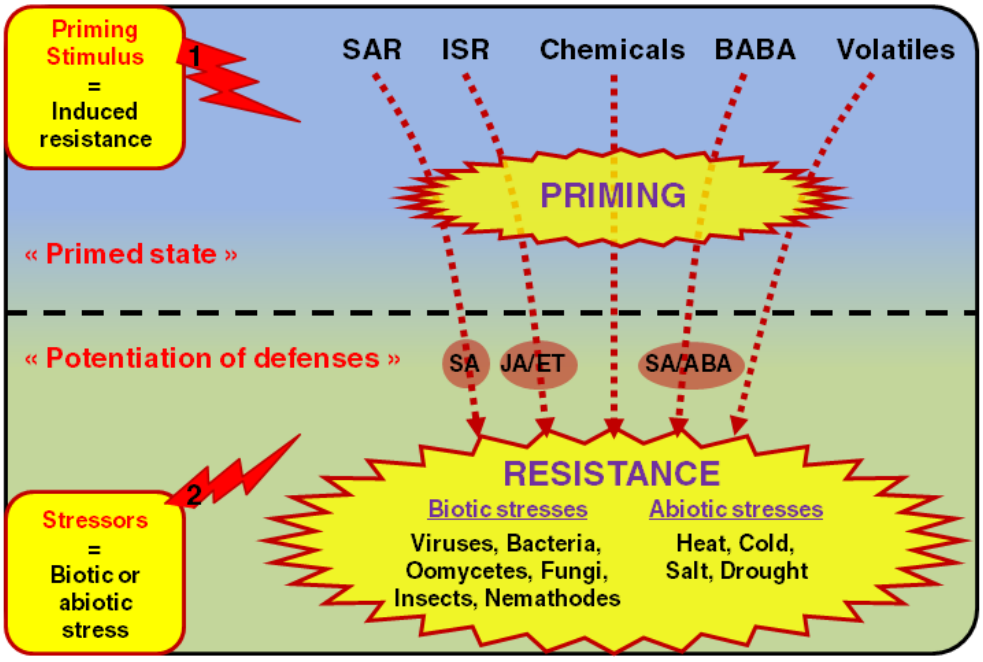

Figure 5. Model of different priming phenomena

Plants are primed by miscellaneous biological or chemical agents known to induce resistance without promoting directly the expression of plant defenses. In a first phase they induce the « primed state ». In a second phase, plants are exposed to a given abiotic or biotic stress. This triggers the boosted expression of a set of specific defenses depending on the priming agent and the stressor. This leads to the efficient protection against a wide range of biotic and abiotic stresses. Adapted from Goellner *et al*., 2008.

Priming against herbivore insects has recently been observed in plants treated with volatile organic compounds (VOCs). VOCs are molecules involved in plant-plant communication (Baldwin *et al.* 2006) and are responsible for the attraction of parasites or natural enemies of herbivore insects (Pare and Tumlinson 1999). Plants treated with VOCs from neighboring plants or collected from caterpillar-infested plants displayed reduced damage by herbivore insects. This correlated with potentiation of specific defenses (Engelberth *et al.* 2004; Ton *et al.* 2007). VOCs have also been shown to promote priming and reduction of herbivore damage between plant species (Kessler *et al.* 2006). In addition, maize plants challenged at the root level with herbivore insects were primed for enhanced production of metabolic compounds in the aboveground part of the plants upon challenge with herbivore caterpillars (Erb *et al.* 2009).

BABA-induced resistance

Research on priming has been facilitated through the use of the non-protein amino acid β-aminobutyric acid (BABA). This priming agent protects a variety of plants against a broad range of biotic and abiotic stresses (Jakab *et al.* 2001; Ton *et al.* 2005). In recent years, significant advances have uncovered the multiple mechanisms of BABA-induced resistance (BABA-IR) against different category of stresses. This has allowed to discern several specific hormone-related defense mechanisms that are primed distinctively depending on the challenging pathogen or the encountered abiotic stress (Figure 6). Some of these defenses have been dissected in several priming mutants *Impaired in BABA-induced Sterility* (*ibs*) (Ton*, et al.* 2005).

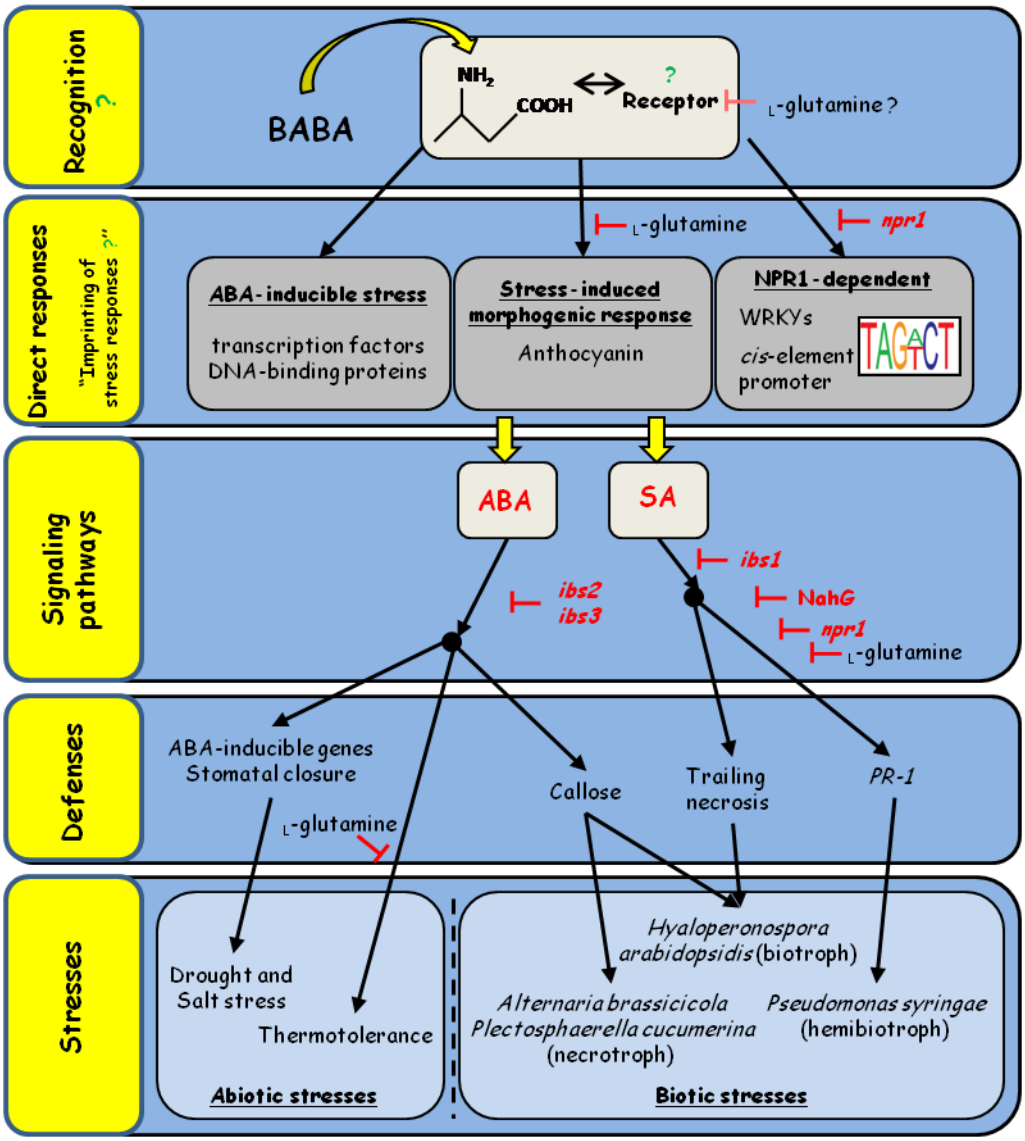

Figure 6. Review of the BABA-induced resistance mechanism

BABA treatment in Arabidopsis potentiates multiple defense responses depending on the stressors. This figure represents the different steps of the BABA-IR, from the assumed recognition by a receptor to the induced resistance against different abiotic and biotic stresses. In-between BABA induces direct stress responses and potentiates defenses that are dependent either on the SA or the ABA pathway. The mutants impaired in specific BABA-induced transduction are indicated in red.

BABA-IR against the hemibiotrophic bacteria *P. syringae* pv. *tomato* DC3000 (*Pst*) phenotypically mimics SAR. It is based on the potentiation of *PR-1* expression that is compromised in several SA-altered mutants and requires a functional cyclin-dependent kinase IBS1 (Zimmerli *et al.* 2000; Ton, *et al.* 2005). Conversely, BABA-IR against the necrotrophic

pathogens *Alternaria brassicicola* and *Plectosphaerella cucumerina* is based on ABA-dependent callose deposition (Ton and Mauch-Mani 2004; Ton*, et al.* 2005) but is dependent of the SA pathway against *Botrytis cinerea* (Zimmerli *et al.* 2001). Interestingly, BABA-IR against the biotrophic oomycete *Hyaloperonospora arabidopsidis* operates through a remarkable complex mechanism involving several plant defense pathways. The oomycete spread is halted through SA-dependent formation of trailing necrosis requiring the gene *IBS1* and by ABA-dependent callose deposition whose mechanism is regulated at different levels through *IBS2* and *IBS3/ABA1*, respectively (Zimmerli*, et al.* 2000; Ton*, et al.* 2005). IBS2 is involved in the phosphoinositide pathway and IBS3 in the biosynthesis of ABA. The ABA pathway also plays a central role in the potentiation of stress-inducible genes and stomatal closure during BABA-induced tolerance against drought and salt stress (Jakab *et al.* 2005; Ton*, et al.* 2005). BABA treatment has also been shown to promote acquired thermotolerance through an elusive mechanism involving ABA (Zimmerli *et al.* 2008).

The astonishing plasticity of BABA to promote multiple defenses and specific resistance against a wide panel of unrelated stresses renders this xenobiotic an intriguing subject of study. However, the mechanism enabling the priming of ABA-dependent responses against abiotic stresses, necrotrophic fungi and *H. arabidopsidis* on the one hand and SA-dependent responses against *P. syringae*, *H. arabidopsidis* and *B. cinerea* on the other hand is not well understood (Zimmerli*, et al.* 2000; Zimmerli*, et al.* 2001; Ton and Mauch-Mani 2004; Jakab*, et al.* 2005; Ton*, et al.* 2005; Flors *et al.* 2008; Zimmerli*, et al.* 2008).

Although BABA pre-treatment does not provoke substantial expression of defenses, BABA induces directly a panel of stress responses. Global gene expression analysis revealed that BABA induces accumulation of transcription factors and DNA-binding protein transcripts related to ABA-inducible stress (Zimmerli*, et al.* 2008). Quantitative PCR-based genome-wide screens revealed that BABA induces the accumulation of NPR1-dependent WRKYs

transcription factors of which 21 conserve the same *cis*-element in their promoter (Van der Ent *et al.* 2009). Recently BABA was shown to induce the stress-induced morphogenic response and accumulation of anthocyanin, both characteristic of a stress response (Wu *et al.* 2010). Theses stresses can be circumvented by a treatment with another amino-acid, L-glutamine. The authors suggested that BABA induces an imprinting of stress responses that enables the potentiation of specific defenses upon exposure of a plant to unrelated stresses. In addition they also demonstrated that BABA-IR against *Pst* and heat stress were both blocked by L-glutamine supporting thus the existence of a receptor for BABA that could be antagonized by L-glutamine.

Cost of induced resistance and benefit of priming

Induced resistance against pathogens and herbivores is an expensive mechanism that affects plant growth and yield (Heil and Baldwin 2002; Walters and Heil 2007; Bolton 2009). Expression of plant defenses is associated with the re-allocation of the primary metabolism resources. This energy diversion includes among others, modulation of photosynthetic processes, and regulation of the metabolisms of nitrogen, amino acids and carbohydrates (Heil and Baldwin 2002; Walters and Heil 2007; Bolton 2009). Treatments of wheat with BTH and of Arabidopsis with exogenous SA are both associated with reduced growth and seed production (Heil *et al.* 2000; Cipollini 2002). Arabidopsis mutants constitutively expressing SAR are dwarfed and display reduced fitness phenotypes (Heidel *et al.* 2004). Therefore, the negative impact on plant growth, development and seed production inflicted by induced resistance represents an evident disadvantage especially if the enemy pressure is low. In contrast to directly inducing plant defenses, priming of plant resistance does not imply significant allocation of plant resources in enemy-free conditions but rather prepares plants to

express suitable defenses upon disease pressure (van Hulten*, et al.* 2006; Beckers and Conrath 2007; Goellner and Conrath 2008; Hammerschmidt 2008; Walters and Fountaine 2009; Walters 2010). Priming soybean with INA or BTH promoted reduced symptoms severity caused by the fungus *Sclerotinia sclerotiorum* and increased seed yield under disease pressure (Dann *et al.* 1998). Priming Arabidopsis with low doses of BTH or BABA did not involve substantial reduction of plant growth and seed production under either enemy-free conditions or challenge with *Pst* or *H. arabidopsidis* (van Hulten*, et al.* 2006). These priming agents conferred similar resistance against these pathogens than the constitutive SAR over-expressing mutant *cpr1*. Conversely, inducing resistance by high doses of BABA or BTH resulted in fitness reduction. More recently, priming barley against *Rhynchosporium secalis* using saccharin resulted in fitness benefit under high fungus pressure (Walters*, et al.* 2008). These studies support the benefit of priming on plant fitness associated with efficient protection. Thereby, this renders priming an interesting induced-resistance mechanism to apply in crop cultures (Beckers and Conrath 2007).

Thesis outline

The aim of the thesis was to investigate both the mechanism of plant defenses and the mechanism of bacterial virulence during the interaction between the model plant *Arabidopsis thaliana* and the bacterial pathogen *Pseudomonas syringae*. Studies on plant-pathogen interactions and the establishment of resistance rely on the accurate scoring of disease development and monitoring of plant defense reactions. On the one hand, disease development can be achieved by the evaluation of pathological symptoms, microscopic or visual observations or by measuring the pathogen biomass. Numerous methods are available, some of which are not suitable for studies in laboratories especially for molecular biology.

On the other hand, the evaluation of plant defenses can be completed by the quantification of specific hormones or defensive compounds and by measuring the expression of genes or the amount of the related proteins involved in the establishment of plant resistance. The methodologies and tools in molecular biology are evolving rapidly, enabling to enhance progressively the sensitivity and the range of analyses. The real-time PCR-based methodology presented in the second chapter enables to measure accurately the development of pathogens in plants, as well as the expression of defense genes in the same samples. This technique also permits to measure the plant hormones in the same samples. This methodology has been employed during the studies presented in this thesis to investigate the mechanism of virulence used by *Pseudomonas syringae* and the mechanism of BABA-IR and to describe the events leading to resistance or susceptibility. This allowed to link accurately the plant phenotypes and their specific defense reactions.

Plant pathogens and in particular bacteria have evolved distinct mechanisms to manipulate or bypass plant defenses. The outcome of *Arabidopsis/Pseudomonas* interaction involves a sensitive interplay between the cross-communicating defense hormones and the bacterial virulence mechanism that required to be better described. These virulence mechanisms can be overcome by using priming agents such as BABA. BABA-IR against biotic or abiotic stresses is also based on the potentiation of multiple defense responses that are regulated by a complex hormonal network The second aim of the study was thus to investigate the interaction of Arabidopsis with the bacterial pathogen *P. syringae* during both compatible interaction and BABA-IR. The transcriptional and hormonal plant reactions during both phenomena were analyzed. Arabidopsis mutants altered in the different defense pathways and bacterial strains compromised in virulence mechanisms were used to dissect the events leading to successful infection or establishment of resistance. The results presented in the third chapter enable a

better understanding of how the bacteria manipulate the plant's hormonal network to achieve its invasion. In complement, the results describe how BABA can prepare the plants to counteract this phenomenon and reverse the final outcome resulting in efficient protection.

The investigations and the sensitive methodologies employed to understand the mechanism of BABA-IR against *P. syringae* led to unexpected results concerning the role of the SA pathway and its signalization. BABA was found to induce a specific signal signature on the SA pathway. This phenomenon may explain how BABA prepares plants to potentiate SA-inducible defenses upon *P. syringae* infection while at the same time promoting only few detriments on plant fitness. This phenomenon was thus examined and the preliminary results are presented and discussed in the general conclusion.

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Chapter 2

Adaptation and improvement of real-time PCR methods to monitor microbial pathogen growth and accumulation of plant defense transcripts in plants

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Adaptation and improvement of real-time PCR methods to monitor microbial pathogen growth and accumulation of plant defense transcripts in plants

ABSTRACT

Background

Quantification of pathogen growth and transcripts level is a critical step to investigate the outcome of plant-pathogen interactions and to understand the implication of specific defense pathways. Monitoring pathogen growth is usually based on symptom display that is often not representative of pathogen biomass and can be time consuming. Generally, the accumulation of transcripts is quantified using northern blotting, semi-quantitative or real-time PCR. The reliability of the analysis relies on accurate normalization of data usually based on the expression of traditional housekeeping genes that are not specifically validated for plant-pathogen interactions.

Results

The objective of this study was to develop a rapid and accurate method to measure the accumulation of plant defense transcripts during infection coupled to the quantification of *Pseudomonas syringae*, *Hyaloperonospora arabidopsidis* and *Botrytis cinerea* biomass in Arabidopsis. Quantification by real-time PCR for transcripts accumulation or pathogen biomass was realized using calibration curves obtained from cloned PCR products. For each pathogen the PCR quantification method correlated well with the traditional quantification methods. The accumulation of defense transcripts was normalized between samples by the

sensitive dye-based quantification of cDNA. This enabled to obtain similar results as when using several reference genes that we validated by using the software geNorm.

Conclusions

This improved and reliable methodology enables the accurate and rapid quantifications of pathogen growth and accumulation transcripts in Arabidopsis. The protocols can easily be applied to other plant species and pathogens and could be useful for large-scale analysis.

BACKGROUND

The availability of well-defined Arabidopsis-microbe pathosystems including biotrophic, necrotrophic and hemi-biotrophic interactions has created the opportunity for in-depth studies of these systems at the molecular level. A pre-requisite for a correct assessment of the sometimes subtle molecular changes occurring during these interactions is the availability of a methodology allowing the rapid, accurate monitoring of pathogen development and the related responses in the plant. This is especially important in view of the often minute differences in wild type and mutant responses to stress situations.

Quantification of disease development has been largely based on visual symptom observation or on pathogen development using microscopy. These methods are useful and sufficient when the outcome of an interaction is either fully compatible or incompatible as visualized by extensive pathogen development or total absence of pathogen growth. However, in addition to these clear phenotypes, an array of varying degrees of infection can be found when assessing various pathogen strains, Arabidopsis mutants or accessions, and different degree of induced resistance (IR). Another drawback of visual quantification is the discrepancy that can occur between symptom display and actual pathogen development [1-3]. In addition, traditional quantification methods are often time-consuming and destructive. This renders

samples unavailable for additional molecular analysis. Furthermore these methods are not always suited for large-scale screening. Measuring bacterial growth, such as the virulent *P. syringae* pv. *tomato* DC3000 (*Pst*), is traditionally based on plate counting [4]. This laborious method, commonly called internal growth counting (IGC), gives an accurate evaluation of the bacterial development in the plant. However, it requires numerous replicates and a good sampling approach since bacterial growth is not always homogeneous in the plant. Furthermore, directly measuring growth of virulent obligate biotrophic organisms such as the oomycete *H. arabidopsidis* is not possible since in the initial phases of the interaction virtually no symptoms are visible. Scoring the development of this pathogen is performed by assessing conidia production at a representative time after inoculation or by microscopic observation using appropriate staining [5]. For *B. cinerea* as well, traditional methods consist of measuring lesions diameter, micro- or macroscopically. However, the fungus is not always growing concentrically and this approach is not suited for the early phases of development.

Several approaches have been developed to override the inconveniences of these traditional methods. Recently, a procedure for direct quantification of bacterial growth using the bioluminescence of a transformed strain of *Pst* has been described [6]. This method appeared to be efficient and useful. However, it necessitates the transformation of each bacterial strain and mutant that is crucial to dissect a given plant defense response. *H. arabidopsidis* biomass has been monitored by Northern blot quantification of its RNA [7]. Fungal development has been scored by quantifying fungal molecules such as ergosterol or chitin [8, 9], by using immunological methods [10, 11] or by the quantification of bacterial *UidA* gene in transformed organisms [12, 13]. However, these techniques are not sensitive enough or hedged by internal plant factors. In addition these methods are also not universally applicable. The availability of polymerase chain reaction (PCR) was a first step towards a more accurate diagnosis of plant pathogens [14]. It was followed in the 1990s by the sensitive quantitative

real-time PCR (qPCR), allowing to measure a wide range of microbial organisms in several plant species [15-18]. Recently, qPCR has been used to measure the development of several pathogens in Arabidopsis. Theses pathogens include the bacteria *Pst* and *Pectobacterium carotovorum* (Previously *Erwinia carotovora*), the oomycete *H. arabidopsidis* and the necrotrophic fungi *B. cinerea* and *Alternaria brassicicola* [19-21]. Even though these methods are specific, rapid, and efficient, they are still poorly used to assess the development of pathogens in Arabidopsis. This could be linked to the relatively high costs of DNA extraction and qPCR. In addition, only few comparisons between these qPCR and traditional methods have been realized. Based on these previous studies mentioned above, we improved qPCR-based quantification to score the development of the frequently used pathogens *Pst*, *H. arabidopsidis* and *B. cinerea* in Arabidopsis. Our method is based on the quantification of well-defined and specific gene products from the pathogens. The sensitive quantification takes advantage of calibration curves established from known copy numbers of cloned PCR products. The normalization between samples is realized by the quantification of an internal control, a plant gene representative of the plant biomass. The results, expressed by the ratio of the copy number of the pathogen gene and the copy number of the plant gene, proved that the quantifications are specific, rapid and as reliable as traditional quantifications. This universal method, well suited for large scale analysis, offers several advantages that are discussed.

In addition, quantitative real-time reverse transcription PCR (qRT-PCR) provides the most powerful and sensitive method to measure the accumulation of transcripts of genes of interest (GOIs) [22]. This technique, even though still underused, is progressively being adopted by the plant science community [16]. It is slowly replacing non-quantitative or semi-quantitative RNA gel blots and RT-PCR analysis due to its ability to circumvent several inconveniences associated with quantitative transcripts accumulation studies [23, 24]. However, this procedure is barely used accurately by plant scientists and recently three letters to the editor of

The Plant Cell described the correct experimental rules to follow in order to validate qRT-PCR analyses [25-27].

Therefore, we evaluated the expression stability of several reference genes in Arabidopsis infected with *Pst*, *H. arabidopsidis* and *B. cinerea* as representatives of frequently used hemibiotrophic, biotrophic and necrotrophic pathogens. We compared the stability of traditional housekeeping genes as well as genes that had already been reported to be stable during biotic stresses [28]. For accurate qRT-PCR measurements, the primary goal of using reference genes that display stable expression is to normalize the variable amount of cDNA in each sample [24]. This amount represents the quantity of mRNA used for the reverse transcription that is expected to be stable between tissues, organs and treatments [29]. We thus developed an absolute qRT-PCR method (aqRT-PCR). The quantification of each specific gene transcript is based on calibration curves traced from known copy numbers of cloned PCR products. The normalization is based on the sensitive quantification of the total amount of cDNA in each sample. This normalization procedure is closely related to the use of several stable reference genes that we validated for our experimental conditions. It is more accurate compared to the use of one traditional housekeeping genes while offering several advantages.

RESULTS

Assay procedures for the PCR-based quantification of pathogens growth

Colonization of plant tissues by pathogens was quantified in each sample by measuring the amount of both pathogen and plant genomic DNA (gDNA). Both gDNA were quantified by qPCR using the non specific SYBR® Green I mix. Serial dilutions of plasmids, each containing a PCR product cloned from a genomic region of each pathogen and Arabidopsis,

respectively, were used to relate their qPCR signal to construct calibration curves. In order to clone representative and specific genomic regions from *Pst*, *H. arabidopsidis* and *B. cinerea* we used the primers previously designed and tested by Brouwer et al. (2003). We also designed specific primers to amplify and clone a genomic region of the *beta tubulin* gene from Arabidopsis (*AtTUB4*). The clones were sequenced and verified by multiple sequence alignment to confirm that the plasmids carried the correct gDNA region from each organism (data not shown). The amount of pathogen in each sample was finally expressed as the ratio between copy numbers of the pathogen gene and copy numbers of the plant gene. Quantity of the pathogen gene represents the pathogen biomass. Quantity of the plant gene represents the plant biomass and is used as internal calibrator to avoid variations stemming from starting material and yield occurring during DNA extraction from one sample to another. All primers used for pathogen quantification are listed in Table S1.

Quantification of *P. syringae* by real-time PCR

The accuracy and sensitivity of the qPCR method was evaluated. First, the efficiency of the primers used to amplify the *oprf* genomic gene from *P. syringae* (*Psoprf*) was tested by using different DNA sources. Because we used clones containing the PCR products from *Psoprf* as a template to design calibration curves, we anticipated that the qPCR efficiency as well as the PCR product could be modified by interference with gDNA from Arabidopsis or the bacteria. However, the qPCR efficiency was the same using gDNA from infected Arabidopsis leaves, pure *Pst* gDNA or *Psoprf* clones (Figure 1a). In addition, the melting curve analysis representing a peak for the dissociation of the PCR products confirmed that only one and same product was amplified using the three different DNA sources (Figure 1b). The qPCR quantifications are linear between the DNA sources for concentration scales including ranges of low amounts of the bacteria (initial inocula) to high amounts (most infected samples).

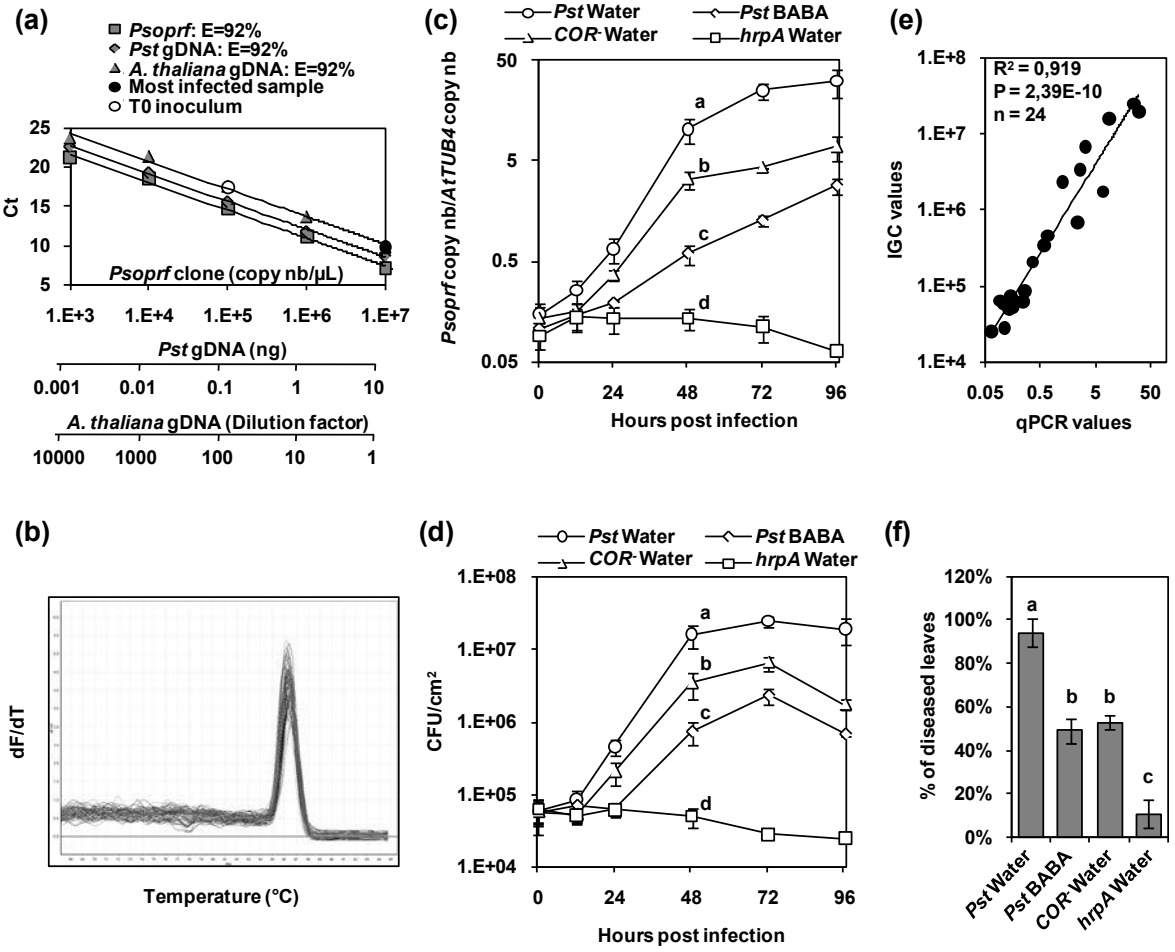

Figure 1. Validation assay of the qPCR-based quantification of *Pseudomonas syringae*

(a) The amplification efficiency of the specific primers was compared by using different DNA templates. qPCR threshold cycles (Ct) obtained from characteristic concentrations ranges of pure *Pst* gDNA (grey circle), *Psoprf* clones (grey square) or total gDNA from the most infected Arabidopsis sample (*Pst* + Water 96 hours post infection) (grey triangle) were used to design calibration curves. The closed circle represents the most infected sample while the open circle represents the initial inoculum (T0). Each value represents a technical triplicate and the percentage of the amplification efficiency is indicated for each DNA template. (b) All qPCR products were analyzed after the qPCR run with a melting curve. The growth of *Pst* in water-treated (open circle) or BABA-treated (open diamond) Arabidopsis plants was compared with the growth of the coronatine deficient (*COR*$^-$) strain (open triangle) and the non-pathogenic *hrpA* mutant (open square) in water-treated Arabidopsis using internal growth counting (IGC) or qPCR. (c) qPCR quantification data are expressed in *Psoprf* copy number per copy number of the Arabidopsis gene (*AtTUB4*). (d) IGC data represent the number of colony forming units (CFU) per surface unit. Data for each time point represent the average ±SD from 4 biological replicates. Letters indicate statistical bacterial different amounts (ANOVA, Student-Newman-Keuls, n=4, $P<0.001$) 48 hours post infection (hpi) that remained until 96 hpi for both quantification methods. (e) Correlation of qPCR and IGC methods using the data from (c) and (d) (Pearson product moment correlation: coefficient R, P Value and number of samples n are indicated). (f) disease rate observed 96 hpi. Data represent the average ±SD of the percentage of diseased leaves from height plants (ANOVA, Student-Newman-Keuls, n=8, $P<0.001$).

Thus, *Psoprf* clones can be used as a template to design calibration curves from which the amount of *P. syringae* in unknown infected samples is determined.

We then decided to compare the PCR method with the traditional IGC procedure and with the observation of symptom display. To this end, we quantified the growth of *Pst* in Arabidopsis plants treated with water or with β-aminobutyric acid (BABA). We also quantified the growth of *Pst COR*$^-$ (*COR*$^-$) strain and the *Pst hrpA* (*hrpA*) mutant over a four days time course period (Figure 1c to f). BABA is a priming agent known to reduce significantly the growth of *Pst* in Arabidopsis [30]. The *COR*$^-$ strain is deficient in the production of the phytotoxine coronatine rendering it less virulent when inoculated by dipping [31] due to the stomatal opening activity of the coronatine [32]. The *hrpA* mutant is a non-host strain due to its compromised type three secretion system [33]. Results confirmed that the qPCR quantification allows to quantify the logarithmic growth of the wild-type bacteria in Arabidopsis from 24 hours post infection (hpi) similarly as the IGC method. In addition, and as for IGC, the qPCR method allowed to observe accurately the reduced growth of the wild type bacteria in BABA-treated plant, as well as the reduced growth of the *COR*$^-$ strain and the non proliferation of the *hrpA* mutants from 48 hpi. These results were supported by statistical analysis enabling to observe significant growth differences between the four infection settings from 48 hpi with both quantification methods. We also observed a significant correlation between both methods (Figure 1e) confirming the accuracy of the qPCR quantification. The symptom display observations (Figure 1f) enabled to observe similar results but did not allow to discriminate the differential growth of *Pst* in BABA-treated plants and *COR*$^-$ in water-treated plants, confirming that this procedure is not sensitive enough to observe slight growth differences.

Quantification of *H. arabidopsidis* by real-time PCR

It was previously demonstrated that the efficiency of the specific primers used to quantify the genomic region *AW737077* of the NOCO strain of *H. arabidopsidis* (*HaAW*) was not modified by the presence of Arabidopsis gDNA [19]. We completed this analysis and compared the qPCR quantification by using different sources of DNA. Results confirmed that the qPCR efficiency was the same by using gDNA from infected Arabidopsis leaves, pure *H. arabidopsidis* gDNA or *HaAW* clones and allowed to amplify only one and the same product (Figure 2a and b). The qPCR quantifications are linear between the DNA sources for concentration scales including ranges of low amounts of the oomycete (initial inocula) to high amounts (most infected samples). Thus, *HaAW* clones can be used as a template to design calibration curves from which the amount of *H. arabidopsidis* in unknown infected samples is determined.

We measured the growth of the virulent oomycete strain NOCO in water- or BABA-treated Col-0 plants over a five day time course period using the qPCR method (Figure 2c). Since BABA was shown to reduce the development of *H. arabidopsidis* [30], it was used as a control to observe the differential growth between a compatible interaction and during IR. By using a normal or logarithmic scale, the results permitted to observe the growth of the NOCO strain from 72 hpi in water-treated plants, while revealing the decrease of NOCO biomass in BABA-treated plants. Furthermore, statistical analysis showed a significant differential growth of the oomycete between the virulent interaction and during BABA-IR from 48 hpi until the end of the time course period. This result was confirmed by scoring the sporulation rate 122 hpi which was also significantly different between the samples (Figure 2d).

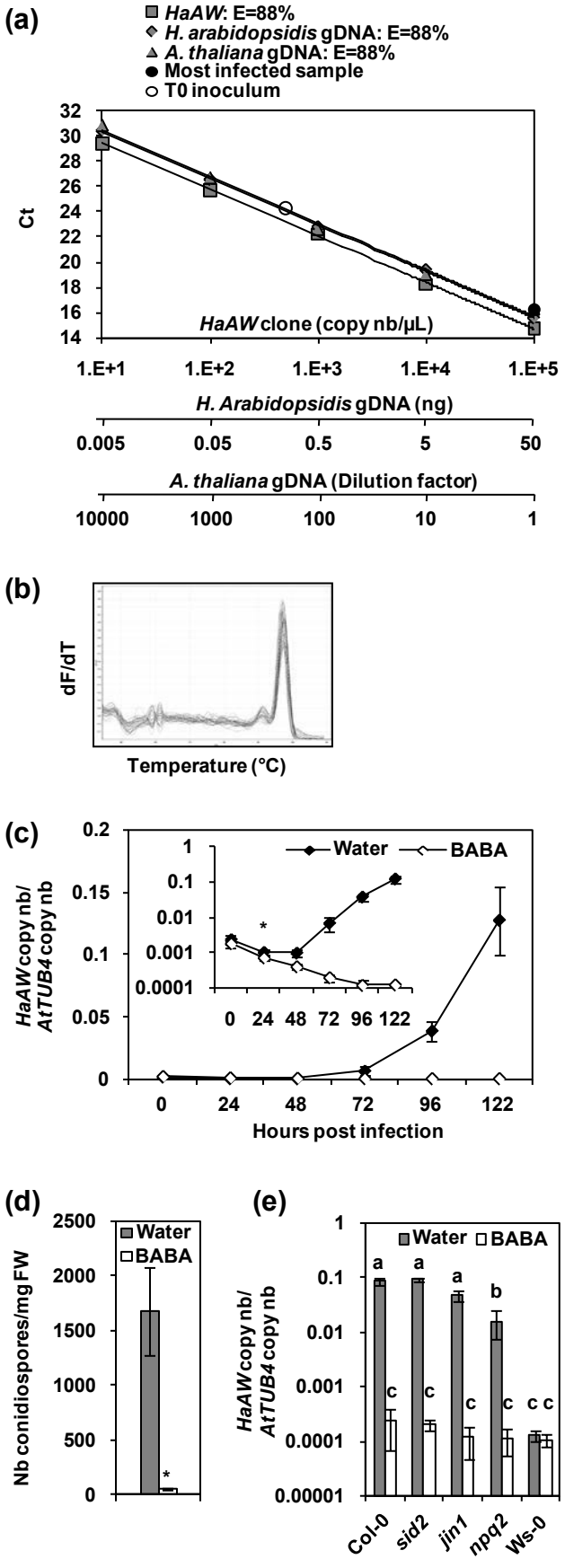

Figure 2. Validation assay of the qPCR-based quantification of *Hyaloperonospora arabidopsidis*

(a) The amplification efficiency of the specific primers was compared by using different DNA templates. qPCR threshold cycles (Ct) obtained from characteristic concentrations ranges of pure *H. arabidopsidis* gDNA (grey circle), *HaAW* clones (grey square) or total gDNA from the most infected Arabidopsis sample (*H. arabidopsidis* + Water 122 hours post infection) (grey triangle) were used to design calibration curves. The closed circle represents the most infected sample while the open circle represents the initial inoculum (T0). Each value represents a technical triplicate and the percentage of the amplification efficiency is indicated for each DNA template. (b) All qPCR products were analyzed after the qPCR run with a melting curve.

(c) The growth of *H. arabidopsidis* in water-treated (closed diamond) or BABA-treated (open diamond) Arabidopsis Col-0 plants was quantified by qPCR and expressed in *HaAW* copy number per copy number of the Arabidopsis gene (*AtTUB4*) (log scale in inserted window). Data for each time point represents the average ±SD from 3 biological replicates. The asterisk represents statistical oomycete growth differences (t-test, n=3, P=0.018) 48 hours post infection (hpi) that remained until 122 hpi (P=0.001). (d) Sporulation rate 122 hours post *H. arabidopsidis* infection in water- or BABA-treated plants (t-test, n=2, P=0.029). (e) qPCR quantification of *H. arabidopsidis* growth 122 hours post infection in water- and BABA-treated Arabidopsis Col-0 (compatible interaction), Ws-0 accessions (incompatible interaction) and in Arabidopsis mutants affected in SA (*sid2*), JA (*jin1*) and ABA (*npq2*) defense pathways. Data for each time point represents the average ±SD from 3 biological replicates. Letters indicate statistical oomycete different amounts (ANOVA, Student-Newman-Keuls, n=3, P<0.001).

We then used the qPCR method to screen for the development of the strain NOCO at a representative time point (122 hpi) in Arabidopsis Col-0 (susceptible) and Ws-0 (resistant) accessions and in Arabidopsis mutants deficient in defense including *sid2* for the salicylic acid (SA), *jin1* for the jasmonate (JA) and *npq2* for the abscisic acid (ABA) pathway (Figure 2e). As expected, the method allowed to verify that the NOCO strain cannot develop in Ws-0 in opposite to Col-0. We used statistical analysis to compare the development of the oomycete in the plant defense deficient mutants treated with BABA or water. This allowed to create groups with significant different infection rates and to confirm the reduced growth of the oomyceete in the water-treated *npq2-1* mutant. We did not observe differential growth of the oomycete in any mutants during BABA-IR.

Quantification of *B. cinerea* by real-time PCR

Similar qPCR-based methods were already well described previously to measure the growth of *B. cinerea* in plants. We however undertook to test the primers used to quantify the *tubulin* gene from *B. cinerea* gDNA (*Bctubu*) by using different source of DNA. Results confirmed that the qPCR efficiency was similar even though varying slightly using gDNA from infected Arabidopsis leaves (E=90%), pure *B. cinerea* gDNA (E=92%) or *Bctubu* clones (E=91%) (Figure 3a) and allowed to amplify only one and the same product (Figure 3b). The qPCR quantifications are linear between the DNA sources for concentration scales including ranges of low amounts of the fungus (initial inocula) to high amounts (most infected samples). Thus, *Bctubu* clones can be used as a template to design calibration curves from which the amount of *B. cinerea* in unknown infected samples can be determined.

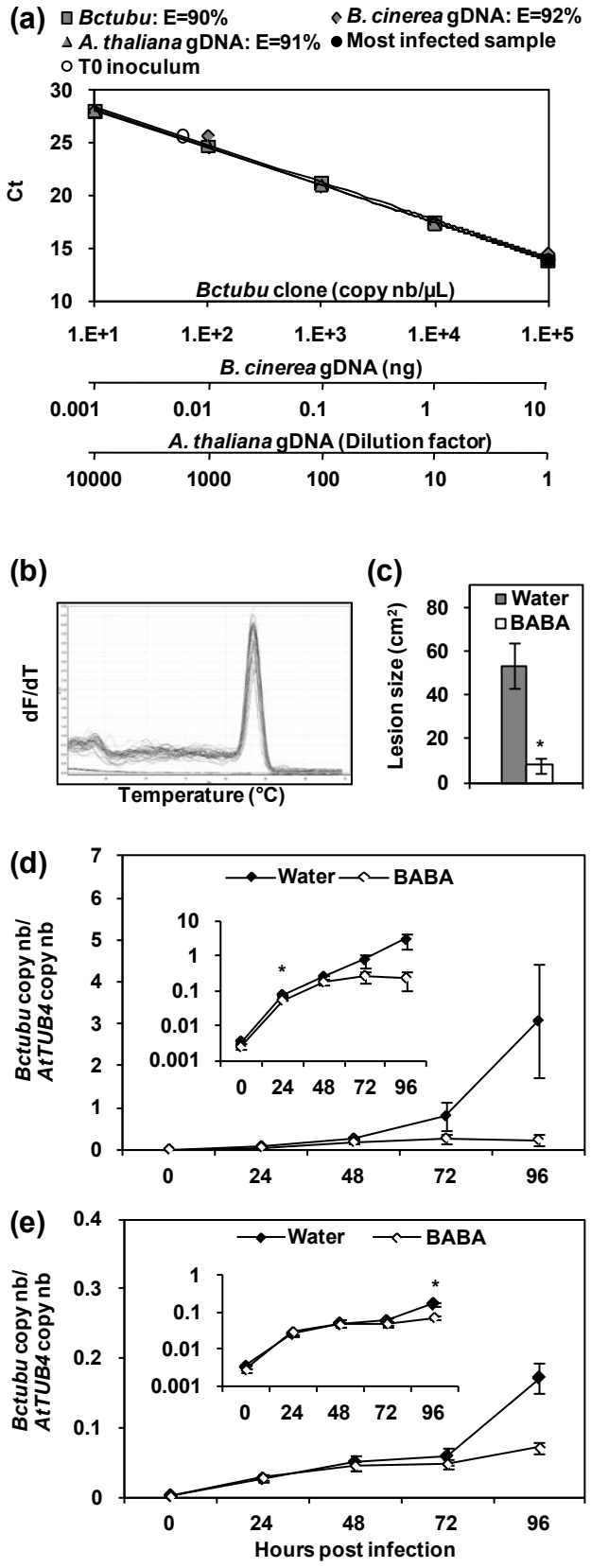

Figure 3. Validation assay of the qPCR-based quantification of Botrytis cinerea

(a) The amplification efficiency of the specific primers was compared by using different DNA templates. qPCR threshold cycles (Ct) obtained from characteristic concentrations ranges of pure *B. cinerea* gDNA (grey circle), *Bctubu* clones (grey square) or total gDNA from the most infected Arabidopsis sample (*B. cinerea* + Water 96 hours post infection) (grey triangle) were used to design calibration curves. The closed circle represents the most infected sample while the open circle represents the initial inoculum (T0). Each value represents a technical triplicate and the percentage of the amplification efficiency is indicated for each DNA template. (b) All qPCR products were analyzed after the qPCR run with a melting curve.

(c) Lesion size of water- and BABA-treated plants were measured 96 hours post *B. cinerea* droplets infection (t-test, n=20, $P \leq 0.001$).

The growth of *B. cinerea* inoculated either with droplets (d) or by spray (e) was quantified in water- (closed diamond) or BABA-treated (open diamond) Arabidopsis plants by qPCR and expressed in *Bctubu* copy number per copy number of the Arabidopsis gene (*AtTUB4*) (log scale in inserted windows). Data for each time point represents the average ±SD from 4 biological replicates. The asterisks represent statistical fungal growth difference between water- and BABA-treated plants from 24 to 96 hpi when inoculated with droplets (t-test, n=4, $P \leq 0.05$) and at 96 hpi when inoculated by spray (t-test, n=4, $P \leq 0.001$).

We measured the growth of *B. cinerea* in water- or BABA-treated plants over a four days time course period using the qPCR method (Figure 3d and e). Since BABA was shown to reduce the development of *B. cinerea* [34], it was used as a control to observe the differential growth between a compatible interaction and during IR. We also compared two inoculation procedures by inoculating plants either with one droplet per leaves or by spraying the whole plant with the same fungal inoculum. Droplet inoculation allows to observe single circular development of the fungus while spraying enables to inoculate full leaves homogenously. By using a logarithmic scale, the results permitted to monitor the exponential growth of the fungus from 24 hpi with both inoculation procedures in water- and BABA-treated plants. The quantity of pathogen gDNA increased exponentially in water-treated plants until the end of the time course while it was stable in BABA-treated plants. Significant differential amounts could be discerned between water- and BABA-treated plants from 24 hpi to 96 hpi in plants infected with droplets and 96 hpi in plants infected by spray. Although the expansion of *Botrytis* was about 10 fold lower when inoculated by spray compared with droplet inoculation, exponential growth and significant differential amounts of pathogen between water- and BABA-treated plants were still clearly visible. Results were confirmed by scoring lesion diameters 122 hpi which were also significantly different in samples inoculated with droplets (Figure 3c).

Assay procedures to quantify the accumulation of GOIs transcripts and to validate stable references genes

To measure the accumulation of GOIs transcripts, we quantified in each sample the amount of specific transcripts by qRT-PCR using the non specific SYBR® Green I mix and the total cDNA concentration by using a sensitive dye-based quantification. Serial dilutions of plasmids, each containing a PCR product cloned from the cDNA of each transcript, were used to relate their qPCR signal to construct calibration curves. The clones were sequenced and verified by multiple sequence alignments (data not shown). We then quantified in each sample the total cDNA amount by using the ultrasensitive fluorescent dye PicoGreen® specific to double-stranded DNA. The absolute quantification of GOIs transcripts level in each sample was obtained by the ratio between copy numbers of each transcript, representative of the mRNA amount transcribed from a specific GOI, and the total amount of cDNA representative of the plant transcriptome and used as the internal calibrator rather than using reference genes. This normalization procedure prevents characteristic errors from variable reverse-transcription efficiency, RNA starting amounts and pipetting errors. However, in order to validate the absolute quantification of GOIs transcripts level, a comparison with the traditional relative quantification normalized with validated stable reference genes was essential. Recently, important studies have highlighted the need to systematically validate reference genes in plant studies in order to normalize accurately the measurements of GOIs transcripts accumulation in specific experimental systems rather than using only one traditional housekeeping gene [24, 25, 28, 35]. New stable genes in Arabidopsis were previously identified from a broad range of varying experimental conditions by using large public set of Affymetrix studies [28]. Even though these reference genes are still barely used, this approach has been questioned because not completely adapted to identify stably expressed genes to normalize qRT-PCR analyses [24]. However, several robust

algorithms, now widely used by the scientific community, have been developed to process qRT-PCR data and to identify stable references genes [36-38]. Stably expressed reference genes have since been validated by qRT-PCR analysis with these algorithms in Arabidopsis during development stages in different organs [35] and during exposure to metal concentrations [39]. However, new reference genes have never been validated with such algorithms in Arabidopsis interacting with commonly used pathogens and during IR. Therefore, in order to compare our aqRT-PCR method with the traditional relative quantification we evaluated the stability of eight reference genes, including traditional housekeeping genes (*ACT2*, *EF1alpha*, *SSR16*, *TUB4* and *UBQ10*) and some new reference genes (*CACTA-like*, *SAND* and *TIP41-like*) previously identified from compiled Affymetrix studies as stably expressed during general biotic stresses [28]. The expression stability of these reference genes as well as the number required to obtain a satisfying normalization factor were tested using qRT-PCR raw data with the algorithm geNorm [36] during BABA-IR and compatible interactions with *Pst*, *H. arabidopsidis* and *B. cinerea* over representative and broad time course periods corresponding to the day of inoculation until full infection or establishment of resistance (See the details of the time course experiments in the labeling of samples included in Table S2). All primers used to measure the accumulation of GOIs transcripts are listed in Table S1.

Characterization of Arabidopsis reference genes during BABA-IR and compatible interactions with *P. syringae*, *H. arabidopsidis* and *B. cinerea*

The algorithm geNorm determines the average expression stability M by comparing the expression of every reference gene, two by two, and with all other genes tested [36]. This results in a ranking where the two genes having the lowest M value are the most stably expressed during the experimental conditions tested. In addition, the pair-wise variation V

enables to consider the contribution of using additional reference genes on the normalization factor. Lower pair-wise variations V by using n reference genes ($V_{n/n+1}$) illustrate improved normalization factors to quantify the accumulation of GOIs transcripts.

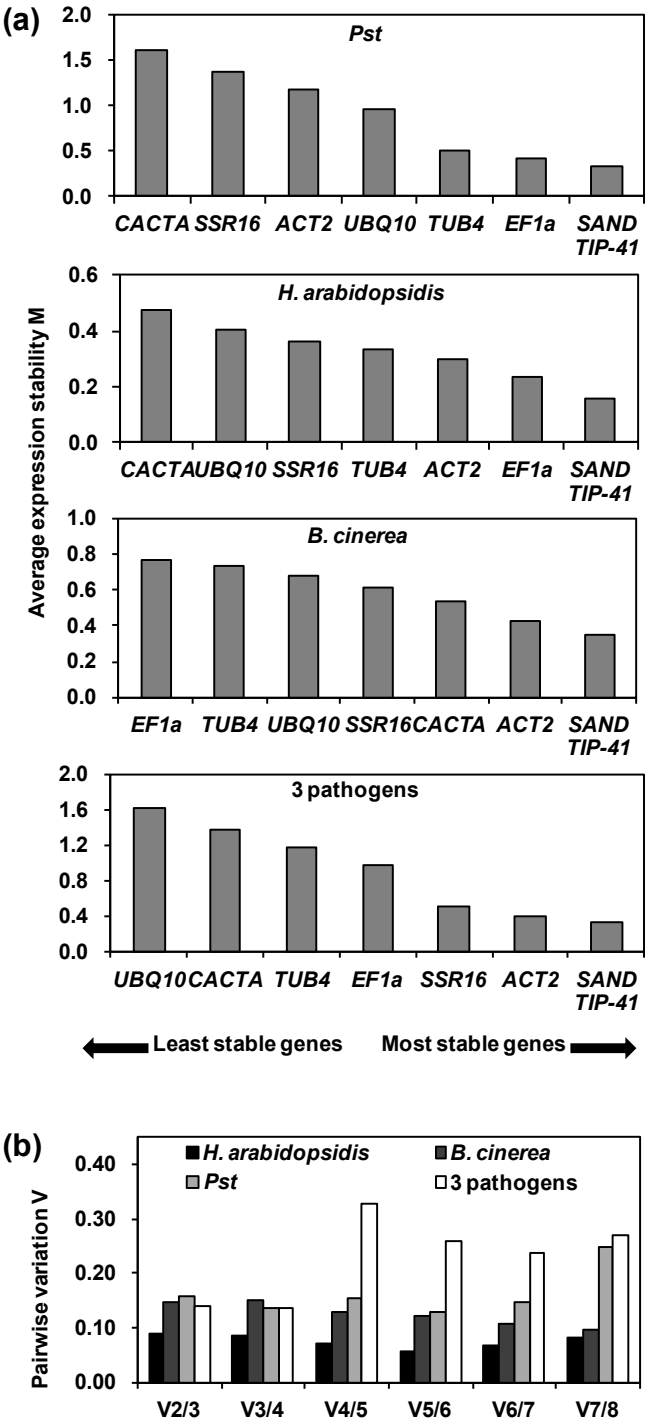

Figure 4. Evaluation of Arabidopsis reference genes during BABA-IR and compatible interactions with different pathogens

The expression stability of height candidate genes was investigated in water- and BABA-treated Col-0 plants challenged either with *Pst*, *H. arabidopsidis* and *B. cinerea* over representative time course periods corresponding to the day of inoculation until full infection (See the details of the time course experiments in the labeling of samples and data included in Table S2).

(a) Ranking of the 8 reference gene candidates based on their average expression stability M calculated with the sofware geNorm giving the most stably expressed reference genes with the lowest M value. (b) The pairwise variation V level illustrates the effect of adding further reference genes on the normalization of gene expression. Lower V values illustrate higher normalization factor. Supplementary results are presented in Table S3 and Figure S1.

In our experimental design, the results identified *SAND* and *TIP41*, two of the newly chosen reference genes over the eight genes tested, as the most stably expressed during both BABA-IR and compatible interactions and by taking into account data from the three pathogen infections separately or together (Figure 4a). These genes were also ranked as the most stable genes by analyzing independently data from samples of compatible interactions or BABA-IR, respectively, in plants infected with *Pst* and *H. Arabidopsis* (See Table S3). However, they were not identified as the most stable genes by taking into account separately data from these two kinds of interactions in plants infected with *B. cinerea* even though they were ranked among the best reference genes. Interestingly, they also were not the best reference genes in the mutant plants *jin1* and *npq2* infected with *Pst*. Additionally, the use of these two reference genes appeared to be sufficient to normalize correctly GOIs expression as illustrated by the pairwise variation $V_{2/3}$ (Figure 4b). For each pathogen and treatment the $V_{2/3}$ values were lower or around 0.15, value used as the cut-off level below which an additional reference gene is not required [36], and were not much reduced by adding a third reference gene ($V_{3/4}$). The high expression stability of *SAND* and *TIP41* compared to the traditional housekeeping genes could be expected since their expression was about 10 to 100 fold less important than the traditional housekeeping genes (See Figure S1 a to c). As already pointed out by Czechowski and collegues [28], references genes having a lower expression than the traditional housekeeping genes affect less the normalization factor to monitor GOIs transcripts level. Additionally, the take-off range of the qRT-PCR amplification curves including all samples from the time course experiments with the three pathogens was more wide for *UBQ10*, than for *SAND* and *TIP41* (See Figure S1d to f). These differences confirm the superior expression stability of *SAND* and *TIP41* compared to *UBQ10*. Thus, we used both *SAND* and *TIP41* as the reference genes to compare the relative quantification and the aqRT-PCR methods to quantify the transcripts accumulation of chosen GOIs.

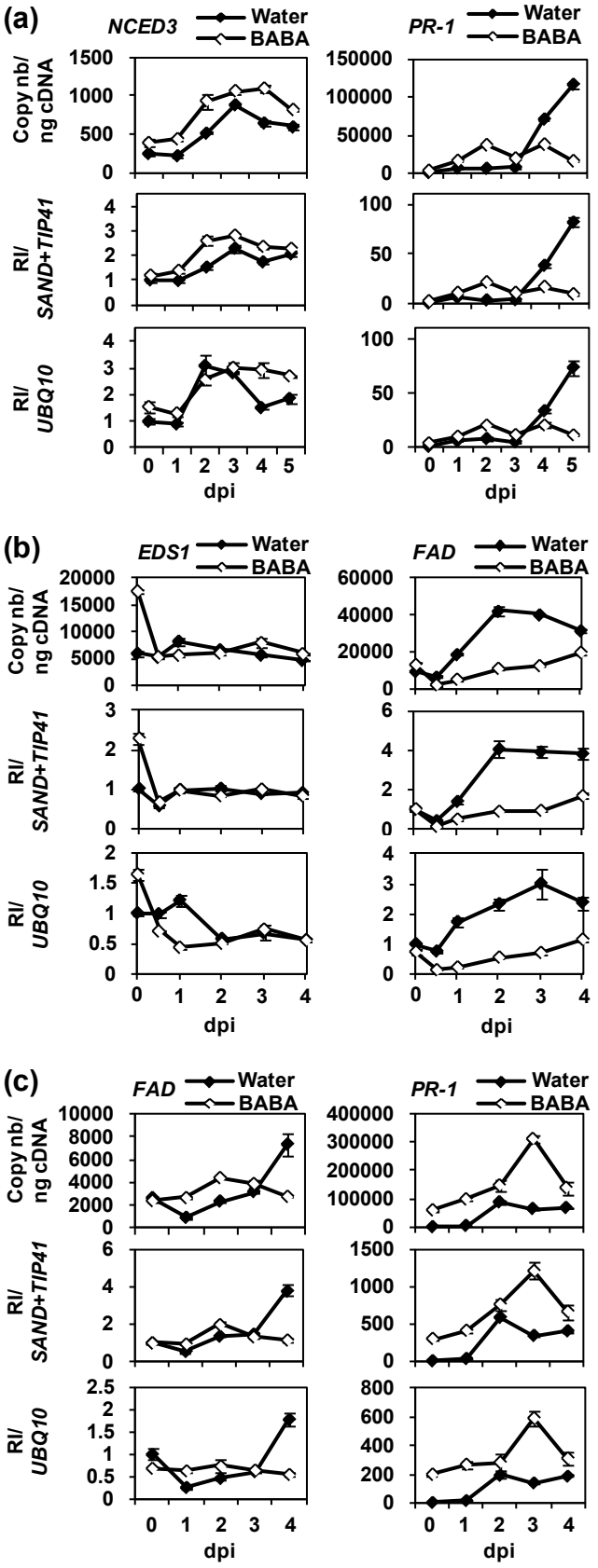

Figure 5. Comparison of GOIs expression by using reference genes or cDNA concentration for the normalization

The expression of several GOIs was compared by using relative qRT-PCR either normalized with both validated reference genes (*SAND+TIP41*) or with one traditional housekeeping gene (*UBQ10*) and with the aqRT-PCR normalized with the quantification of total cDNA. GOIs expression was measured in Arabidopsis plants during BABA-IR and compatible interactions with *Pst*, *H. arabidopsidis* and *B. cinerea* at the indicated times (Days post infection). Absolute quantification in each sample was obtained by the ratio of the copy number of each GOI cDNA per amount of total cDNA. Relative expression was calculated with the software qBase, expressed in relative intensity (RI) and calibrated to the sample treated with water 0 DPI (RI =1). (a) Accumulation of *NCED3* and *PR1* transcripts in Arabidopsis plants challenged with *H. arabidopsidis*. (b) Accumulation of *EDS1* and *FAD* transcripts in Arabidopsis plants challenged with *Pst*. (c) Accumulation of *FAD* and *PR1* transcripts in Arabidopsis plants challenged with *B. cinerea*. Bars represent errors from technical triplicates. The expression of further GOIs is presented in Figure S2 and correlations between the data obtained with the different normalization methods are presented in Table S4.

Comparison between absolute and relative quantification of gene expression

We monitored the transcripts accumulation of five GOIs during time course periods in Arabidopsis plants treated with water or BABA and infected with *Pst*, *H. arabidopsidis* or *B. cinerea*. We chose GOIs which are components or marker genes involved at different steps of the major plant defense pathways controlled by hormones and coding for proteins displaying different functions such as biosynthesis enzymes or direct defenses. *EDS1* encodes a component of the R-gene mediated resistance against *H. arabidopsidis* and functions upstream of the SA signaling pathway [40, 41]. *FAD* encodes a protein with putative protein binding activity and is induced by JA-dependent control of COI1 [42]. *NCED3* encodes for a key enzyme involved in ABA biosynthesis and is induced by virulent *Pst* [43, 44]. *PR-1* is coding for the expression of the pathogenesis-related (PR) 1 protein induced by SA stimuli and is a commonly used marker of the systemic acquired resistance while *PDF1.2* is a plant defensin induced by both JA and ethylene signalling pathways [45-47]. We compared and correlated the quantification of these transcripts either by aqRT-PCR normalized on the total amount of cDNA or by using both *SAND* and *TIP41* as reference genes or *UBQ10* alone (Figure 5, see Figure S2 and Table S4). The relative quantifications of the GOIs transcripts normalized with one or several references genes were calculated using the software qBase that enables to take into account gene specific qPCR efficiency correction and multiple reference genes normalization [48]. Since the expression of *SAND* and *TIP41* was validated as very stable during BABA-IR and compatible interactions of Arabidopsis with the three pathogens tested, the relative quantification of the GOI transcripts level using these two genes was used as a positive control. We also used independently *UBQ10* as a reference gene because it was identified as the most stably expressed gene among the traditional housekeeping genes tested during biotic stresses in a previous study [28] and because it is still commonly used as a unique reference gene.

In most interactions and treatments, both normalization methods enabled to observe similar gene expression profiles during the time course periods and depending on the treatment. In addition, the accumulation measurements of the five GOIs transcripts using the aqRT-PCR method significantly correlated with their relative quantification normalized with both *SAND* and *TIP41* (See Table S4), despite the accumulation of *EDS1* transcripts in water-treated plants infected either by *Pst* or *B. cinerea*. The use of *UBQ10* as a unique reference gene allowed to observed similar profiles of GOIs transcripts accumulation in general. Nevertheless, the correlations analysis indicates that the quantification is generally more accurate when using the aqRT-PCR than using *UBQ10* alone as illustrated by generally lower correlation coefficients of which some were not significant (See Table S4). Furthermore, some differential transcripts accumulation at specific time points were different when using *UBQ10* and especially to normalize the low accumulations observed for *EDS1*, *NCED3* and *FAD* transcripts. These differences were less obvious for *PR-1* and *PDF1.2* transcripts for those the accumulation variations during infections were significantly much higher. These results support that the aqRT-PCR is a reliable method enabling to quantify the accumulation of GOI transcripts as efficiently as the relative quantification using validated references genes. Obviously, and as expected, an improved normalization factor is more important to validate measurements of low transcripts level and for those the induction is weak, than for transcripts which accumulate highly and whose induction is higher.

DISCUSSION

Arabidopsis is a powerful system to dissect the complex network of plant defense pathways controlled by phytohormones [49]. The availability of the sequenced genome [50] and knock-out mutants [51] for most of the coding genes has rendered Arabidopsis a model plant to study the signaling cascades resulting from interactions with pathogens, innate immunity and IR [52, 53]. Thus, accurate and sensitive tools are essential to monitor the parameters of these interactions. The goal of this study was to extend qPCR features in order to develop reliable and universal protocols for the quantification of commonly used pathogens in the field of plant-pathogen interactions in Arabidopsis and to measure accurately the accumulation of GOIs transcripts. Our results demonstrate that the improved qPCR protocols we developed enable to quantify *Pst*, *H. arabidopsidis* and *B. cinerea* colonization in Arabidopsis accurately and rapidly and often more conveniently as the use of standard protocols. We also validated the expression stability of two reference genes to increase the accuracy of plant gene expression measurements in Arabidopsis interacting with these three pathogens during compatible interactions and IR. Additionally, we demonstrated that the normalization of GOIs expression using total cDNA concentration as an internal control is as reliable as the relative quantification using validated reference genes and offers several advantages.

qPCR-based quantification of pathogen development

Quantification of pathogen development is a critical step to link plants phenotypes to their genotypes and to confirm the outcome of interactions with different pathogens. Traditional methods can be tedious, not suited for quantifications at early steps of the infection and not completely representative of pathogen biomass. These time-consuming methods are not always well suited for large scale analyses and often, samples cannot be used for further analyses. Alternatively, collecting whole plants and freezing them in nitrogen for later DNA

extraction followed by qPCR analyses appeared to be more convenient. After the sampling period, samples can be processed at any time. Only small amounts of material are needed for DNA extraction (about 80 mg), and samples can be processed again if needed. The remaining material can be used for RNA or plant hormone extractions thus allowing to directly link plant phenotype with the accumulation of both GOI transcripts and hormone from the same sample. In addition, DNA extraction and qPCR can be done quickly for a large amount of samples by using kits or automatized extractions and qPCR plates preparation with robots. The cost of DNA extraction and qPCR technologies are progressively more affordable especially with the use of Sybr® Green I mix. Furthermore, our qPCR protocol uses a final volume of only 8µL for each measurement requiring only 3µL of Sybr® Green I mix reducing thus the cost of the experiment without reducing qPCR quality. For each pathogen, the method is sensitive and linear enabling to measure accurately very low amounts of pathogens in plant extracts representing at least 10 fold less amount than the initial inocula used (Figure 1, 2 and 3). The quantification remains accurate and linear up to high amounts of pathogen gDNA representing late infection steps. qPCR methods have already been well described previously using similar approaches to quantify the development of several fungi in Arabidopsis by the use of calibration curves [20, 21]. In addition and to our knowledge, only one study has described qPCR technology to quantify bacterial and oomycetes growth in Arabidopsis [19]. However, these methods on which we based our work are still barely used in plant studies even though they overcome certain inconveniencies from which standard methods suffer. In this study we suggest to use plasmids, each containing a specific genomic region from the pathogen or from the plant in order to design calibration curves from which the amounts of pathogen and plant gDNA are determined by qPCR in each sample. This approach appears to be very useful and easy to carry out since the use of plasmids does not change the dynamic range of the quantifications (Figure 1, 2 and 3). The quantification of the plant gDNA is

essential to reduce variations from one sample to another. However, plant gDNA can decay during late steps of necrotrophic pathogen infections due to massive tissue maceration as previously reported [19, 20]. Therefore, results from qPCR-based quantification of necrotrophic pathogens should be interpreted at early steps of infection, especially when inoculated with droplets of inoculum as described for *B. cinerea* (Figure 3c and d) where plants were completely macerated at the end of the infection. Alternatively, the spray inoculation we tested allowed homogenous infections. The initial inocula were about the same as the ones measured with the droplet inoculations but resulted in lower final fungal amount compared to droplet inoculation, probably due to reduced concentrations of fungal spores at specific locations (Figure 3d and e). However, fungal development was accurately monitored by qPCR (Figure 3e). This procedure did not result in complete tissue maceration within 96 hpi and was thus more adapted for aqRT-PCR measurements of transcripts accumulation.

We finally also tested the additional primers designed by Brouwer and colleagues [19] to quantify the growth of *H. arabidopsidis* and *B. cinerea* enabling to obtained similar results than those presented in this study (data not shown).

aqPCR-based quantification of transcripts accumulation

The quantification of gene transcripts is essential to assess the role of specific genes in the process of specific molecular mechanisms. In plant defense studies, the accumulation of several gene transcripts has been shown to be dependent of specific plant pathways controlled by hormones that lead to the virulence of microbial pathogens or resistance of the plant. Thus, monitoring correctly the transcripts level is essential to understand their role in the outcome of the interactions. Accurate quantification relies on the sensitivity of the method and on the normalization step that is required to reduce variations of the total mRNA amount between samples. In plant studies, Northern blotting and semi-quantitative RT-PCR analysis are

progressively replaced by qRT-PCR to monitor the accumulation of specific transcripts. The two first methods were widely used for qualitative analysis of highly expressed genes such *PR-1* in order to illustrate yes or no responses. Thus, the fairly accurate normalizations applied to these methods, mainly based on the quantification of total RNAs and/or on the quantification of traditional housekeeping genes appeared not to be critical for the accuracy of the results [24]. qRT-PCR is the most sensitive and powerful method to monitor the accumulation of gene transcripts [23]. In plant studies this method has already been successfully used to quantify very weakly expressed genes [54]. However, it becomes essential to give special attention to the normalization step that should systematically rely on validated reference genes for each experimental condition since the use of traditional housekeeping genes can lead to misinterpretations of the results [24, 35]. Therefore, we re-evaluated the expression stability of several traditional housekeeping genes compared to new reference genes in Arabidopsis plants challenged with *Pst*, *H. arabidopsidis* and *B. cinerea* during compatible interactions or BABA-IR by using a robust and well-recognized algorithm. This analysis was performed over representative and wide time course periods for each pathogen including the day of inoculation until full infection enabling to evaluate the expression stability of the reference genes tested across the infection process and establishment of resistance. According to the results obtained with the software geNorm, *SAND* and *TIP41*, two of the new reference genes, were ranked as the most stably expressed genes during both compatible interaction and BABA-IR in plants, respectively, infected with the three pathogens tested while the traditional housekeeping genes were always ranked behind (Figure 4). In addition, the two new reference genes were also ranked as the most stable by analyzing separately data from compatible interactions or BABA-IR against *Pst* and *H. arabidopsidis,* respectively, or by analyzing all the data including the three pathogens and both kind of interactions. The use of these two genes appeared to be sufficient to obtain a

satisfying normalization factor in our experimental conditions. Interestingly, *SAND* was also found to be one of the most stably expressed reference gene among new and traditional housekeeping genes tested in Arabidopsis exposed to increased heavy metals concentrations [39]. Thus it appears that this gene is quite appropriate to be used for the normalization of gene expression in various experimental designs. However and unexpectedly, the gene *CACTA*, identified by Czechowski and colleagues [28] as the most stably expressed gene during biotic stresses by using large public set of Affymetrix studies and that is even lowly expressed than *SAND* and *TIP41* was always ranked behind traditional housekeeping genes in our conditions. This finding, in addition with recent reports [24, 25], supports the necessity to validate systematically and specifically the stability of supposed reference genes for qPCR analysis in specific experimental designs.

Reference genes are used as normalizers because their stable expression is supposed to represent the constant total mRNA concentration in every sample. Thus, an accurate normalization should be obtained by the quantification of the total cDNA amount in each sample. This method has been successfully used to measure the absolute quantity of specific gene transcripts in human samples [29]. It is based on the specific quantification of double-stranded DNA molecules in each cDNA sample. We compared the accuracy of this method with the relative quantification method using the two validated reference genes or only one commonly used traditional housekeeping gene. We measured the accumulation of five GOIs transcripts involved in plant defenses pathways in Arabidopsis plants challenged with *Pst*, *H. arabidopsidis* and *B. cinerea* and during compatible interactions or BABA-IR over representative time course periods (Figure 5, see Figure S3 and Table S4). We measure the levels of highly and lowly transcribed genes displaying different functions and we compared the effect of the normalization procedure on the results. Interestingly, the absolute quantification was easy to use and gave similar results as the use of the two validated stable

reference genes. Additionally, this method gave almost always improved reliable results compared with the use of one traditional housekeeping gene, especially for the transcripts that were lowly accumulated and displaying low level variations. The aqRT-PCR method provides absolute values of the transcripts accumulation which are expressed in copy number of a specific transcript per quantity of the total amount of cDNA. The use of standard curses that take into account the amplification efficiencies allow a precise observation of both basal transcripts accumulation and their induction levels in plants interacting with different pathogens and submitted to different treatments beyond the basic fold change expression related to a calibrator sample (relative expression = 1) that is obtained using relative quantification. The principal inconvenience of the method is the required cloning step of the PCR product from the cDNA of each gene of which the expression needs to be analyzed. However, it appeared that this procedure was quite simple regarding the relative short time spent to clone little RT-PCR fragments. However, the normalization procedure using total cDNA amount could be biased by the presence in the RNA fraction of non-host mRNA belonging to eukaryotic pathogens. mRNA polyadenylation is a widespread and special feature in eukaryotic organisms that can however occurs in prokaryotic organism but is more likely to promote RNA degradation [55, 56]. Thus bacterial mRNA in plants would barely be reverse transcribed and false the aqRT-PCR normalization. However, we addressed the question by analyzing the plant gDNA concentration in the total DNA extracts across the infection process of the three pathogens (See Figure S3). The concentration of the *AtTUB4* gene in the extracts decreased at the same time as the biomasses of *Pst* and *B. cinerea* inoculated with droplets increased in the plant tissue. On the contrary, it stayed constant in samples infected with *H. arabidopsidis* and *B. cinerea* inoculated by spray. These results correlate well with the quantifications of each pathogen development (Figure 1 - 3) showing important ratios pathogen gene/plant gene at late stages of infection with *P. syringae* (31) and

B. cinerea inoculated with droplets (3) while it stayed relatively low in plants infected with *H. arabidopsidis* (0.13) and *B. cinerea* inoculated by spray (0.17). This illustrates the pathogen cell number per plant cell at the end of the infection. Thus, one could expect a relatively important effect of *B. cinerea* mRNAs in plant tissues inoculated with droplets that could be extracted and reverse transcribed at the same time as plant mRNAs and thus biasing the normalization of the aqRT-PCR. However, these results have to take into account the genome size and number of coding genes representing the supposed transcriptome state of Arabidopsis and *B. cinerea* that is quite more important for Arabidopsis (See Table S5). Thus, all gene transcripts accumulation monitored during interactions between Arabidopsis and *B. cinerea* (Figure 5) were realized in plants inoculated by spray with the fungus. Furthermore, the accumulation of all gene transcripts in plants inoculated by spray with *B. cinerea* and analyzed by aqRT-PCR correlated well with the relative quantification normalized with both validated references genes. This illustrates the relatively weak effect of pathogen mRNAs on the normalization.

CONCLUSIONS

In our hands, the qPCR method enabled to follow accurately the development of *Pst*, *H. arabidopsidis* and *B. cinerea* in Arabidopsis over representative time course periods and to perform statistical analyses that are required to confirm plant phenotypes, treatment efficiency, and the outcome of the interactions. As for the best quantification methods, experiments need to be repeated to confirm the results trend and our protocol enabled a good reproducibility of the results. We decided not design our own primers because the sensitivity obtained was sufficient to measure the pathogens growth in the conditions tested. Nonetheless, the protocol presented in this study could easily be adapted for the use of other primers or with fluorescent primers- and probe-based chemistry that are more sensitive than

the simple DNA-binding dye we used. The methodology we established is easy to carry out and to process, it permits a gain of time and renders less tedious some difficult aspects from which suffer traditional pathogen quantifications. In conclusion, this universal protocol brings together the requirements of sensitivity, accuracy, rapidity and simplicity that render it ideal to be used for routine experiments as well as for large scale analysis. It could easily be adapted to other plants species to measure the growth of any microbial pathogens.

Similarly, the aqRT-PCR method, based on the association of standard curves and the normalization on total cDNA amounts, allows the accurate and reliable quantification of the basal GOIs transcripts accumulation as well as their level changes during specific interactions. Furthermore, it allows to compare independent experiments without the need for a calibrator sample, and the transcripts accumulation between each other beyond their relative fold change intensity. In addition, the final results are obtained more easily compared with the relative quantifications that need the use of complex softwares that takes into account the expression of several reference genes. Another advantage is that this technique can be transposed to every system, thus avoiding the systematic need to validate reference genes for specific experimental designs.

METHODS

Biological material

Wild-type *Arabidopsis thaliana* accession Col-0 was obtained from Lehle Seeds (Round Rock, TX). The Col-0 mutants *npq2-1* was obtained from the Nottingham Arabidopsis Stock Centre (Nottingham,UK). Col-0 mutants *jin1* and *sid2-1* were kindly provided by V. Flors (University of Jaume I, Spain) and C. Nawrath (University of Lausanne, Switzerland), respectively. Wild-type *A. thaliana* accession Ws-0 was kindly provided by John V. Dean (DePaul University, USA). One plant per pot for *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) or *Botrytis cinerea* bioassays and about 30 plants per pot for *Hyaloperonospora arabidopsidis* bioassays were grown in 30 mL Jiffy® peat tablets (Ryomgaard, Denmark), maintained at 20°C day/18°C night temperature with 10 h of light (150 µmol m^{-2} s^{-1}) per 24 h and 70% RH. The virulent strain of *Pst* [4] was grown during 12 to14 hours at 28°C in liquid King's medium B containing 50 µg/mL of rifampicine for the selection. *Pst* strains DC3000 *hrpA* A9 and DC3118 coronatine$^-$ were kindly provided by Sheng Yang He (Michigan State University, USA) and grown in King's medium B containing 50 µg/mL of rifampicine and kanamycin for the selection. *H. arabidopsidis* isolate NOCO was grown as described previously [57]. *B. cinerea* strain BMM1 was grown as described previously [34].

Plant inoculation and sampling procedure

β-aminobutyric acid (BABA) treatment (250 µM) or water (control) were applied as soil drench 48 hours before pathogen inoculation on five-week-old plants for *P. syringae* or *B. cinerea* bioassays as described previously [34, 57]. BABA treatment (120 µM) or water (control) were applied similarly on two-week-old seedlings for *H. arabidopsidis* bioassays as described previously [57].

For the bioassays with *P. syringae*, plants were inoculated by dipping the leaves in a suspension of *Pst* DC3000 or strains DC3000 *hrpA* A9 and DC3118 coronatine$^-$ containing 5 x 10^7 CFU/mL in 10 mM $MgSO_4$ and 0.03% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX). IGC in plants were determined by collecting 5 leave discs from individual leaves from one plant with an 8 mm diameter cork borer at the indicated time points. The remaining plant materials were directly sampled and frozen in nitrogen for later gDNA and RNA extraction. Leave discs were then disrupted and homogenized in 10 mM $MgSO_4$. Serial dilutions were plated on selective King's medium B agar supplemented with 50 mg/L of the appropriate antibiotics according to the bacterial strain used as described above. After incubation at 28°C the number of resistant CFU per surface of infected plant was determined and bacterial proliferation over the 4 day time course was calculated. The operation was repeated with 4 individual plants for each time point, treatment and bacterial strain. Four days after inoculation, the percentage of diseased leaves presenting symptoms was determined on 8 plants for each treatment or bacterial strain. Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased.

For the bioassays with *H.* arabidopsidis, seedlings were inoculated by spraying a suspension containing 5 x 10^4 conidiospores per mL in 10 mM $MgSO_4$ short of runoff. Plants were kept at 100% RH for 1 day to ensure infection. At the indicated time points, seedlings from one pot were collected and directly frozen in nitrogen for later gDNA and RNA extractions. The experiment was repeated 3 times for each treatment, time point or Arabidopsis mutant. Four days post inoculation plants were placed under 100% RH to induce the sporulation of the oomycete. The next day, seedling plants from one pot were collected and weighted before being dipped in a 10 mM $MgSO_4$ in order to gather the conidiospores. The sporulation rate was determined by counting twice the number of conidiospores in a Neubauer-improved counting chamber (Marienfeld, Lauda-Königshofen, Germany) and expressed as the number

of conidiospores per g of seedlings. The operation was repeated with 2 individual seedling pots for each treatment.

For the quantification of *B. cinerea* growth inoculated with droplets, 5 leaves of each plant were inoculated with a 5 µL droplet containing 1 x 10^5 conidia per mL in PDB (12 g/L) or full plant leaves were sprayed short of runoff with the same inoculums. Plants were kept immediately at 100% RH for the rest of the experiment to ensure fungus penetration. At the indicated time points, 5 leave discs surrounding the droplet inoculation sites were collected using a 8 mm diameter cork borer and directly frozen in nitrogen for later total gDNA extraction. The macroscopic evaluation of *B. cinerea* growth was evaluated by measuring the diameter of the lesions from 5 leaves on 4 individual plants 4 days post inoculation. Results were expressed as necrosis size in square millimeters. For the quantification of *B. cinerea* growth inoculated by spray and for the quantification of gene expression, the inoculation was performed by spraying each plant until droplet runoff with a suspension of 1 x 10^5 conidia per mL in PDB (12 g/L). Plants were kept immediately at 100% RH for the rest of the experiment to ensure fungus penetration. At the indicated time points, 4 plants were collected and pooled together for each treatment condition and frozen into nitrogen for later total gDNA and RNA extractions.

Isolation of gDNA, RNA and cDNA biosynthesis

For the bioassays with *H. arabidopsidis* and the quantification of *B. cinerea* (droplet inoculation) growth where the amount of plant material collected was not important, grinding and homogenization of the collected samples were performed by using a 10 mm grinding ball directly in the 12 mL vials and by hand shaking during 15 sec. For the bioassays with *P. syringae* and *B. cinerea* inoculated by spray, grinding and homogenization of the samples

were performed with a Qiagen® tissue lyzer in 10 mL grinding jars with 20 mm grinding balls during 30s at maximum speed.

For the PCR-based quantification of pathogens, 80 mg of finely powdered leaves were submitted to an additional disruption step during 1 min at maximum speed in vials containing a few 1 and 2 mm glass beads and by using a Qiagen® tissue lyzer. Total gDNA was then extracted using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. Total gDNA was finally gathered by 2 elution steps using 200 µL of the TAE buffer.

For the construction of the PCR standard curves using pure pathogen gDNA, samples from *Pst* and *B. cinerea* were isolated from *in vitro* cultures as described above. gDNA from *H. Arabidopsis* was extracted from conidiospores washed from infected Arabidopsis leaves with 10 mM $MgSO_4$ buffer. The conidiospore suspension was filtered on gauze and then purified twice by centrifugation at 5000 rpm during 5 min and suspended in 10 mM $MgSO_4$ buffer.

For the quantification of gene expression, ground samples from the biological replicates were homogenized together. Total RNA was isolated from about 80 mg of the plant material using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. cDNA was obtained from 2 µg of RNA using oligo$(dT)_{18}$ and Superscript III reverse transcriptase (Invitrogen) following the instructions of the manufacturer and then diluted to a final volume of 200 µL in sterile water.

Quantification of gene expression

For each gene, the corresponding cDNA were transformed in the pGEM®-T easy Vector (Promega). Primers were designed with Primer 3© excepted for *NCED3* and *ACT2* [43]. Copy number of each clone was calculated as follows: Copy number (molecule/g) = molecular weight of the full (plasmid + cDNA) dsDNA sequence (g/mol) / Avogadro's

number. Concentration of each clone solution was performed using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) according to the manufacturer's guidelines. Briefly, 10 µL of each clone solution were diluted with 40 µL of TE 1X buffer and 50 µL of 200 times diluted PicoGreen® in 96 ELISA well plates. Standard DNA provided by the manufacturer (Invitrogen) was used to construct a calibration curve (from 0 to 500 ng/mL). After a 2 min waiting period, samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instrument). Fluorescence emission intensity was then plotted versus DNA concentration.

Quantification of gene expression was performed by real time PCR in a Rotor Gene 6000 (Corbett Life Science) and plates were prepared with the ROBOT CAS1200 (Corbett Robotics). 2µL of diluted cDNA were amplified using 3µL of SensiMixPlus SYBR Kit (Quantace) with 250 nM primers in a final volume of 8 µL adjusted with sterile water. Cycling conditions were 95°C for 10 min followed by 40 cycles at 95°C for 10s, 60°C for 15s and 72°C for 20s, followed by a melting curve analysis (the negative first derivative of the fluorescence is plotted as a function temperature) from 55°C to 95°C. For each GOI, a standard curve (Copy number/log Ct) was constructed using serial dilutions of known copy number of the corresponding clone (10^8 to 10^1 copies/µL). Concentration of total cDNA in each sample was performed using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) as described below. aqRT-PCR results of GOI expression were expressed as copy number of a specific transcript per ng of total cDNA. Since technical triplicates were performed for both PCR and cDNA quantification, the combined error was calculated as: (mean of the number of target cDNA/mean total cDNA) × ((S.D. of number of target cDNA /mean number of target cDNA) + (S.D. of total cDNA /mean total cDNA)).

The stability of the reference gene expression was tested with geNorm v3.4 by using unnormalized raw data expressed in transcript copy number per µL of cDNA. The relative expression of the 5 GOIs was normalized either with both *SAND* and *TIP41,* or with *UBQ10* alone by using the software qBase [48] that takes into account gene specific PCR efficiency and multiple reference genes normalization. To measure the plant gDNA stability, total gDNA from infected leaves was measured using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) as described below.

Quantification of pathogens growth

Quantifications of pathogen and plant gDNA were performed by real-time PCR with 2 µL of diluted gDNA by using the same protocol as for the quantification of gene expression. Standard curves of specific genes (pathogens and plant) were constructed with clones obtained as described for cDNA standard curves or by using pure pathogens gDNA. Primers were taken according to Brouwer et al. (2003) except for the At*TUB4*and *AtCACTA* genes for those the primers were designed with Primer 3©. PCR results for the pathogen quantification were expressed as copy number of the pathogen gene per copy number of the plant gene.

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SUPPLEMENTARY MATERIALS

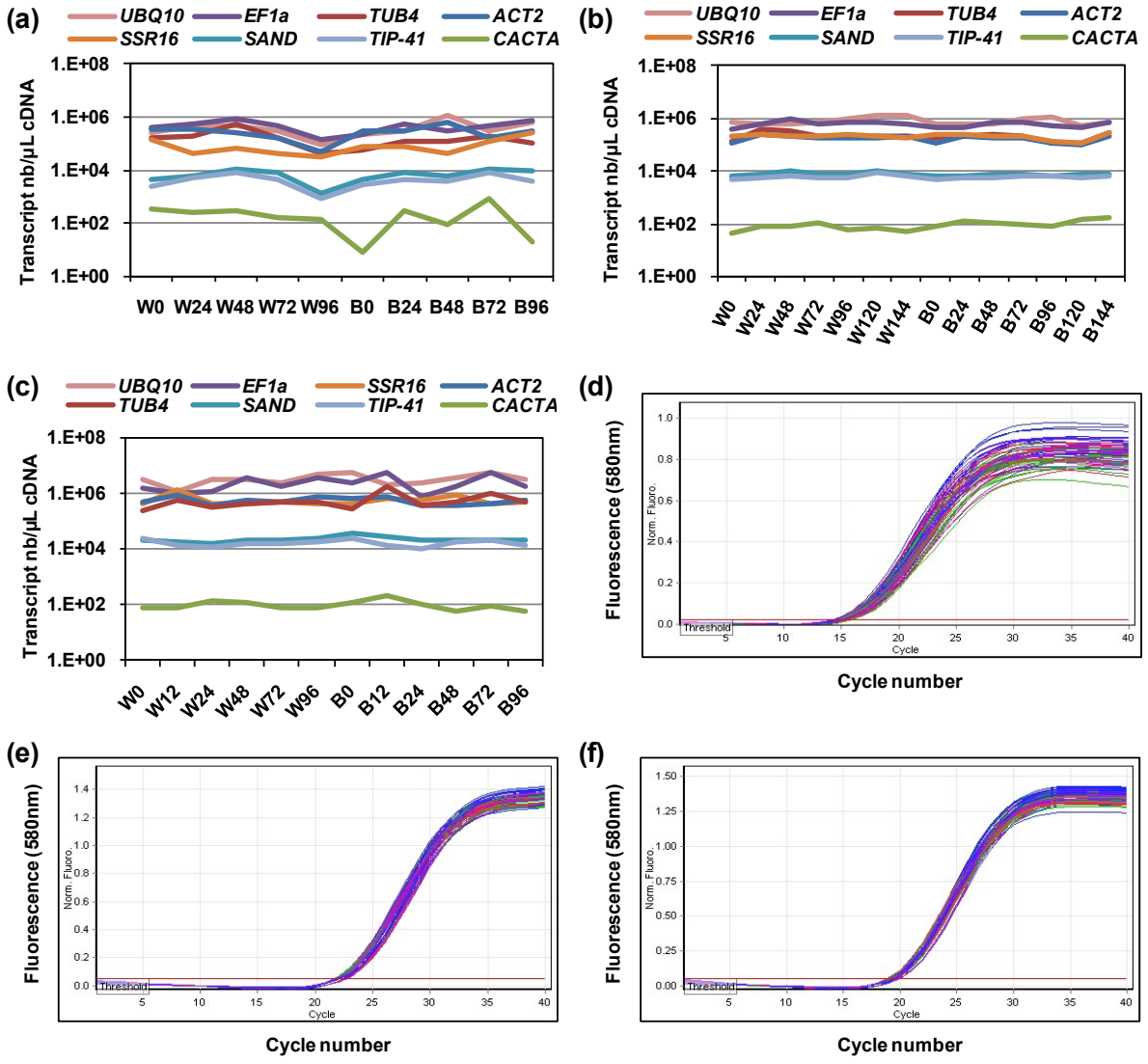

Figure S1. Quantification of the level of reference genes transcripts by qRT-PCR in Arabidopsis during BABA-IR and compatible interactions with *Pst*, *H. arabidopsidis* and *B. cinerea.*

Quantification of the transcripts level of the 8 reference genes tested in Arabidopsis plants challenged with *Pst* (a), *H. arabidopsidis* (b) and *B. cinerea* (c). Quantifications of the transcripts level were not normalized and expressed in transcripts number per µL of cDNA. Water (W) or BABA (B) treatments, and times post infections are indicated for each sample under the graphs. qPCR amplification curves of the transcripts *UBQ10* (d), *SAND* (e) and *TIP-41* (f) over 40 cycles corresponding to all the samples presented in A, B and C from water- and BABA-treated plants challenged with *Pst*, *H. arabidopsidis* or *B. cinerea*.

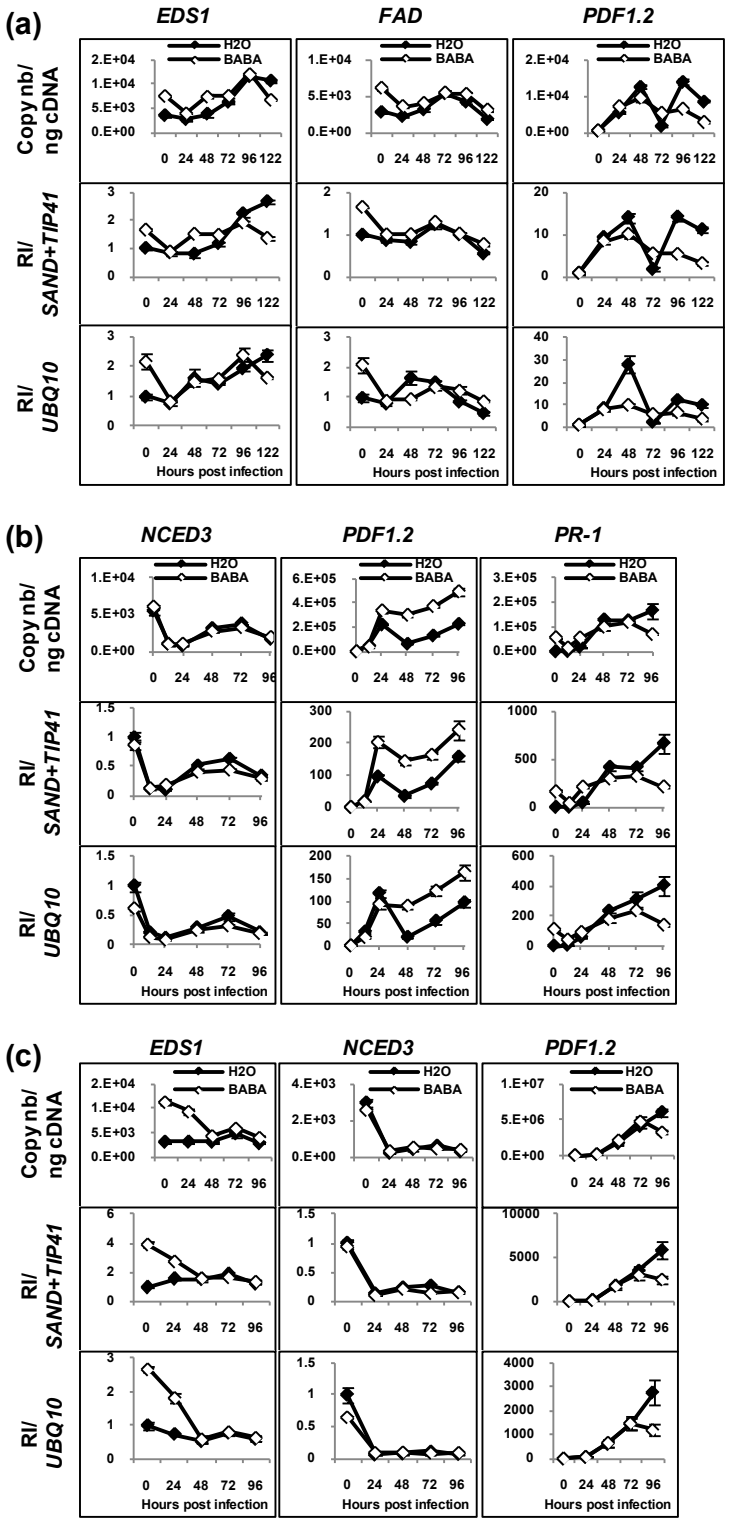

Figure S2. Expression of additional GOIs by qRT-PCR. For more information see legend of Figure 5.
(a) Accumulation of *EDS1, FAD* and *PDF1.2* transcripts in Arabidopsis plants challenged with *H. arabidopsidis*. (b) Accumulation of *NCED3, PDF1.2* and *PR-1* transcripts in Arabidopsis plants challenged with *Pst*. (c) Accumulation of *EDS1, NCED3* and *PDF1.2* transcripts in Arabidopsis plants challenged with *B. cinerea*. Bars represent errors from technical triplicates. Correlation between the normalization methods are presented in the Table S4.

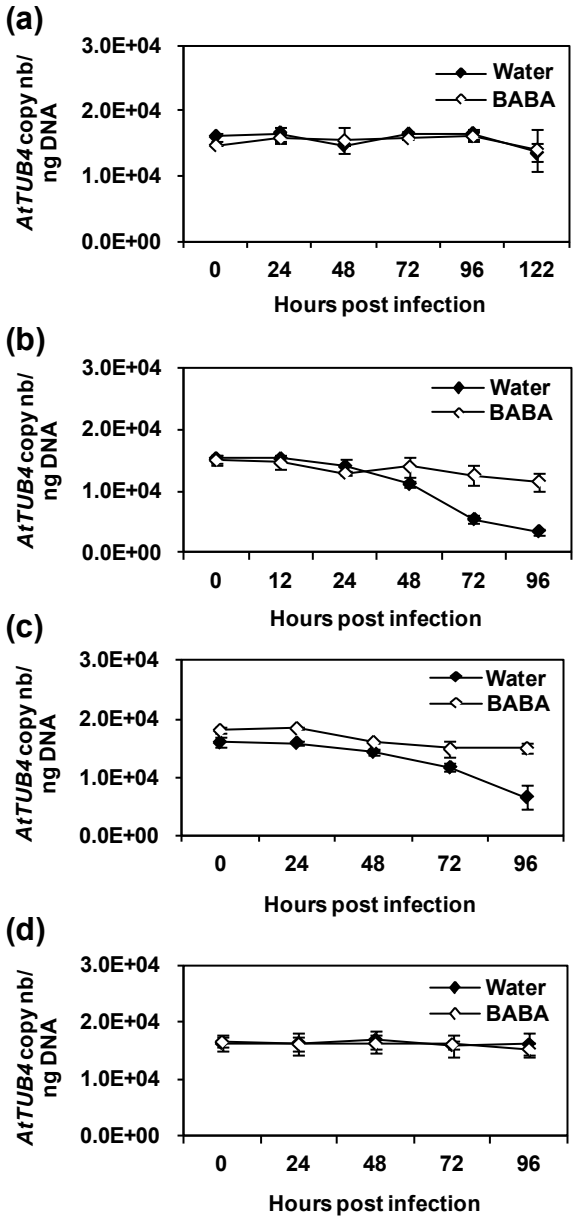

Figure S3. Stability of the concentration of plant gDNA in total DNA extracts from Arabidopsis plants infected with pathogens *Pst*, *H. Arabidopsidis*, and *B. cinerea*.

Concentration of Arabidopsis gDNA in each extract was measured by the ratio of the copy number of the *AtTUB4* gene quantified by qPCR and the total amount of total DNA quantified by spectrofluorescence and using the dye PicoGreen®. *AtTUB4* concentration was measured in total DNA extracts from Arabidopsis plants treated with water or BABA and challenged with *H. arabidopsidis* (a), *Pst* (b), and *B. cinerea* inoculated with droplets (c) or by spray (d). Data for each time point represent the average ±SD from 4 (b, c and d) or 3 (a) biological replicates.

Table S1. Gene-specific primers used for the qPCR quantifications of pathogens and GOIs expression

Primers used for pathogens DNA cloning and for qPCR quantifications

Name	Organism	Origin	Primer forward	Primer reverse
AtTUB4	*A. thaliana*	*TUB4* gene	GCGAACAGTTCACAGCTATGTTCA	GAGGGAGCCATTGACAACATCTT
Bctubu	*B. cinerea*	tubulin gene	CCGTCATGTCCGGTGTTACCAC	CGACCGTTACGGAAATCGGAAG
HaAW	*H. arabidopsidis*	AW737077 (GenBank accession number)	CACGTCCCTTATGTCGAGG	CAAGTTGTCGCAGATCACG
Psoprf	*Pst* DC3000	*oprf* gene	AACTGAAAAACACCTTGGGC	CCTGGGTTGTTGAAGTGGTA

Primers used for reference genes cloning and for qPCR quantifications

Name	AGI	Funtion	Primer forward	Primer reverse
ACT2	AT3G18780	Structural constituent of cytoskeleton	AGTGGTCGTACAACCGGTATTGT	GATGGCATGAGGAAGAGAGAAAC
CACTA-like	AT1G42220	Unknown	GGAACCTCATTCATGGACTTTT	TCCTGGATGACACTTCCTTGT
EF1alpha	AT5G60390	Translation elongation factor activity	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA
SAND	AT2G28390	Unknown	GGATTTTCAGCTACTCTTCAAGCTA	CTGCCTTGACTAAGTTGACACG
SSR16	AT4G34620	Structural constituent of ribosome	ACAAATCGAGGTGTTAGGCTTT	GGTTGAGCTCCAACAGATAACC
TIP41-like	AT4G34270	Unknown	GAACTGGCTGACAATGGAGTG	ATCAACTCTCAGCCAAAATCG
TUB4	AT5G44340	Structural constituent of cytoskeleton	GCGAACAGTTCACAGCTATGTTCA	GAGGGAGCCATTGACAACATCTT
UBQ10	AT4G05320	Protein binding targeting for degradation	GGCCTTGTATAATCCCTG	AAAGAGATAACAGGAACGGAAACATAG

Primers used for the cloning of GOIs involved in plant defence responses and for qPCR quantifications

Name	AGI	Annotation	Primer forward	Primer reverse
EDS1	AT3G48090	Component of R gene-mediated disease resistance	CTCAATGACCTTGGAGTGAGC	TCTTCCTCTAATGCAGCTTGAA
FAD binding	AT2G34810	Response to jasmonic acid stimulus	CCGTCACCGAAACTCACA	TCCAAACGGTTTCGTCTT
NCED3	AT3G14440	Abscisic acid biosynthetic process	CGTCTTCTCAAAGCTCCGAC	TGAATCTTCGGCGTATTTGTCT
PR-1	AT2G14610	Systemic acquired resistance marker	AAAACTTAGCCTGGGGTAGCGG	CCACCATTGTTACACCTCACTTTG
PDF1.2a	AT5G44420	Response to ethylene and jasmonic acid stimulus	AGAAGTTGTGCGAGAAGCCAAG	GTGTGCTGGGAAGACATAGTTGC

Table S2. qRT-PCR raw data used for the evaluation of the reference genes expression stability

qRT-PCR raw data used to evaluate the expression stability of 8 reference genes during BABA-IR and compatible interactions with *Pst* , *H. arabidopsidis* and *B. cinerea* with geNorm. Labels of the samples including the time of infection and treatments are listed. Pathogens and genes name are indicated. Raw data are expressed in gene copy number per µL of cDNA samples

***Pst* time course infection**

Sample list	*ACT2*	*CACTA*	*EF1a*	*SAND*	*SSR16*	*TIP-41*	*TUB4*	*UBQ10*
Col-0 Water t0	3.27E+05	3.47E+02	4.31E+05	4.41E+03	1.42E+05	2.32E+03	1.64E+05	2.56E+05
Col-0 Water t24	3.52E+05	2.66E+02	5.25E+05	6.41E+03	4.32E+04	5.02E+03	1.93E+05	5.11E+05
Col-0 Water t48	2.78E+05	2.98E+02	8.87E+05	1.14E+04	6.85E+04	7.89E+03	5.15E+05	4.84E+05
Col-0 Water t72	1.60E+05	1.76E+02	4.97E+05	8.20E+03	4.32E+04	4.40E+03	1.76E+05	3.20E+05
Col-0 Water t96	5.20E+04	1.37E+02	1.47E+05	1.31E+03	3.35E+04	8.93E+02	4.30E+04	9.69E+04
Col-0 BABA t0	2.89E+05	8.53E+00	2.18E+05	4.31E+03	8.37E+04	2.88E+03	6.02E+04	2.10E+05
Col-0 BABA t24	3.20E+05	2.81E+02	5.41E+05	7.83E+03	8.02E+04	4.67E+03	1.27E+05	3.06E+05
Col-0 BABA t48	6.18E+05	8.53E+01	3.07E+05	6.23E+03	4.16E+04	4.08E+03	1.31E+05	1.14E+06
Col-0 BABA t72	1.69E+05	8.10E+02	4.81E+05	1.04E+04	1.31E+05	7.61E+03	1.94E+05	3.01E+05
Col-0 BABA t96	3.16E+05	2.10E+01	7.45E+05	9.26E+03	2.47E+05	4.10E+03	1.08E+05	6.41E+05
jin1 Water t0	3.99E+05	5.19E+02	1.76E+06	1.62E+04	2.21E+05	3.27E+03	4.86E+05	9.49E+04
jin1 Water t24	6.58E+05	5.20E+02	8.62E+05	1.29E+04	1.32E+05	3.31E+03	3.14E+05	1.78E+05
jin1 Water t48	6.46E+05	3.93E+02	9.33E+05	1.59E+04	1.04E+05	4.99E+03	3.24E+05	2.27E+05
jin1 Water t72	4.04E+05	3.06E+02	1.05E+06	1.51E+04	1.22E+05	5.25E+03	4.58E+05	2.26E+05
jin1 Water t96	4.18E+05	6.89E+02	1.44E+06	1.58E+04	1.42E+05	5.44E+03	4.87E+05	1.92E+05
jin1 BABA t0	3.68E+05	3.63E+02	1.38E+06	3.10E+04	2.10E+05	9.28E+03	2.45E+05	2.38E+05
jin1 BABA t24	4.15E+05	4.73E+02	7.64E+05	1.61E+04	2.13E+05	6.28E+03	1.85E+05	2.30E+05
jin1 BABA t48	6.57E+05	1.64E+02	8.19E+05	2.34E+04	1.39E+05	7.10E+03	1.82E+05	3.60E+05
jin1 BABA t72	6.60E+05	7.98E+01	6.99E+05	7.48E+03	1.68E+05	5.81E+03	1.22E+05	2.97E+05
jin1 BABA t96	7.78E+05	4.63E+01	4.57E+05	8.22E+03	1.41E+05	3.91E+03	1.37E+05	3.29E+05
npq2 Water t0	4.13E+05	2.80E+02	3.04E+06	2.68E+04	3.84E+05	5.92E+03	5.55E+05	1.71E+05
npq2 Water t24	4.31E+05	3.56E+02	1.49E+06	1.53E+04	2.45E+05	6.04E+03	5.34E+05	1.51E+05
npq2 Water t48	6.85E+05	3.63E+02	1.82E+06	2.73E+04	2.27E+05	9.46E+03	5.85E+05	2.45E+05
npq2 Water t72	4.22E+05	1.53E+02	1.05E+06	2.12E+04	1.41E+05	5.42E+03	4.50E+05	2.08E+05
npq2 Water t96	6.24E+05	5.70E+02	1.51E+06	1.49E+04	2.35E+05	6.37E+03	5.57E+05	2.16E+05
npq2 BABA t0	2.33E+05	2.44E+02	1.50E+06	2.08E+04	3.83E+05	1.01E+04	2.24E+05	1.81E+05
npq2 BABA t24	3.83E+05	2.63E+02	4.63E+05	7.42E+03	1.48E+05	6.19E+03	1.33E+05	2.82E+05
npq2 BABA t48	4.38E+05	1.02E+02	8.76E+05	1.09E+04	2.37E+05	6.73E+03	2.55E+05	3.04E+05
npq2 BABA t72	6.65E+05	1.51E+02	3.62E+05	1.88E+03	1.07E+05	1.75E+03	7.66E+04	2.02E+05
npq2 BABA t96	4.42E+05	2.97E+02	9.56E+05	1.52E+04	2.72E+05	7.66E+03	3.48E+05	1.90E+05

***B. cinerea* time course infection**

Sample list	*ACT2*	*CACTA*	*EF1a*	*SAND*	*SSR16*	*TIP-41*	*TUB4*	*UBQ10*
Col-0 Water t0	4.59E+05	7.94E+01	1.59E+06	2.07E+04	4.51E+05	2.28E+04	2.36E+05	3.01E+06
Col-0 Water t12	8.91E+05	7.75E+01	1.02E+06	1.88E+04	1.38E+06	1.29E+04	5.34E+05	1.08E+06
Col-0 Water t24	3.65E+05	1.34E+02	1.10E+06	1.55E+04	3.93E+05	1.13E+04	3.27E+05	3.02E+06
Col-0 Water t48	5.28E+05	1.18E+02	3.47E+06	2.16E+04	3.94E+05	1.58E+04	4.15E+05	3.25E+06
Col-0 Water t72	4.73E+05	7.90E+01	1.71E+06	2.15E+04	4.64E+05	1.55E+04	5.15E+05	2.43E+06
Col-0 Water t96	7.60E+05	7.33E+01	3.87E+06	2.56E+04	4.02E+05	1.76E+04	4.80E+05	4.80E+06
Col-0 BABA t0	6.72E+05	1.22E+02	2.49E+06	3.49E+04	3.99E+05	2.55E+04	2.86E+05	5.80E+06
Col-0 BABA t12	7.38E+05	2.17E+02	5.39E+06	2.77E+04	6.88E+05	1.44E+04	1.83E+06	2.13E+06
Col-0 BABA t24	3.57E+05	1.02E+02	7.71E+05	2.14E+04	5.37E+05	9.74E+03	3.46E+05	2.21E+06
Col-0 BABA t48	3.70E+05	5.78E+01	1.85E+06	2.07E+04	8.05E+05	1.79E+04	4.56E+05	3.46E+06
Col-0 BABA t72	4.26E+05	9.43E+01	5.80E+06	2.06E+04	4.26E+05	2.02E+04	9.62E+05	5.47E+06
Col-0 BABA t96	5.43E+05	5.47E+01	1.84E+06	2.03E+04	5.12E+05	1.43E+04	4.79E+05	2.94E+06

***H. arabidopsidis* time course infection**

Sample list	*ACT2*	*CACTA*	*EF1a*	*SAND*	*SSR16*	*TIP-41*	*TUB4*	*UBQ10*
Col-0 Water t0	1.24E+05	4.54E+01	3.98E+05	6.49E+03	2.19E+05	4.90E+03	1.39E+05	7.17E+05
Col-0 Water t24	2.46E+05	8.52E+01	6.68E+05	7.51E+03	2.42E+05	5.87E+03	4.03E+05	6.17E+05
Col-0 Water t48	2.23E+05	8.23E+01	9.95E+05	1.11E+04	2.07E+05	6.91E+03	3.41E+05	6.66E+05
Col-0 Water t72	1.89E+05	1.15E+02	6.60E+05	7.73E+03	2.31E+05	6.04E+03	2.25E+05	7.42E+05
Col-0 Water t96	1.94E+05	5.75E+01	7.07E+05	7.98E+03	2.59E+05	5.80E+03	2.11E+05	1.03E+06
Col-0 Water t120	1.93E+05	6.54E+01	7.12E+05	1.12E+04	2.01E+05	9.41E+03	2.15E+05	1.26E+06
Col-0 Water t144	2.05E+05	4.97E+01	6.36E+05	7.27E+03	1.89E+05	6.14E+03	2.22E+05	1.25E+06
Col-0 BABA t0	1.24E+05	8.02E+01	4.67E+05	6.34E+03	2.46E+05	5.09E+03	1.91E+05	6.54E+05
Col-0 BABA t24	2.20E+05	1.24E+02	4.33E+05	6.50E+03	2.40E+05	5.46E+03	2.20E+05	5.90E+05
Col-0 BABA t48	1.98E+05	1.13E+02	7.32E+05	7.36E+03	2.30E+05	5.91E+03	2.51E+05	5.85E+05
Col-0 BABA t72	1.96E+05	8.77E+01	7.34E+05	7.78E+03	2.03E+05	6.96E+03	2.30E+05	1.01E+06
Col-0 BABA t96	1.26E+05	8.27E+01	5.58E+05	6.87E+03	1.31E+05	6.78E+03	1.26E+05	1.08E+06
Col-0 BABA t120	9.97E+04	1.49E+02	4.41E+05	7.66E+03	1.13E+05	5.56E+03	1.16E+05	5.42E+05
Col-0 BABA t144	2.22E+05	1.71E+02	7.36E+05	7.82E+03	2.70E+05	6.13E+03	2.75E+05	7.08E+05

Table S3. Complete geNorm analysis of the expression stability of 8 reference genes

The expression stability of 8 reference genes was calculated in wild -type Arabidopsis during BABA-IR and compatible interaction with *H. arabidopsidis* and *B. cinerea* and in wild-type Arabidopsis and the mutants *jin1* and *npq2* during BABA-IR and compatible interaction with *Pst* . Ecotype, pathogen, treatment, number of sample used, average expression stability M, genes ranking and pairwise variation V are indicated. The best reference genes pairs are highlighted in bold.

Ecotype	Pathogen	Treatment	Nb	Average expression stability M							Pairwise variation V					
Col-0	*Pst*	Water	5	*SSR16*	*CACT*	*ACT2*	*UBQ1*	*TUB4*	*EF1a*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.7	0.59	0.5	0.39	0.35	0.32	0.25	0.11	0.08	0.08	0.11	0.11	0.12
Col-0	*Pst*	BABA	5	*CACT*	*SSR16*	*UBQ1*	*ACT2*	*EF1a*	*SAND*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				1.28	0.85	0.76	0.66	0.41	0.28	0.22	0.1	0.13	0.2	0.15	0.14	0.31
Col-0	*Pst*	Water+BABA	10	*CACT*	*SSR16*	*ACT2*	*UBQ1*	*TUB4*	*EF1a*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				1.13	0.82	0.71	0.63	0.5	0.41	0.25	0.16	0.14	0.15	0.13	0.15	0.25
Col-0	*H. arabidopsidis*	Water	5	*UBQ1*	*CACT*	*TUB4*	*SSR16*	*ACT2*	*EF1a*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.43	0.38	0.35	0.31	0.27	0.24	0.15	0.09	0.07	0.07	0.06	0.06	0.07
Col-0	*H. arabidopsidis*	BABA	5	*CACT*	*UBQ1*	*SSR16*	*TUB4*	*ACT2*	*EF1a*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.45	0.42	0.38	0.36	0.34	0.24	0.14	0.09	0.1	0.07	0.06	0.07	0.06
Col-0	*H. arabidopsidis*	Water+BABA	10	*CACT*	*UBQ1*	*SSR16*	*TUB4*	*ACT2*	*EF1a*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.48	0.4	0.36	0.34	0.3	0.24	0.16	0.09	0.08	0.07	0.06	0.07	0.08
Col-0	*B. cinerea*	Water	6	*SSR16*	*UBQ1*	*EF1a*	*CACT*	*TIP-41*	*SAND*	***ACT2/TUB4***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.73	0.67	0.63	0.54	0.47	0.42	0.4	0.13	0.12	0.12	0.12	0.09	0.11
Col-0	*B. cinerea*	BABA	6	*TUB4*	*EF1a*	*CACT*	*SSR16*	*UBQ1*	*TIP-41*	***ACT2/SAND***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.82	0.76	0.65	0.58	0.48	0.41	0.29	0.15	0.13	0.13	0.12	0.13	0.11
Col-0	*B. cinerea*	Water+BABA	12	*EF1a*	*TUB4*	*UBQ1*	*SSR16*	*CACT*	*ACT2*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.77	0.73	0.67	0.61	0.54	0.43	0.35	0.15	0.15	0.13	0.12	0.11	0.09
Col-0	*B. cinerea* + *Pst* + *H. arabidopsis*	Water	18	*UBQ1*	*TUB4*	*CACT*	*EF1a*	*SSR16*	*ACT2*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				1.62	1.41	1.3	1.05	0.52	0.42	0.34	0.14	0.14	0.36	0.27	0.22	0.27
Col-0	*B. cinerea* + *Pst* + *H. arabidopsis*	Water+BABA	36	*UBQ1*	*CACT*	*TUB4*	*EF1a*	*SSR16*	*ACT2*	***SAND/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				1.61	1.37	1.19	0.97	0.5	0.41	0.34	0.14	0.13	0.33	0.26	0.24	0.27
jin1	*Pst*	Water	5	*UBQ1*	*ACT2*	*TIP-41*	*CACT*	*SAND*	*TUB4*	***EF1a/SSR16***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.53	0.49	0.43	0.37	0.3	0.27	0.24	0.09	0.08	0.09	0.08	0.08	0.08
jin1	*Pst*	Water+BABA	10	*CACT*	*TUB4*	*EF1a*	*SAND*	*SSR16*	*TIP-41*	***ACT2/UBQ10***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.88	0.75	0.69	0.66	0.61	0.56	0.47	0.19	0.15	0.13	0.11	0.11	0.15
npq2	*Pst*	Water	5	*CACT*	*EF1a*	*SSR16*	*SAND*	*TUB4*	*UBQ1*	***ACT2/TIP-41***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.5	0.44	0.41	0.33	0.25	0.23	0.2	0.07	0.06	0.09	0.09	0.07	0.08
npq2	*Pst*	Water+BABA	10	*ACT2*	*UBQ1*	*CACT*	*SSR16*	*TIP-41*	*SAND*	***TUB4/EF1a***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.84	0.8	0.73	0.66	0.64	0.54	0.5	0.17	0.17	0.11	0.13	0.13	0.1
Col-0+*jin1*+*npq2*	*Pst*	Water	15	*UBQ1*	*CACT*	*SSR16*	*ACT2*	*TIP-41*	*SAND*	***TUB4/EF1a***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				0.8	0.69	0.62	0.55	0.49	0.37	0.36	0.11	0.14	0.11	0.11	0.11	0.13
Col-0+jin1+npq2	*Pst*	Water+BABA	30	*CACT*	*UBQ1*	*ACT2*	*SSR16*	*TIP-41*	*SAND*	***TUB4/EF1a***	V2/3	V3/4	V4/5	V5/6	V6/7	V7/8
				1.06	0.92	0.81	0.72	0.64	0.56	0.49	0.18	0.16	0.15	0.14	0.16	0.17

Table S4. Correlation of the 5 GOIs expression depending the normalization procedure

The relative expression of the 5 GOIs normalized with *SAND+TIP41* was compared either with their relative expression normalized with *UBQ10* or with their expression measured by aqPCR (See the graphs in Figure 5 and additional Figure 2). Conditions of the experiments, GOIs name, correlation coefficients r, and P Values are indicated. Highest r and Pvalues are highlighted in bold, non significant correlations are highlighted in red.

Conditions (Pathogen, treatment, sample nb)	GOI	RI (*SAND+TIP41*) / RI (UBQ10)		RI (SAND+TIP41) / aqPCR	
		r	P Value	r	P Value
H. arabidopsidis + Water (n=6)	*EDS1*	0.859	0.029	**0.949**	**0.004**
	FAD	0.565	0.243	**0.872**	**0.024**
	NCED3	0.657	0.156	**0.958**	**0.003**
	PR-1	**0.990**	**<0.001**	0.991	<0.001
	PDF1.2	0.806	0.053	**0.966**	**0.002**
H. arabidopsidis + BABA (n=6)	*EDS1*	**0.957**	**0.003**	0.938	0.006
	FAD	**0.948**	**0.004**	0.875	0.023
	NCED3	0.936	0.006	**0.948**	**0.004**
	PR-1	**0.965**	**0.002**	0.964	0.002
	PDF1.2	**0.984**	**<0.001**	0.972	0.001
Pst + Water (n=6)	*EDS1*	-0.124	0.815	**0.487**	**0.327**
	FAD	0.932	0.007	**0.975**	**<0.001**
	NCED3	0.937	0.006	**0.993**	**<0.001**
	PR-1	0.987	<0.001	**0.989**	**<0.001**
	PDF1.2	0.865	0.026	**0.946**	**0.004**
Pst + BABA (n=6)	*EDS1*	0.934	0.006	**0.986**	**<0.001**
	FAD	0.982	<0.001	**0.992**	**<0.001**
	NCED3	0.985	<0.001	**0.997**	**<0.001**
	PR-1	0.929	0.007	**0.975**	**<0.001**
	PDF1.2	0.953	0.003	**0.981**	**<0.001**
B. cinerea + Water (n=5)	*EDS1*	-0.371	0.538	**0.740**	**0.153**
	FAD	0.895	0.040	**0.987**	**0.002**
	NCED3	0.993	<0.001	**0.999**	**<0.001**
	PR-1	0.972	0.006	**0.990**	**<0.001**
	PDF1.2	0.994	<0.001	**0.996**	**<0.001**
B. cinerea + BABA (n=5)	*EDS1*	**0.994**	**<0.001**	0.973	0.005
	FAD	0.593	0.292	**0.916**	**0.029**
	NCED3	0.995	<0.001	**0.999**	**<0.001**
	PR-1	0.936	0.019	**0.979**	**0.004**
	PDF1.2	**0.991**	**<0.001**	0.986	0.002

Chapter 3

Effector-triggered activation of the interconnected Arabidopsis JA and ABA pathways promotes disease susceptibility to *Pseudomonas syringae* and MeSA emission

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Effector-triggered activation of the interconnected Arabidopsis JA and ABA pathways promotes disease susceptibility to *Pseudomonas syringae* and MeSA emission

ABSTRACT

In Arabidopsis, disease susceptibility to the virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) results from the delivery of pathogen effectors into the host cell. *Pst* effectors such as coronatin (COR) and proteins of the type three secretion system target both Arabidopsis jasmonic acid (JA) and abscisic acid (ABA) signaling pathways that antagonize salicylic acid (SA)-inducible defenses. While the cross-talk between the effector-triggered JA pathway and the SA-dependent defenses has been well described, the interplay between JA and ABA pathways has received little attention. By using the mutant *jin1*deficient in the JA signaling pathway and the mutant *npq2* affected in the biosynthesis of ABA we showed that both JA and ABA pathways co-regulate each other during the infection of Arabidopsis with the virulent *P. syringae.* In particular, the aboundance of the oxylipins OPDA, JA and Ile-JA as well of those of ABA were severely reduced in these mutants compared to wild-type when challenged with *Pst.* Our results highlight the inter-dependency between the effector-triggered activation of both JA and ABA pathways in Arabidopsis during *Pst* infection. We demonstrate that MeSA emission from Arabidopsis challenged with the bacteria results from the secretion of pathogen effectors and is dependent of effector-triggered induction of both JA and ABA pathways. The compatible interaction between Arabidopsis and *Pst* can be circumvented by inducing resistance with β-aminobutyric acid (BABA) that potentiates SA-inducible responses through repression of the COR-mediated responses. Reduced *Pst*

colonization in BABA-treated plants correlated with reduced effector-triggered activation of both JA and ABA pathways and MeSA emission.

INTRODUCTION

The outcome of plant-microbe interactions is determined by the complex hormonal interplay induced by the virulence factors of pathogens on the one side and by the plant defense responses on the other side (Lopez *et al.* 2008, Pieterse *et al.* 2009). During infection of Arabidopsis by the virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), both SA-dependent and independent basal defenses are activated as a result of the pathogen-associated molecular patterns- (PAMPS) triggered immunity (PTI) (Jones and Dangl 2006, Nürnberger and Kemmerling 2009, Tsuda *et al.* 2008). Although the SA pathway mediates resistance against *Pst*, the bacteria antagonize both SA-dependent and independent defenses. This is achieved through delivery into the host cell of an arsenal of virulence effectors such as the phytotoxin coronatin (COR) and protein effectors of the type III secretion system (TTSS) (Kim *et al.* 2008, Nomura *et al.* 2005). The role of COR to suppress plant defenses is known for long (Kloek *et al.* 2001, Mittal and Davis 1995). The mechanisms by which COR and TTSS effectors promote bacterial virulence share common features such as the suppression of SA-independent PTI (Li *et al.* 2005) and suppression of SA-mediated basal defenses through induction of the JA pathway (DebRoy *et al.* 2004, Kim, et al. 2008, Nomura, et al. 2005). COR was shown to mimic jasmonoyl-isoleucine (Ile-JA), the amino acid-conjugated form of JA identified as the signal molecule of the JA pathway. Ile-JA binds to the F-box protein CORONATINE-INSENSITIVE 1 (COI1) and subsequently promotes the ubiquitin-dependent degradation of JA ZIM-domain (JAZ) proteins that repress the JA-induced transcription factor MYC2/JIN1 (for JASMONATE INSENSITIVE 1) (Chini *et al.* 2007, Thines *et al.* 2007). Thus, during Arabidopsis infection by virulent *Pst*, COR-mediated degradation of JAZ protein leads to the MYC2-mediated expression of a subset of JA-dependent genes that repress SA-inducible defenses (Dombrecht *et al.* 2007, Laurie-Berry *et al.* 2006, Lorenzo *et al.* 2004, Nickstadt *et al.* 2004). In addition to JA, COR was shown to trigger ethylene (ET),

auxin (AUX) and ABA pathways (Thilmony *et al.* 2006, Uppalapati *et al.* 2005) and to promote the re-opening of stomata that are closed upon recognition of several PAMPs by the plants (Melotto *et al.* 2006). Some TTSS effectors also interfere with ABA signaling, resulting in the repression of the SA pathway (de Torres-Zabala *et al.* 2007, Zabala *et al.* 2009).

Disease susceptibility of Arabidopsis to virulent *Pst* can be overturned by treating plants with β-aminobutyric acid (BABA). This priming agent is a non-protein amino acid conferring resistance against a broad range of biotic and abiotic stresses in a variety of plants (Cohen 2002, Jakab *et al.* 2001, Prime-A-Plant Group 2006). BABA-induced resistance (BABA-IR) against *Pst* requires a functional cyclin-dependent kinase IBS1 and is characterized by the potentiation of SA-inducible defenses (Ton *et al.* 2005, Zimmerli *et al.* 2000) as a result of the BABA-induced repression of COR-mediated responses (Tsai *et al.* 2011).

According to the recent findings about hormone networks during the establishment of bacterial disease or plant resistance as described above we investigated the interplay between the effector-triggered activation of both JA and ABA pathways in Arabidopsis challenged with the virulent *Pst*. We monitored the interplay between the JA, ABA and SA pathways during compatible interaction between Arabidopsis and the virulent *P. syringae* and during BABA-IR in wild-type Arabidopsis plants and in two mutants altered in the JA and ABA pathways, respectively. We also monitored the interplay between these hormones in Arabidopsis challenged with a COR-deficient strain or with the non-host *HrpA* strain. We finally considered the role of methyl-salicylate (MeSA) that was first described as a potential mobile signal for the establishment of SAR (Liu *et al.* 2010, Park *et al.* 2007, Vlot *et al.* 2008a, Vlot *et al.* 2008b) but was recently shown to be emitted in Arabidopsis during *Pst* infection as a result of COR-triggered responses.

Our results highlight the interdependency between the effector-triggered activation of both JA and ABA pathways in Arabidopsis during *Pst* infection as a result of both COR and TTSS effectors. This simultaneous induction of the JA and ABA pathways leads to MeSA emission. These trends are reduced in a SA-dependent manner in BABA-treated plants, in which the SA-inducible defenses are activated before *Pst* infection. These results confirm that disease susceptibility of Arabidopsis to the virulent *Pst* is due to the effector-triggered interference on both the ABA and JA pathways, resulting in the antagonism on SA-inducible defenses. This virulence mechanism can be reduced if the SA-inducible responses are activated prior to the infection.

RESULTS

Arabidopsis JA and ABA pathways induced by *Pseudomonas syringae* are interdependent to antagonize the SA pathway

As a strategy to investigate the interplay between the Arabidopsis JA and ABA pathways we monitored the transcriptional and hormonal profiles of both signaling pathways in the Arabidopsis mutants *jin1* and *npq2* challenged with virulent *Pst* (Figure 1). *jin1* is altered in the activity of MYC2, a transcription factor involved downstream of the JA signaling pathway (Lorenzo, et al. 2004). *npq2* is an ABA-deficient mutant altered in the biosynthesis of ABA with a deficient zeaxanthin epoxidase ABA1 (Niyogi *et al.* 1998). We used treatment with the priming agent BABA (Ton, et al. 2005, Zimmerli, et al. 2000) to investigate the effect of resistance establishment on the effector-triggered induction of both JA- and ABA-dependent responses.

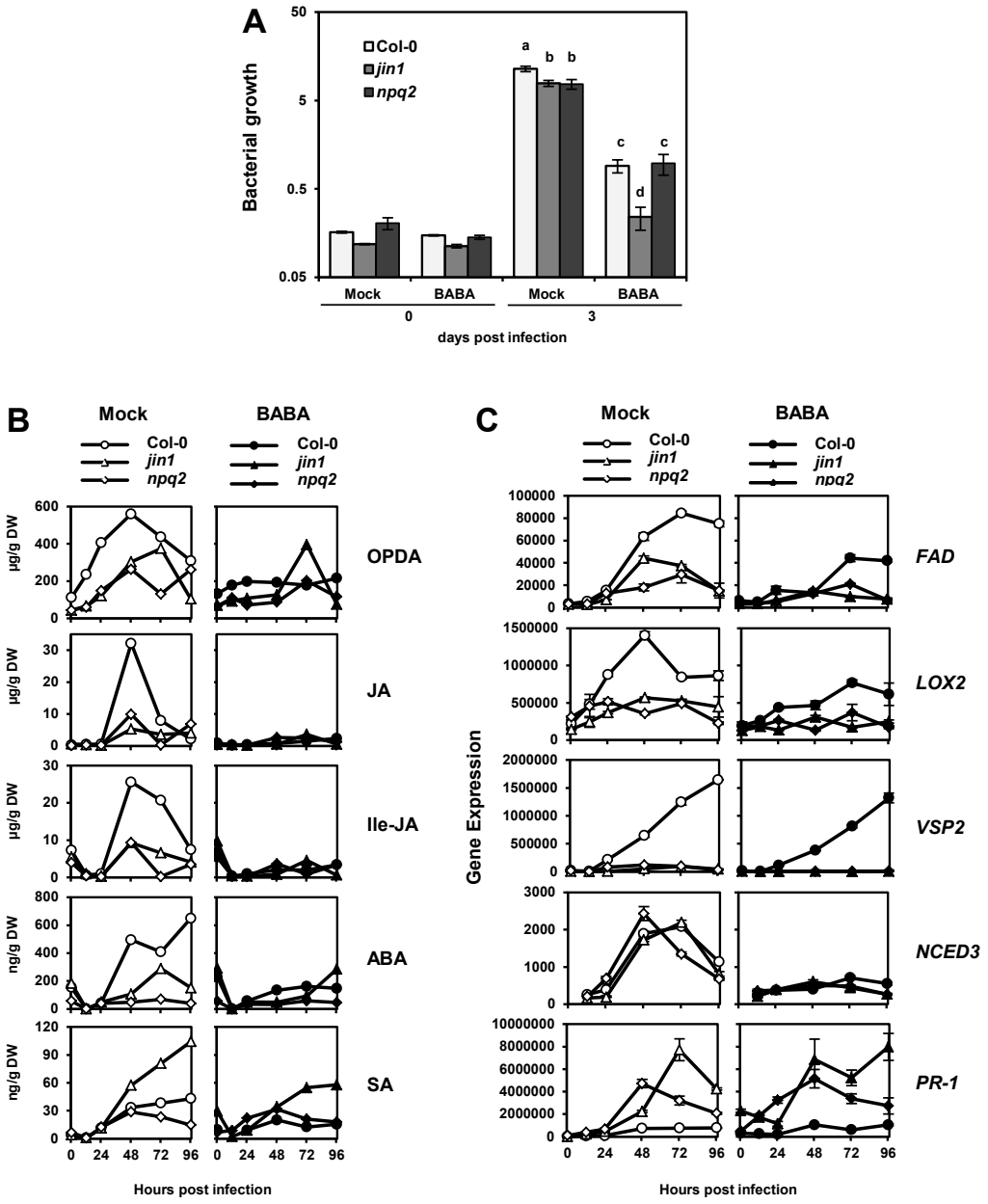

Figure 1. Interplay between the Arabidopsis JA and ABA pathways during *Pseudomonas syringae* infection.

Five-weeks old wild type, *jin1* and *npq2* plants were treated with water or BABA (250 µM final) and challenged two days later with the virulent *Pst* at 5 x 10^7 colony-forming units mL^{-1}. Experiments were reproduced twice with similar results.

(A) Bacterial growth was monitored by real-time PCR at 0 and 3 days post infection. Data represent the mean (±SD) of the ratio *Pst* gene copy number/Arabidopsis gene copy number. Different letters represent significant growth differences (Student-Neuman-Keuls ANOVA, n=4, P<0.001).

(B) Accumulation of OPDA, JA, Ile-JA, ABA and SA was measured at the indicated time post *Pst* infection by UPLC-MS.

(C) Expression of FAD, *LOX2*, *VSP2* and *NCED3* was measured at the indicated time post *Pst* infection by real-time RT-PCR. Data represent the mean (±SD) from technical replicates of the ratio transcript copy nb/ng cDNA.

As previously described (Laurie-Berry, et al. 2006, Zabala, et al. 2009), *jin1* and *npq2* mutants were more resistant than the wild-type plants towards *Pst* (Figure 1.A). As expected the accumulation of the JA precursor OPDA (12-oxophytodienoic acid), JA and Ile-JA were reduced in *jin1* compared to the wild-type during the infection (Figure 1.B). As well, the expression of two genes involved in JA biosynthesis, *Fatty Acid Desaturase* (*FAD*) and *Lipoxygenase2 (LOX2),* and the expression of the JA/ET marker genes *Vegetative Storage Protein 2* (*VSP2)* were also reduced in *jin1* compared to wild-type plants (Figure 1.C). In the mutant *npq2*, *Pst* did not trigger the accumulation of ABA (Figure 1.B) as in the wild-type while the expression of the ABA biosynthetic gene *9-cis-epoxycarotenoid dioxygenase* 3 (*NCED3*), placed downstream of ABA1, was unchanged (Figure 1.C). Surprisingly, the accumulation of ABA, but not *NCED3* transcripts, was strongly reduced in *jin1* compared to wild-type plants (Figure 1.B and C). This suggests that during *Pst* infection the JA-dependent transcription factor MYC2 positively regulates the accumulation of ABA but not *NCED3* expression. Conversely, the accumulation of OPDA, JA, Ile-JA and the related JA-dependent transcripts *FAD*, *LOX2* and *VSP2* were almost totally abolished in the ABA-deficient mutant *npq2* (Figure 1.B and C). This indicates that ABA positively regulates the whole JA pathway. The accumulation of SA and the expression of the SA-inducible gene *Pathogenesis Related 1* (*PR-1*) where higher in *jin1* compared to wild-type plants (Figure 1B and C). Only *PR-1* expression was higher in *npq2*. These results confirm that both JA and ABA pathways antagonizes the SA-dependent pathway in Arabidopsis during *Pst* infection.

As expected, BABA treatment resulted in reduced bacterial growth in wild-type plants (Figure 1A). However and contrarily to previous studies (Ton, et al. 2005, Tsai, et al. 2011, Zimmerli, et al. 2000) this correlated with enhanced SA accumulation and *PR-1* expression prior to the infection and not during the infection (Figure 1A and B). BABA-IR against *Pst* also correlated with reduced *Pst*-triggered accumulation of both, JA and ABA and the

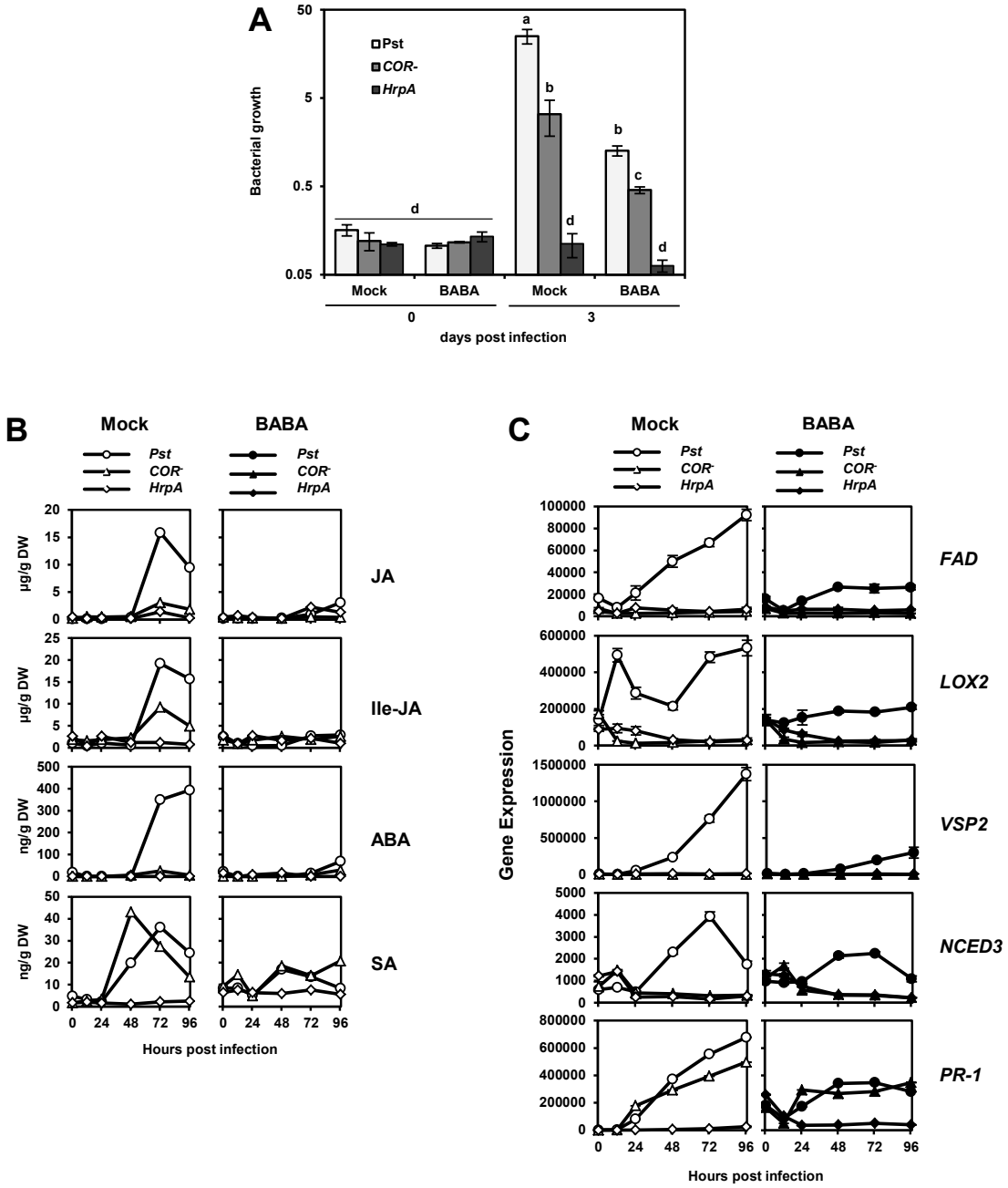

Figure 2. Coronatine- and TTSS effectors-triggered activation of the JA and ABA pathways in Arabidopsis challenged with the virulent *Pst*.

Five-weeks old wild-type Col-0 plants were treated with water or BABA (250 µM final) and challenged two days later with the wild-type strain *P. syringae* pv. *tomato* DC3000 (*Pst*), the coronatine-deficient strain (*COR*$^-$) or with the mutant compromised in TTSS (*HrpA*) at 5 x 10^7 colony-forming units mL^{-1}. Experiments were reproduced twice with similar results.

(A) Bacterial growth was measured 0 and 3 days post infection by real-time PCR. Data represent the mean (±SD) of the ratio *Pst* gene copy number/Arabidopsis gene copy number. Letters represent significant growth differences (t-test, n=4, $P<0.05$).
(B) Accumulation of JA, Ile-JA, ABA and SA were measured at the indicated time by UPLC-MS.
(C) Expression of *FAD*, *LOX, VSP2, NCED3* and *PR-1* was measured at the indicated time by real-time RT-PCR. Data represent the mean (±SD) from technical replicates of the ratio transcript copy nb/ng cDNA.

expression of their respective marker transcripts (Figure 1B and C). In addition, BABA treatment further reduced *Pst* growth and the *Pst*-triggered activation of the JA and ABA pathways in *jin1* and *npq2* mutants compared to untreated mutants (Figure 1A-C). These results confirm that the reduced *Pst* growth in BABA-treated plants correlates with reduced effector-triggered activation of the JA and ABA pathways.

Effector-triggered activation of both Arabidopsis JA and ABA pathways is mainly due to the secretion of coronatin

The role of COR and TTSS effectors in the establishment of the networking between both JA and ABA pathways during *Pst* infection was investigated. Bacterial growth, transcriptional and hormonal profiles of the JA, ABA and SA pathways were monitored and compared in between wild-type plants challenged with the virulent wild-type *Pst* strain, with a *Pst* strain unable to secrete COR (*COR*$^-$) (Ma *et al.* 1991, Mittal and Davis 1995) or with a non-host *Pst* strain deficient in both TTSS and COR production (*HrpA*) (Roine *et al.* 1997, Zabala, et al. 2009). BABA treatment was used to verify its effect on the bacterial effectors. As expected, the reduced growth of *COR*$^-$ compared to *Pst* in wild-type plants (Figure 2A and Figure S1) correlated with only few accumulation of JA, Ile-JA and ABA (Figure 2B) and no induction of their respective marker transcripts *FAD, LOX, VSP2* and *NCED3* (Figure 2C). SA and *PR-1* transcripts accumulated 24 hours earlier in Arabidopsis plants challenged with *COR*$^-$ compared to those challenged with *Pst*. As expected the *HrpA* strain did not colonize the plant tissues (Figure 2A and Figure S1) and did not trigger the accumulation of any hormones (Figure 2B) or marker transcripts (Figure 2C). These results suggest that mainly COR, and

TTSS effectors to a lower extend, mediate the synergistic induction of both Arabidopsis JA and ABA pathways during *Pst* infection.

In addition and conversely to a previous study (Tsai, et al. 2011), BABA treatment further inhibited the growth of *COR*$^-$ compared with mock-treated plants (Figure 2A). This result suggests that in our condition BABA-IR against *Pst* was not only due to a direct inhibition of the COR-triggered activation of the Arabidopsis JA and ABA pathways. Indeed, JA, Ile-JA, ABA and the marker transcripts *FAD, LOX, VSP2* and *NCED3* accumulated barely in both BABA-and mock- treated plants challenged with *COR*$^-$ compared to the levels observed in *Pst* challenged plants (Figure 2B and C). BABA treatment had no significant influence on the growth of the *HrpA* strain (Figure 2A and Figure S1).

Effector-triggered activation of both Arabidopsis JA and ABA pathways is restored in SA-deficient mutants treated with BABA

To investigate the role of SA-inducible defenses expressed during BABA-IR on the *Pst*-triggered activation of both ABA and JA pathways, we have monitored bacterial growth, transcriptional and hormonal profiles of the JA, ABA and SA pathways in several SA-deficient mutants treated with water or BABA. As expected (Zimmerli, et al. 2000), BABA-IR against *Pst* and the associated accumulation of SA and expression of *PR-1* were compromised in the transgenic NahG plants and in the *npr1* mutant (Figure 3 and S2). *npr1* displays a disrupted ability to express *PR-1* while transgenic NahG plants do not accumulate SA (Cao *et al.* 1994, Delaney 1994). In addition, BABA-IR was strongly reduced in both *sid1* and *sid2* mutants (for SA Induction-Deficient), respectively deficient in EDS5 (for Enhance disease Susceptibility 5) and ISOCHORSMATE SYNTHASE 1, two protein involved respectively in the signalization and biosynthesis of SA (Figure 3A and Figure S2). These mutants are known to accumulate reduced levels of SA and *PR-1* transcripts during compatible and incompatible interactions with *Pst* (Nawrath and Metraux 1999). The

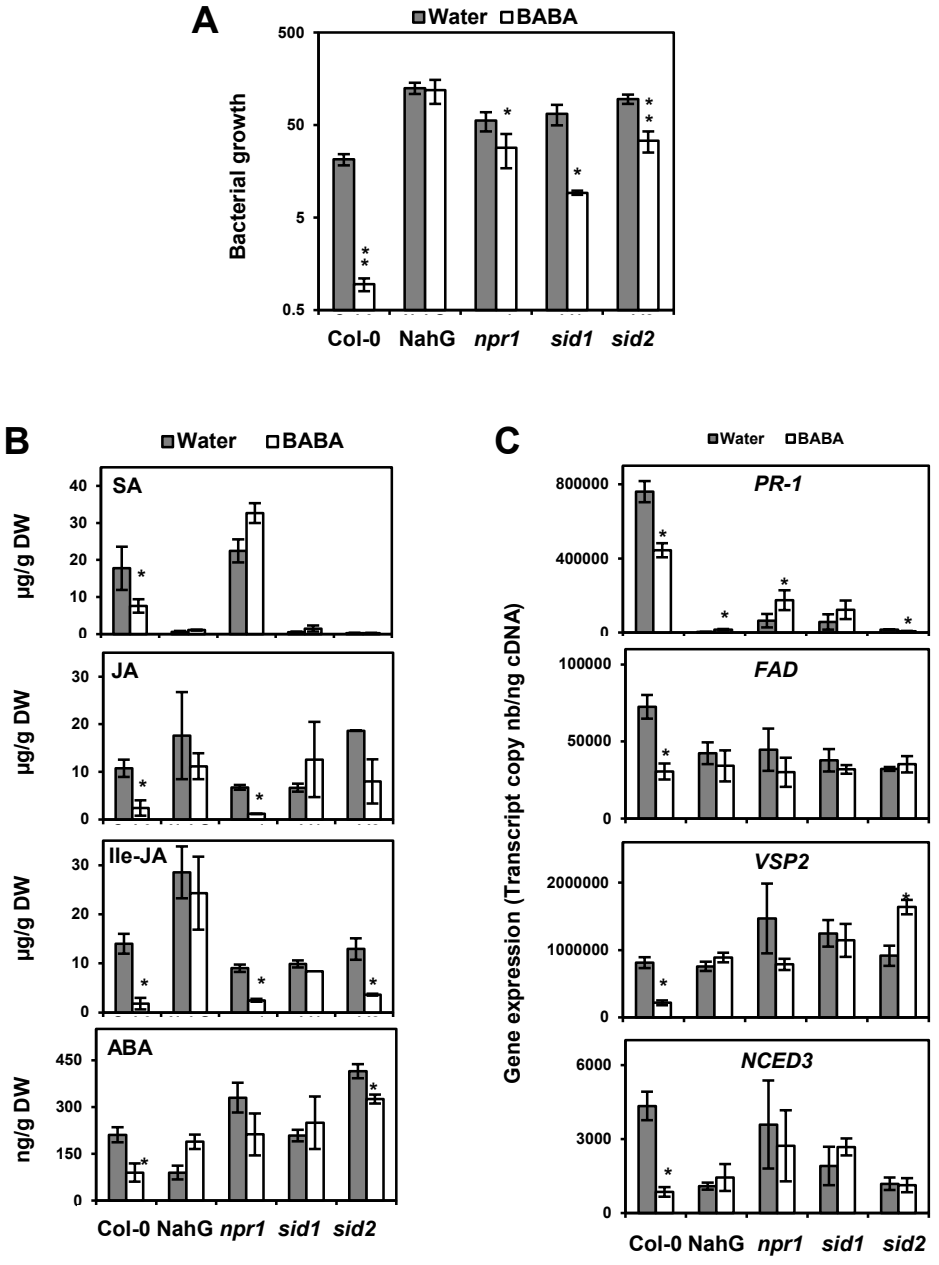

Figure 3. Effector-triggered activation of both Arabidopsis JA and ABA pathways is restored in BABA-treated SA-deficient mutants

Five-weeks old Col-0, NahG, *npr1*, *sid1* and *sid2* plants were treated with water or BABA (250 µM final) and challenged two days later with *Pst* at 5 x 10^7 colony-forming units mL^{-1}.
(A) Bacterial growth was measured three days post infection by real-time PCR. Data represent the mean (±SD) the ratio *Pst* gene copy number/Arabidopsis gene copy number. Asterisks represent significant growth differences between water- and BABA-treated plants (t-test, n=4, * : $P<0.05$, ** $P\leq0.001$).
(B) Internal levels of SA, JA, Ile-JA and ABA were measured three days post infection by UPLC-MS. Data represent the mean (±SD) of biological replicates. Asterisks represent significant amount differences between water- and BABA-treated plants (t-test, n=2, $P<0.05$).
(C) Expression of plant defense genes were measured three days post infection by real-time RT-PCR. Data represent the mean (±SD) of biological replicates. Asterisks represent significant expression differences between water- and BABA-treated plants (t-test, n=4, $P<0.05$).

deficiency of BABA-IR in SA-altered mutant plants correlated with reduced induction of SA-dependent responses. This also correlated with restored effector-triggered activation of JA- and ABA-dependent responses (Figure 3B and C). These results suggest that the SA-inducible defenses in BABA-treated plants reduce *Pst* growth leading to reduced effector-triggered activation of both Arabidopsis ABA and JA pathways.

MeSA emission is due to the effector-triggered activation of both ABA and JA pathway

MeSA produced locally upon challenge with an avirulent *Pst* strain was proposed to act as a mobile signal of SAR (Liu, et al. 2010, Park, et al. 2007, Vlot, et al. 2008a, Vlot, et al. 2008b). In addition, MeSA emission from Arabidopsis challenged with *Ps*t was shown to be dependent of COR secretion (Attaran *et al.* 2009). To precise the significance of MeSA production, we monitored its emission in Arabidopsis mutants deficient in SA, JA and ABA pathways challenged with *Pst*, and in wild-type plants challenged with the *COR*$^{-}$ and *HrpA* strains. MeSA production was strongly reduced in both *jin1* and *npq2* mutants challenged with *Pst* compared to wild type plants and was not detectable from wild type plants challenged with the *COR*$^{-}$ strain (Figure 4A). Consistent with these findings the expression of *BSMT1,* the gene coding the *Benzoic acid and Salicylic acid Methyl Transferase* (Chen *et al.* 2003), was almost abolished in *jin1* and *npq2* mutants challenged with *Pst* (Figure 4B). In addition, *BSMT1* expression remained at the basal level in Col-0 plants challenged with both *COR*$^{-}$ or *HrpA* strains (Figure 4C). As already observed for the effector-triggered induction of both JA- and ABA-dependent responses, MeSA emission and *BSMT1* expression were strongly reduced in BABA-treated wild-type plants compared to water-treated controls (Figure 4A-C). MeSA emission could not be detected from water- nor BABA-treated *sid2* mutants challenged with the bacteria (Figure 4A). However the expression of *BSMT1* was less

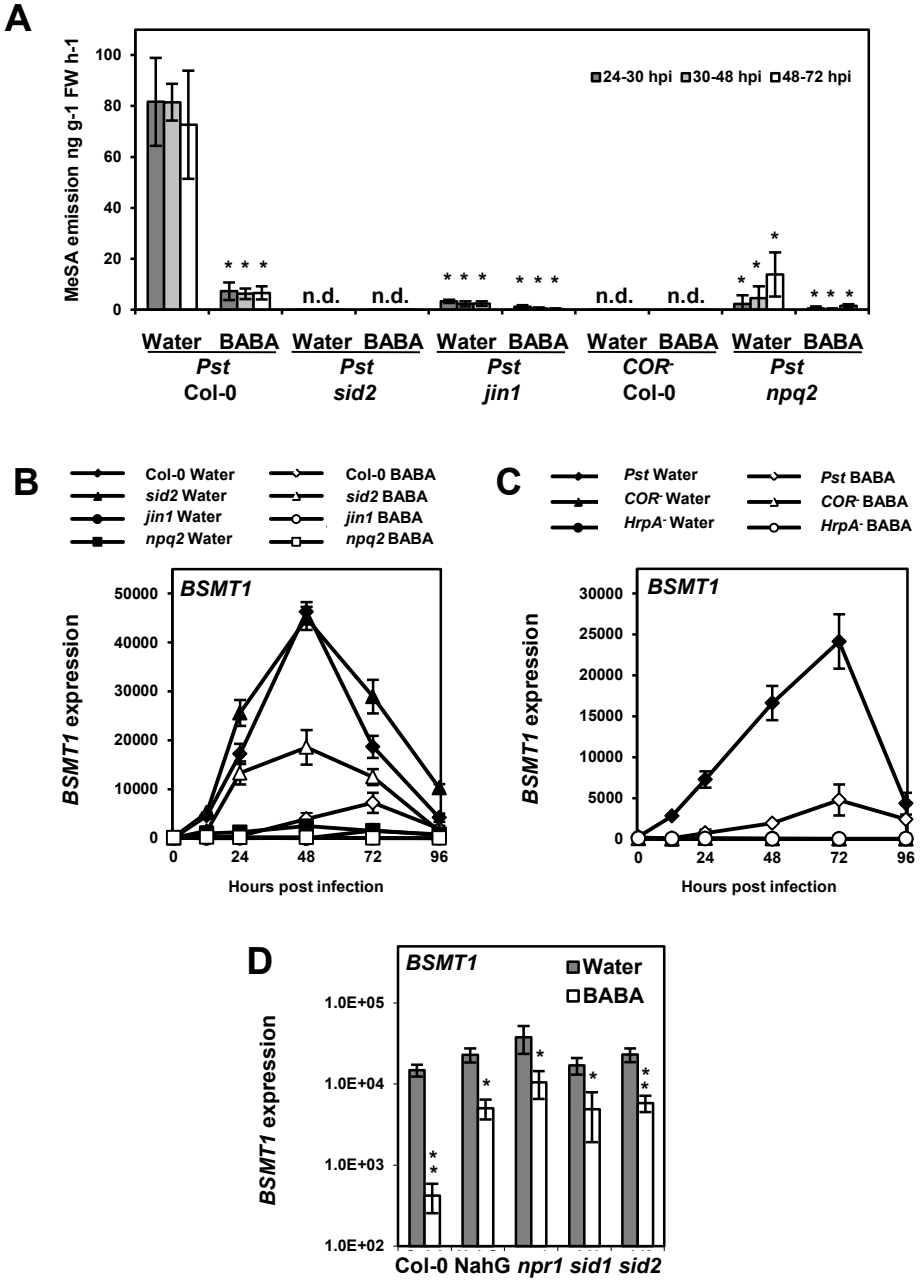

Figure 4. Effector-triggered emission of MeSA requires both JA and ABA pathways

Five-weeks old wild type, *jin1* and *npq2* plants were treated with water or BABA (250 µM final) and challenged two days later with the virulent *Pst* at 5 x 10^7 colony-forming units mL^{-1}. Experiments were reproduced twice with similar results.

(A) Emission of MeSA was measured by GC-MS. Data represent the mean (±SD) of MeSA emitted during the indicated period post bacterial infection in Col-0, *sid2*, *jin1* and *npq2* plants challenged with *Pst*, or in Col-0 plants challenged with *COR*-. n.d. notes indicate that MeSA emission was not detectable. Asterisks represent significant emission differences compared to water-treated Col-0 plants (t-test, n=3, P<0.05).

(B) Expression of *BSMT1* was measured by real-time RT-PCR at the indicated times in Col-0, *sid2*, *jin1* and *npq2* plants challenged with *Pst* and **(C)** in Col-0 plants challenged either with *Pst* or with the strains *COR*- and *HrpA*. Data represent the mean (±SD) from technical replicates (n=3) of the ration transcript copy nb/ng cDNA.

(d) Expression of *BSMT1* was measured by real-time RT-PCR in Col-0, *npr1*, *sid1*, *sid2*, and in NahG plants three days post *Pst* infection, Data represent the mean (±SD) of the ration transcript copy nb/ng cDNA. Asterisks represent significant expression differences between water- and BABA-treated plants (t-test, n=4, * : P<0.05, ** P≤0.001).

reduced in several BABA-treated Arabidopsis lines altered in the SA-pathway and in the transgenic NahG line than in BABA-treated Col-0 plants challenged with *Pst* (Figure 4B and D). These results support that the production of MeSA in plants challenged by *Pst* is dependent on both functional JA and ABA pathways, both induced mainly by the secretion of COR.

DISCUSSION

In this study we investigated the interplay between the Arabidopsis JA and ABA pathways impacted by bacterial effectors and SA-inducible defenses. Our results enable a better understanding of the interconnected networks between both Arabidopsis JA and ABA pathways induced by the bacterial COR and TTSS effectors and the potential role of MeSA. In addition we confirm that BABA-IR against *Pst* is SA-dependent and leads to reduced effector-triggered activation of both Arabidopsis JA- and ABA-dependent responses. These results are summarized in figure 5.

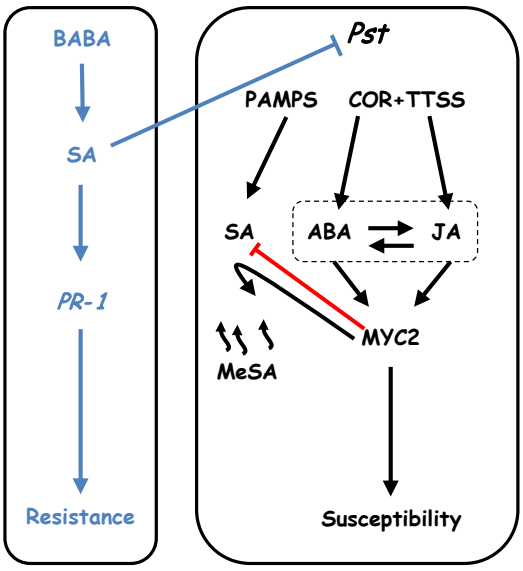

Figure 5. Representation of the interplay between effector-trigerred activation of both JA- and ABA-dependent responses and the PAMPs- or BABA-induced SA-dependent responses,

Interplay between the effector-triggered activation of both JA and ABA pathways during *P. syringae* infection

Our results support that COR is the major effector impacting the ABA pathway in Arabidopsis during *Pst* infection. Indeed, the *Pst* strain deficient in COR secretion barely induced both JA and ABA signaling pathways contrarily to the virulent wild-type *Pst* (Figure 2). The ABA pathway, in addition to JA, was also shown to be induced as results of COR and TTSS effectors in Arabidopsis challenged with *Pst* (de Torres-Zabala, et al. 2007, Zabala, et al. 2009). Previous global gene expression studies already reported that both COR and TTSS effectors target the ABA pathway (Thilmony, et al. 2006, Truman *et al.* 2006). Furthermore, the *Pst* effector AvrPtoB has been identified to target the ABA pathway (de Torres-Zabala, et al. 2007). Thus the reduced growth of the *COR*$^{-}$ strain compared to the virulent *Pst* might also result in reduced delivery in Arabidopsis cells of TTSS effectors impacting both JA and ABA pathways.

In addition, our study supports the existence of a strong interdependency between the Arabidopsis JA and ABA pathways during *Pst* infection whose induction leads to plant susceptibility. We show that both Arabidopsis ABA and JA pathways co-regulate each other during *Pst* infection. Indeed, the effector-triggered accumulation of ABA in Arabidopsis was strongly reduced in the mutant *jin1* (Figure 1). This indicates that the JA-dependent transcription factor MYC2 positively regulates the biosynthesis of ABA in Arabidopsis challenged with *Pst*. Conversely, the reduced accumulation of OPDA, JA, JA-Ile and the JA-dependent transcripts *FAD*, *LOX2* and *VSP2* in the ABA-deficient mutant *npq2* (Figure 1) also suggests that the effector-triggered accumulation of ABA in Arabidopsis challenged with *Pst* positively regulates both JA biosynthesis and JA-signaling pathway. Previous reports have indicated that ABA positively regulates JA biosynthesis and the expression of MYC2/JIN1-

dependent genes (Adie *et al.* 2007, Anderson *et al.* 2004, Fan *et al.* 2009). However, the exact mechanism of this regulation remains to be determined.

Mechanism leading to MeSA emission in Arabidopsis challenged with *P. syringae*

Our findings support that the emission of MeSA in Arabidopsis during *Pst* infection is the result of COR-triggered activation of both JA and ABA pathways. *BSMT1* expression and MeSA emission were strongly induced in wild-type plants challenged with *Pst* (Figure 4) but were compromised in both *jin1* and *npq2* mutants challenged with *Pst* as well as in wild type plants challenged with the *COR*$^{-}$ and *HrpA* strains. In Arabidopsis, the expression of *BSMT1* and the emission of MeSA were first reported to be induced by wounding, herbivores, and MeJA (Chen, et al. 2003). Intriguingly, jasmonates and MeSA have both been proposed to be the mobile signal of SAR (Liu, et al. 2010, Park, et al. 2007, Truman *et al.* 2007, Vlot, et al. 2008a, Vlot, et al. 2008b). However, a recent study attested that neither JA signaling nor MeSA emission were critical for the establishment of SAR in Arabidopsis (Attaran, et al. 2009). Instead, Attaran *et al.* (2009) showed that MeSA production during *Pst* infection was almost totally lost in the atmosphere, a phenomenon resulting from the COR-triggered activation of the JA pathway (Attaran, et al. 2009). However the role of this mechanism is not totally understood.

Influence of the effector-triggered activation of both JA and ABA pathways and the resulting MeSA emission on the SA-inducible defenses

Our observations suggest that the activity of bacterial effectors result in the positive co-regulation between both JA and ABA pathways leading to the expression of genes regulated by the transcription factor MYC2. Such results are in line with previous report showing the

regulation by MYC2 of the development of symptoms and the repression of SA-inducible defenses during *Pst* infection (Laurie-Berry, et al. 2006). It is also well established that COR and TTSS effectors both target the JA pathway which results in the suppression of SA-inducible defenses (Brooks *et al.* 2005, DebRoy, et al. 2004, He *et al.* 2004, Laurie-Berry, et al. 2006, Nickstadt, et al. 2004, Thilmony, et al. 2006, Uppalapati, et al. 2005, Uppalapati *et al.* 2007, Zhao *et al.* 2003). Similarly, evidences that ABA down-regulates SA-inducible defenses in Arabidopsis, tobacco and rice are emerging (Jiang *et al.* 2010, Kusajima *et al.* 2010, Mohr and Cahill 2007, Yasuda *et al.* 2008). For example, the *Pst* effector AvrPtoB known to target the ABA pathway (de Torres-Zabala, et al. 2007), was shown to suppress SA-inducible defenses (Fan, et al. 2009, Zabala, et al. 2009). Our experiment confirmed that the expression of the SA-inducible *PR-1* gene was strongly enhanced in both *jin1* and *npq2* mutants challenged with *Pst* (Figure 1). In addition, SA accumulated earlier in Arabidopsis challenged with the *COR*$^{-}$ strain compared to plants challenged with *Pst*. Such results strongly suggest that *Pst* use both COR and TTSS effectors to promote JA- and ABA-dependent antagonism on SA-inducible defenses, thereby resulting in plant susceptibility.

The involvement of MeSA emission as a result of the effector-triggered activation of both JA and ABA pathways is a subject of controversy. On one hand, transgenic Arabidopsis overexpressing *BSMT1* were reported to be more susceptible than wild-type plants when challenged with *Pst* and with the fungus *Golovinomyces orontii* (Koo *et al.* 2007, Liu, et al. 2010). Theses phenotypes coincided with enhanced MeSA production and reduced accumulation of SA and *PR-1* transcripts. Furthermore, Attaran *et al.* (2009) suggested that the COR-triggered activation of the JA pathway resulting in MeSA emission in the atmosphere contributed to the JA-mediated suppression of SA-inducible defenses. On the other hand, several Arabidopsis *bsmt1* knock-out mutants were found unable to exhibit enhanced basal resistance against *Pst* or *Pseudomonas syringae* pv. *maculicola* compared to

wild-type plants (Attaran, et al. 2009, Liu, et al. 2010). It seems thus that the effector-triggered production of MeSA contributes to reduce SA-inducible defenses but might not be required for disease susceptibility.

The hormonal balance rules the outcome of Arabidopsis/*Pseudomonas syringae* interaction

Although SA-dependent basal defenses are activated by PTI in Arabidopsis upon recognition of *Pst* (Tsuda, et al. 2008), both COR and TTSS effectors are secreted sufficiently early to overcome plant immunity (Kim, et al. 2008, Nomura, et al. 2005). In this study we confirmed the importance of the effector-triggered activation of both JA and ABA pathways to antagonize the SA-inducible defenses. However, the susceptibility of Arabidopsis to the virulent *Pst* can be overturned by the help of BABA treatment. Recently, BABA-IR against *Pst* was shown to be due to the specific inhibition of BABA on COR-triggered responses and thus that BABA treatment could not further reduce the growth of the *COR*$^-$ strain (Tsai, et al. 2011). Tsai *et al*. (2011) proposed that the BABA-induced inhibition of COR-triggered responses resulted in the repression of the COR-mediated antagonism of SA-inducible defenses, thus leading to the potentiation of SA-inducible defenses. In our hands, BABA could still reduce the growth of the *COR*$^-$ strain and we show that the BABA-induced reduction of COR-triggered activation of both JA and ABA pathways was mainly SA-dependent (Figure 4). In addition, during our experiments the SA pathway was induced by BABA prior to *Pst* infection and not potentiated during the infection (Figure 1, 2 and 4). The discrepancies observed between our results and those of Tsai *et al*. (2011) might be explained by protocol details. Indeed we used a higher BABA concentration (250 µM) to treat plants asTsai *et al*. (2011) (200 µM). In addition, the work of Tsai *et al*. (2011) demonstrated that

BABA treatment induced the expression of several SA-inducible genes. Thus it seems that in our case BABA treatment induced sufficient accumulation of SA and its marker transcripts *PR-1* in Arabidopsis prior to the infection resulting in reduced bacterial growth and subsequent reduced effector-triggered responses. This phenomenon was compromised in several SA-deficient mutants (Figure 4). These results suggest that BABA-IR could be due to an antagonism of BABA-induced SA-dependent defenses on the COR-triggered activation of both JA and ABA pathways. It is well known that SA can antagonize the JA pathway (Beckers and Spoel 2006, Bostock 2005, Glazebrook *et al.* 2003, Koornneef and Pieterse 2008, Kunkel and Brooks 2002, Pieterse, et al. 2009, Rojo *et al.* 2003). Recent studies also reported the antagonism of SA on the ABA pathway (Jiang, et al. 2010, Yasuda, et al. 2008). The outcome between SA-ABA interaction is not well understood but involves mutual antagonism (Jiang, et al. 2010, Kusajima, et al. 2010, Mohr and Cahill 2007, Yasuda, et al. 2008) and synergy (Mosher *et al.* 2010). In addition, Tsai *et al*. (2011) demonstrated that BABA inhibited the COR-triggered stomatal re-opening leading to restriction of the bacterial spread.

Thus it is likely that in our experiment BABA-IR against *Pst* resulted from BABA-induced SA-dependent responses leading either to pathogen restriction or to an antagonism against the effector-triggered activation of both the JA and ABA pathway leading to the failure of the bacteria to induce COR-triggered opening of the stomata and to colonize plant tissues.

In conclusion our results illustrate the fine-tuning of the balance of defense-related hormones in plants whose modulation leads either to pathogen spread or plant resistance. We could show the strong interdependency between the JA and ABA pathways whose induction by the bacteria was essentially due to the secretion of COR. For the first time we demonstrate that

both JA and ABA pathways are essential to trigger MeSA emission in Arabidopsis challenged with *Pst.* This later phenomenon might thus contribute to the COR-triggered antagonism on the SA-inducible defenses. Finally, we show that this virulence mechanism can be countered by the help of BABA through induction of the SA-inducible defenses.

MATERIALS AND METHODS

Biological material

Wild-type *Arabidopsis thaliana* accession Col-0 was obtained from Lehle Seeds (Round Rock, TX). The Col-0 mutants *npr1, sid1*, *sid2-1* and the transgenic NahG line were kindly provided by C. Nawrath (University of Lausanne, Switzerland). The Col-0 mutants *jin1* and *npq2-1* were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham,UK). One plant per pot was grown in 30 mL Jiffy® peat tablets (Ryomgaard, Denmark), maintained at 20°C day/18°C night temperature with 10 h of light (150 µmol m^{-2} s^{-1}) per 24 h and 70% relative humidity. The virulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Whalen *et al.* 1991) was grown during 12 to 14 hours at 28°C in liquid King's medium B containing 50 µg/mL of rifampicine. *Pst* strains DC3000 *hrpA* A9 (*HrpA*) and DC3118 *coronatine*$^-$ (*COR*$^-$) were kindly provided by Sheng Yang He (Michigan State University, USA) and grown in King's medium B containing 50 µg/mL of rifampicine and kanamycin.

Plant treatment, inoculation and sampling procedures

Five-week-old plants were treated with 250 µM final with β-aminobutyric acid (BABA) or water (mock) as soil drench as described previously (Ton, et al. 2005). Two days later, plants

were inoculated by dipping the leaves in *Pst*, *HrpA* or *COR*$^-$ strains suspensions containing 5 x 10^7 CFU/mL in 10 mM MgSO4 and 0.03% (v/v) Silwet L-77 (Lehle Seeds, Round Rock, TX). After inoculation, plants were grown at 100% relative humidity until sampling. For *P. syringae* bioassays, the percentage of diseased leaves presenting symptoms was determined on the indicated number of plants at the indicated time points before sampling. Leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored as diseased. At the indicated time points four rosette leaves were either pulled together for the time course experiments or sampled separately for specific time point measurements, frozen in liquid nitrogen and stored at -80°C for later gDNA, RNA, and hormone extractions. Grinding and homogenization of the collected samples was performed using a Qiagen® tissue lyzer in 10 mL grinding jars with 20 mm grinding balls during 30s at maximum speed.

Isolation of gDNA, RNA and cDNA biosynthesis

For gDNA and RNA extraction, 80 mg of fine leave powder were disrupted again 1 min at maximum speed in 1.5 mL vials containing 1 and 2 mm glass beads using a Qiagen® tissue lyzer. Total gDNA was extracted using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was isolated using the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. cDNA was obtained from 2 µg of total RNA using oligo$(dT)_{18}$ (Promega) and Superscript III reverse transcriptase (Invitrogen) following the instructions of the manufacturer.

Real-time PCR-based quantification of *P. syringae* growth and gene expression

The quantification of *Pst* growth and transcripts accumulation were performed on the gDNA and cDNA samples, respectively, by real-time PCR in a Rotor Gene 6000 (Corbett Life

Science). Primer sequences are listed in Table S1. PCR plates were prepared with the ROBOT CAS1200 (Corbett Robotics). 2µL of diluted cDNA or gDNA were amplified using 3µL of SensiMixPlus SYBR Kit (Quantace) with 250 nM primers in a final volume of 8 µL adjusted with ultrapure water. Cycling conditions were 95°C for 10 min followed by 40 cycles at 95°C for 10s, 60°C for 15s (except for *Psoprf*: 58°C for 15s) and 72°C for 20s and followed by a melting curve analysis (the negative first derivative of the fluorescence is plotted as a function of temperature). For the absolute quantification, each gene was amplified using the same primers and the corresponding PCR products were cloned into pGEM®-T Easy Vector (Promega) according to the manufacturer's guidelines. The clone concentrations were measured using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) according to the manufacturer's guidelines. 10 µL of each clone solution was diluted with 40 µL of TE 1X buffer and 50 µL of 200 times diluted PicoGreen® in 96 ELISA well plates. Standard DNA provided by the manufacturer (Invitrogen) was used to construct a calibration curve (from 0 to 500 ng/mL). After a 2 min incubation period, samples were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instrument). Fluorescence emission intensity was then plotted versus DNA concentration. Copy number/µL of each clone solution was calculated as follows: Copy number (molecule/g) = molecular weight of plasmid + cDNA or gDNA sequence (g/mol) / Avogadro's number. 2 µL of diluted clone solution (from 10^8 to 10^1 copies/µL) was amplified as described above to construct the standard curves (Copy number/log Ct). Results of the quantification of *P. syringae* growth were expressed as the ratio between copy numbers of a *Pst* specific gene (*Psoprf*) and the copy numbers of an Arabidopsis specific gene (*AtTUB4*). Normalization for the absolute quantification of transcript accumulation was performed by measuring the total amount of cDNA in each sample using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) as described above.

Gene expression results were expressed as the ratio between copy number of each transcript and the quantity of cDNA in ng. Since technical triplicates were performed for both PCR and cDNA quantification, the combined error was calculated as: (mean of the number of target cDNA/mean total cDNA) × ((S.D. of number of target cDNA /mean number of target cDNA) + (S.D. of total cDNA /mean total cDNA)).

Quantification of internal hormones level

After sampling of material for gDNA and RNA extractions, the remaining frozen material was lyophilized. Before extraction, a mixture of internal standards was added to 50 mg of dried tissue. Samples were immediately homogenized in 2.5 mL of ultrapure water for 1min on ice using a polytron. After centrifugation (5000 g, 40 min), the supernatant was recovered and adjusted to pH ≤ 2,7 with 10% acetic acid, and subsequently partitioned twice against an equal volume of diethyl ether. The organic fraction was dried under N_2 flux at room temperature. The solid residue was re-suspended in 1 ml of a water/methanol (90:10) solution and filtered through a 0.22 lm cellulose acetate filter. A 20 µL aliquot of this solution was then injected into a liquid chromatography tandem mass spectrometry and hormones were quantified as previously described (Flors *et al.* 2008, Pastor *et al.* 2012).

Quantification of MeSA emission

Three Arabidopsis plants per treatment were placed in glass bottles 24 hours post *Pst* infection. A constant airflow of charcoal filtered, humidified air entered the bottle at a rate of 0.6 L/min. Filters containing 25mg of the absorbent SuperQ (ARS) were attached to the outlet of the bottle. The system was hermetically closed, thereby forcing all the headspace volatiles through the filter. At different time points, the filters were detached and the trapped volatile

compounds were eluted with 150 µl $MeCl_2$. 10 µl of a mixture of internal standards (n-Octane and nonyl-actetate, 20ng/ml, Sigma) was added to each sample. All extracts were stored at -80°C until analyses. Volatiles were identified and quantified using a gas chromatograph (Agilent 6890 Series GC system G1530A) coupled to a mass spectrometer (Agilent 5973 Network Mass Selective Detector) that operated in electron impact mode (transfer line 230°C, source 230°C, ionization potential 70 eV, scan range 33–280 m/z). A 2µl aliquot of each sample was injected in the pulsed splitless mode onto an apolar capillary column (HP-1, 30 m, 0.25 mm ID, 0.25 µm film thickness; Alltech Associates). Helium at constant flow (0.9 ml/min) was used as carrier gas. After injection, the column temperature was maintained at 40°C for 3 min and then increased to 100°C at 8°C/min and subsequently to 125°C at 5°C/min followed by a post run of 5 min at 250°C. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 05 library and authentic standards of MeSA (SIGMA). Quantification of MeSA was carried out by comparing the ntegrated total ion peaks with the internal standard nonyl-actetate. After the sampling period, plants were weighed and the emission of volatiles was determined as the mass of MeSA emitted per mass of fresh weight per hour.

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SUPPLEMENTARY MATERIALS

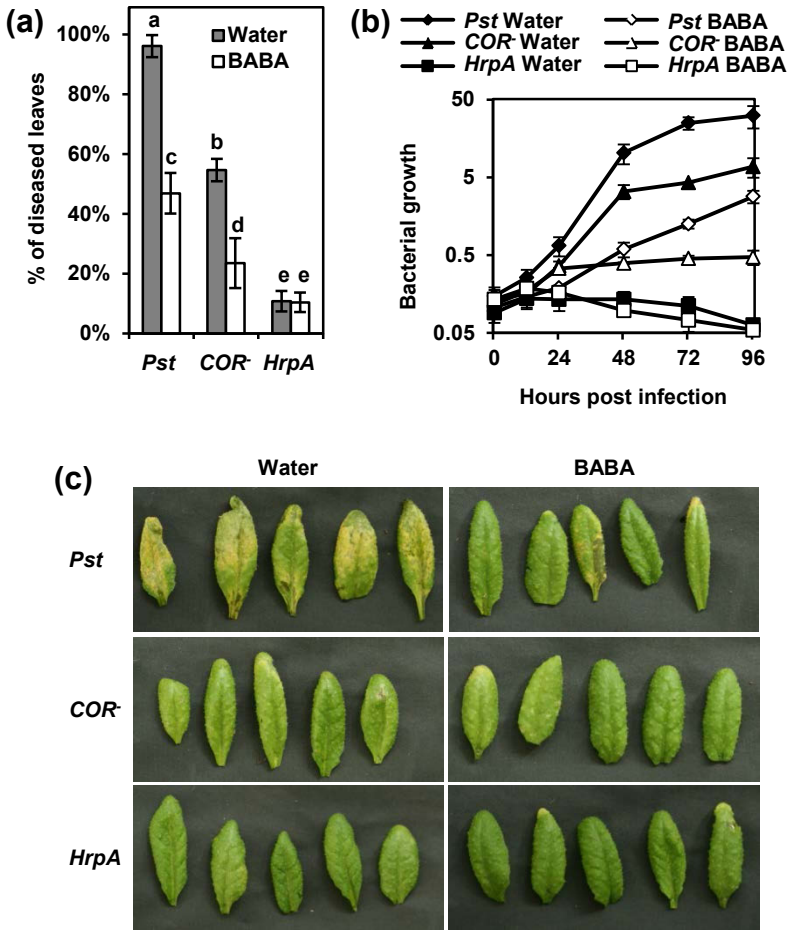

Figure S1. Coronatine- and TTSS effectors-triggered activation of the JA and ABA pathways in Arabidopsis challenged with the virulent *Pst*.
Five-weeks old wild-type Col-0 plants were treated with water or BABA (250 µM final) and challenged two days later with the wild-type strain *P. syringae* pv. *tomato* DC3000 (*Pst*), the coronatine-deficient strain (*COR*$^-$) or with the mutant compromised in TTSS (*HrpA*) at 5 x 10^7 colony-forming units mL^{-1}. Experiments were reproduced twice with similar results.

(a) Disease levels were calculated as the percentage of diseased leaves per plant three days post infection. Data represent the mean (±SD) of biological replicates. Letters represent significant disease level differences (ANOVA SNK, n=8, $P<0.05$).
(b) Bacterial growth was measured at the indicated times by real-time PCR. Data represent the mean (±SD) from technical replicates of the ratio *Pst* gene copy number/Arabidopsis gene copy number.
(c) Pictures show the symptoms of the plants three days post infection.

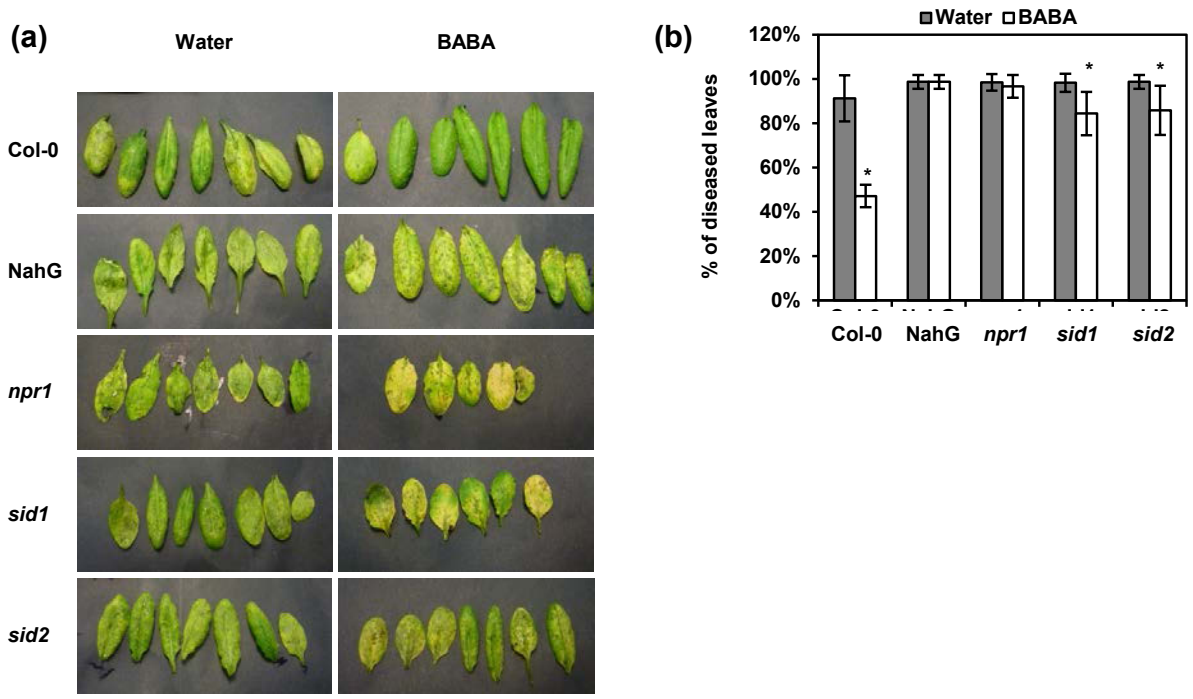

Figure S2. BABA-IR against *Pst* in SA-altered mutants.

Five-weeks old Col-0, NahG, *npr1*, *sid1* and *sid2* plants were treated with water or BABA (250 µM final) and challenged two days later with *Pst* at 5 x 10^7 colony-forming units mL^{-1}.
(a) Pictures show the symptoms of plants three days post infection.
(b) Disease levels were calculated as the percentage of diseased leaves per plant. Data represent the mean (±SD) of biological replicates. Asterisks represent significant disease level differences between water- and BABA-treated plants (t-test, n=6, $P<0.05$).

Table S1. Primer sequences used for the real-time PCR-based quantifications of *P. syringae* growth and gene expression

Primers used for the quantification of *P. syringae* growth

Name	Organism	Primer forward	Primer reverse
AtTUB4	*A. thaliana*	GCGAACAGTTCACAGCTATGTTCA	GAGGGAGCCATTGACAACATCTT
Psoprf	*P. syringae*	AACTGAAAAACACCTTGGGC	CCTGGGTTGTTGAAGTGGTA

Primers used for the quantification of gene expression

Name	AGI	Primer forward	Primer reverse
BSMT1	AT3G11480	TGCGTTTGTGAAAGCTCTATG	CTGGTTTGGCCATTGATAAAA
FAD	AT2G34810	CCGTCACCGAAACTCACA	TCCAAACGGTTTCGTCTT
LOX2	AT3G45140	AACTACGATTGCATGGGT	TGTTTCTGCGATGGGTAT
NCED3	AT3G14440	CGTCTTCTCAAAGCTCCGAC	TGAATCTTCGGCGTATTTGTCT
PR-1	AT2G14610	AAAACTTAGCCTGGGGTAGCGG	CCACCATTGTTACACCTCACTTTG
VSP2	AT5G24770	GAAGCTGCTGGCGTGACCTA	CCCAGGGGTATCCTCAACCA

CHAPTER 4

GENERAL DISCUSSION AND OUTLOOKS

GENERAL CONCLUSION AND OUTLOOKS

Development of the Real-Time PCR method to investigate plant-pathogen interactions

The absolute quantitative real-time PCR (aqRT-PCR) methodology applied during this work and presented in Chapter 2 was particularly suitable for the investigation of plant-pathogen interactions. This allowed numerous sample analysis and quantification of a large set of Arabidopsis responses, including the expression of genes of interest as well as pathogen growth.

The quantification of pathogen development and especially the growth of *P. syringae* were facilitated by the aqRT-PCR method. It allowed to monitor the development of different strains of *P. syringae* in different Arabidopsis mutants and to analyze numerous samples and biological replicates at the same time which would have been impossible by the use of the traditional methods. This PCR-based quantification method of *P. syringae* growth is now used routinely in several laboratories (personal communication) and has been used in a recent publication (Slaughter *et al.* 2012).

The aqRT-PCR methods developed to quantify gene expression is based on the establishment of standard curves using specific primers and clones for each gene and on the normalization between samples by the quantification of cDNA. This made it possible to bypass the tedious requirement of reference genes validation for each experimental set-up. It also allows the accurate comparison of the expression between the monitored genes, which is otherwise not reliable without the use of standard curves and normalization with reference genes. Finally, in comparison with the traditional relative quantification methods, data were obtained very conveniently. For instance, the use of software such as REST and qBase is believed to accelerate and facilitate the management of raw data, instead of using complicated calculation

through Excel sheet. These software are used to manage different PCR runs and to process automatic analysis, in order to quantify the expression of genes whose normalization is based on the expression of several reference genes (Pfaffl *et al.* 2002; Hellemans *et al.* 2007). However, the use of these software was more time-consuming and complex than the use of the aqRT-PCR method.

Furthermore, these methodologies could be additionally improved in order to reduce the number of PCR runs and to improve the sensitivity. This could be realized by multiplex PCR reactions using a fluorescent labeled-probe for each gene. These sequence-specific probes contain a fluorescent reporter at the 5' end combined with a quencher at the 3' end. The fluorescence of the reporter is quenched when the probe is intact. The probe then hybridizes with its complementary sequence between the specific primers. During the elongation phase of the PCR, the exonuclease activity of the polymerase liberates the fluorescent reporter. The emitted signal is then proportional to the amount of amplified products. The different reporter-quencher pairs and their related specific fluorescence signals generally enable to the amplification of up to five different products in a single run. Although the initial cost of the probes may be high, the reduced number of reactions would decrease the experimental cost and time. This improvement would also enable the measurement of the expression of one marker gene for each major plant defense pathway in a single run, which in turn would make it possible to obtain a rapid overview of the state of the complex networking of plant defenses. Finally, this method would also enable the quantification of both the pathogen and the plant genes in a single run.

However, the measurement of gene expression is insufficient to unravel the outcome of specific biological process. The fate of a transcribed messenger RNA is regulated by several post-transcriptional mechanisms. They include specific RNA-binding proteins and small non-coding RNA that leads to translation into the protein or degradation of the transcripts (Moore

2005). Nevertheless, the genes whose the expression was monitored during this work are mostly known marker genes of specific plant defense pathways controlled by plant hormones. To confirm the results obtained by measuring the expression of these marker genes, we also determined the accumulation of the corresponding hormones or the emission of volatile metabolites. The work presented in this thesis is thus based on sensitive and complementary analysis, including the PCR-based assessment of plant phenotypes against pathogens and the resulting transcriptional and hormonal responses.

Study of the Arabidopsis/*P. syringae* interaction

The aqRT-PCR methods developed during this thesis were used to study the interaction between Arabidopsis and the virulent bacteria *Pseudomonas synringae*. The PCR methods permitted to investigate precisely the interplay between a large set of Arabidopsis responses either induced through the virulence mechanisms of *P. syringae* or induced as defense responses in BABA-treated plants (Chapter 3). This enabled the confirmation that *P. syringae* promotes disease susceptibility in Arabidopsis through COR- and TTSS effectors-trigerred activation of both Arabidopsis JA and ABA pathways. We could show that both signaling pathways are highly interconnected and co-regulate each other during *P. syringae* infection. Their activation results in the repression of the SA-mediated basal defenses via the transcription factor MYC2 (Chapter 3) (DebRoy *et al.* 2004; Brooks *et al.* 2005; Laurie-Berry *et al.* 2006; Thilmony *et al.* 2006; Truman *et al.* 2006; Zabala *et al.* 2009). In addition, we confirmed that *P. syringae* induce the expression of the Arabidopsis gene *BSMT1*coding for the BENZOIC ACID and SALICYLIC ACID METHYL TRANSFERASE resulting in massive emission of MeSA in the atmosphere. This phenomenon is also mainly due to the COR-mediated manipulation of both JA and ABA pathways, probably to repress SA-inducible defenses (Koo *et al.* 2007; Attaran *et al.* 2009).

Beyond the SA-JA-ABA network, other plant hormones have been shown to be involved in the Arabidopsis/*P. syringae* interaction. For instance, ET has recently been shown to be an important regulator of the SA/JA antagonism. ET can render the antagonistic effect of SA on JA-dependent responses NPR1 independent (Leon-Reyes *et al.* 2009). On the other side, activation of the JA/ET pathway through ERF1 (ETHYLENE RESPONSE FACTOR) renders the plant insensitive to future SA-mediated suppression of JA-dependent defenses (Leon-Reyes *et al.* 2010). Although ABA was first associated with plant responses against abiotic stresses, its role as a major regulator of plant defense expression against pathogens has

become obvious (Mauch-Mani and Mauch 2005; Asselbergh *et al.* 2008; Ton *et al.* 2009). Interestingly, several studies and this work highlight the importance of ABA for the regulation of both JA biosynthesis and expression of *MYC2* while also repressing *ERF1* (Anderson *et al.* 2004; Adie *et al.* 2007). The mutual antagonism between SA and ABA is increasingly documented but the regulation of this cross-talk is not totally understood (Mohr and Cahill 2007; Yasuda *et al.* 2008; Zabala*, et al.* 2009; Jiang *et al.* 2010; Kusajima *et al.* 2010; Mosher *et al.* 2010). Several evidences support that the growth hormone auxin (AUX) is involved in the regulation of plant defenses (Navarro *et al.* 2006; Chen *et al.* 2007), and can be antagonized by the SA pathway (Wang *et al.* 2007; Zhang *et al.* 2007). AUX produced by bacterial pathogens in plants was shown to enhance disease susceptibility (Spaepen and Vanderleyden 2011) while conjugation of AUX to amino acids was also shown to promote disease development (Gonzalez-Lamothe *et al.* 2012). Interestingly, AUX also positively regulates the JA pathway during flower maturation and represses it in seedlings (Nagpal *et al.* 2005; Liu and Wang 2006), thus it would be interesting to investigate the link between ABA and AUX. In addition, other hormones involved in plant growth regulation such as gibberellins (Navarro *et al.* 2008), cytokinins (Siemens *et al.* 2006; Walters and McRoberts 2006) and brassinosteroids (Krishna 2003; Nakashita *et al.* 2003; Shan *et al.* 2008) have been shown to modulate plant disease resistance. For instance, the role of cytokinins implies synergism with SA and antagonism with AUX (Naseem *et al.* 2012). However the connection of all these hormones with the major defense hormones SA, JA, ET and ABA remains to be clarified.

The activation and fine-tuning of plant defenses are thus regulated by an extensive network of cross-communicating phytohormones. This complex network is more and more detailed and extended (Bari and Jones 2009; Grant and Jones 2009; Pieterse *et al.* 2009; Robert-Seilaniantz *et al.* 2011). Expression of plants defenses implies an ecological fitness cost (Heil and

Baldwin 2002; Walters and Heil 2007; Bolton 2009). This fine regulation would avoid excessive energy loss for the benefit of plant growth (Pieterse*, et al.* 2009). On the other side, virulent pathogens have evolved complex mechanisms such as the use of toxins and/or effectors to manipulate the cross-communication of plant defense pathways. This can result in the suppression of plant defenses and in the manipulation of host cellular functions to optimize growth conditions (Jones and Dangl 2006; Lopez *et al.* 2008; Pieterse*, et al.* 2009). Thus, the outcome of plant-microbe interactions is determined by the complex hormonal interplay between plant defense pathways on the one side and the virulence factors of pathogens on the other side. In conclusion, the work presented in this thesis makes a small contribution that enables a better understanding on this complex network.

Study of the priming mechanism and BABA-Induced Resistance against *P. syringae*

In addition to the contribution of this thesis to better describe the Arabidopsis/*P. syringae* interaction the aim of this project was also to study the mechanism by which BABA induces priming against *P. syringae.* We could show that BABA-IR against the virulent *P. syringae* growth correlated with reduced effector-triggered responses, although the mechanism could not be totally eluded.

BABA-IR against the virulent *P. syringae* was first described as characterized by boosted SA-inducible defenses upon infection (Zimmerli *et al.* 2000). In addition, it was recently shown that BABA specifically inhibits the Arabidopsis response to coronatine induced by the bacteria thus repressing the COR-mediated antagonism on the SA-dependent defenses (Tsai *et al.* 2011). This phenomenon explains the boosted expression of SA-inducible defenses in BABA-treated plants challenged with the viulent *P. syringae*. Previous studies also reported the inability of BABA to induce the expression of *PR-1* in stress free condition but rather potentiates this SA-inducible genes upon infection (Zimmerli*, et al.* 2000; Ton *et al.* 2005; Flors *et al.* 2008; Van der Ent *et al.* 2009). In addition, Tsai *et al* (2011) showed by using transcriptomic analyses that BABA treatment alone induces the expression of genes dependent of the SA pathway such as *PR-2* and *ICS1* but not *PR-1*. Moreover, in this study we observed slight but significant accumulation of SA and *PR-1* transcripts in plants treated with BABA in stress-free condition. This observation suggested that in our conditions, the induction of SA-inducible defenses in BABA-treated plants might be responsible for the resistance against the bacteria and the reduced effector-triggered responses. These discrepancies could be explained by differences in the experimental procedures and by the methodology used to monitor gene expression (See Chapter 2). In this thesis, transcript accumulation was monitored by real-time PCR and coupled to the sensitive measurement of hormones. Previous studies (Zimmerli*, et al.* 2000; Ton*, et al.* 2005; Flors*, et al.* 2008; Van

der Ent, *et al.* 2009) were mostly based on the quantification of gene expression by using Northern blotting of which the lower sensitivity is a result of reduced linear range of amplitude quantification (Guenin *et al.* 2009). In addition BABA is applied by watering soil. This implies that the uptake of the molecule by plant roots depends on both soil and air moisture and further environmental conditions that are difficult to standardize. Thus, it can be suggested that the amplitude of the SA-dependent responses induced by BABA in plants could vary depending on environmental conditions, BABA concentration and the capacity of plants to uptake the molecule applied in the soil.

The ability of BABA to induce the SA pathway (the SA pathway is represented in the Figure 1) was further investigated, although the work presented in this thesis did not permit to completely elude this phenomenon.

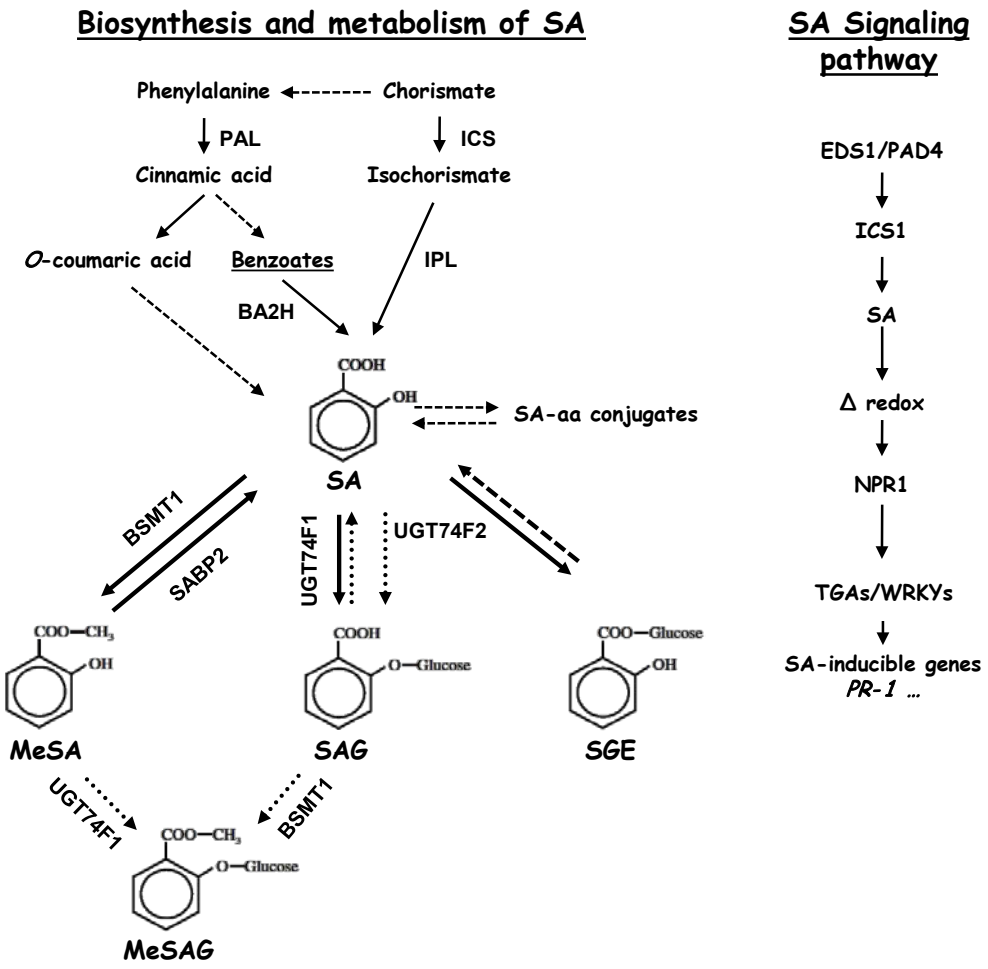

Figure 1. Representation of the SA metabolic and signaling pathway

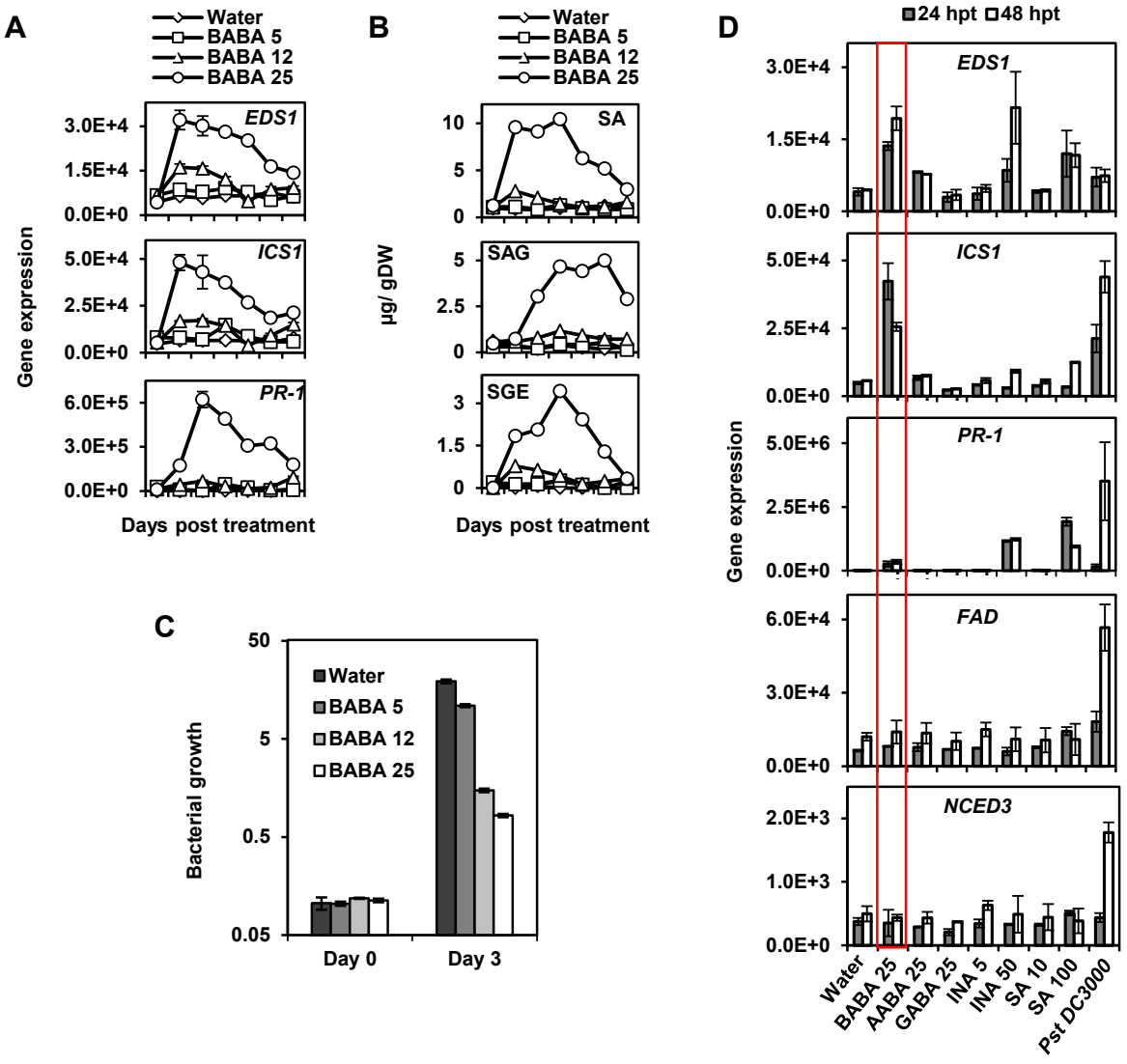

Figure 2. Effect of BABA treatment on the SA pathway. Data are from representative experiments that were repeated twice with similar results. Five-weeks old Arabidopsis were soil-drenched with water or increasing concentrations of BABA (5, 10 and 25 ppm).

(A) Expression of plant defense genes representing key component of the SA pathway (*EDS1*, *ICS1*, *PR-1*) was measured during six days post treatment. Accumulation of transcripts was measured in technical triplicates by real-time PCR. Data represent the mean (±SD) of each transcript copy number per ng of cDNA.

(B) Accumulation of free SA and the conjugated forms SAG and SGE was measured during six days post treatment by UPLC-MS.

(C) *P. syringae* pv. *tomato* DC3000 growth was measured in technical triplicates at the indicated times by real-time PCR in plants challenged two days post treatment with 5 x 10^7 colony-forming units mL^{-1}. Data represent the mean (±SD) of the ratio *Pst* gene copy number /Arabidopsis gene copy number.

(D) Expression of the marker genes *EDS1*, *ICS1*, *PR-1*, *FAD* and *NCED3* was measured at the indicated times (Hours post treatment) in five-weeks old plants soil-drenched with water, BABA, the BABA isomers AABA and GABA, the SAR inducers INA and SA at the indicated concentrations (in ppm) or in water treated plants challenged with *Pst* at 5 x 10^7 colony-forming units mL^{-1}. Accumulation of transcripts was measured in biological duplicates by real-time PCR. Data represent the mean (±SD) of each transcript copy number per ng of cDNA.

Preliminary results supported that BABA induced a dose dependent expression of genes representing key component at different steps of the SA pathway (*EDS1*, *ICS1*, *PR-1*) (Figure 2A) and accumulation of SA (Figure 2B). These dose dependent responses correlated with increasing resistance induction against the virulent *P. syringae* (Figure 2C). Furthermore, we compared the ability of BABA to induce these SA-dependent genes with those of the BABA isomers AABA and GABA enable to induce priming, the SAR inducers INA and SA and by the virulent *P. syringae* (Figure 1D). This enabled to show that only BABA activates a specific signal signature on the SA pathway characterized by a strong induction of the first steps of the SA signaling pathway (*EDS1* and *ICS1*) but a weak expression of the SA-inducible gene *PR-1* compared to the other SA inducers.

The mechanism of priming has been proposed to be associated with the accumulation of latent and inactive signals that would amplify the expression of plant defenses upon a stress situation (Prime-A-Plant Group 2006). Previous studies identified some potential candidates for these signals. Quantitative PCR-based genome-wide screens revealed that BABA induces the accumulation of NPR1-dependent WRKYs transcription factors (Van der Ent*, et al.* 2009). In addition, it was shown that the priming agent BTH induces the accumulation of *MPK3* and *MPK6* transcripts, both required to activate primed defenses (Beckers *et al.* 2009).

We hypothesized that the BABA-induced specific signal signature on the SA pathway could involve a shunt in the signalization leading to the accumulation of latent and inactive priming signals and finally resulting in low expression of the marker gene *PR-1* in stress-free condition. However, the work undertaken during this thesis could hardly link the ability of BABA to induce a specific signal signature on the SA-pathway with the priming mechanism. Recognition of the bacteria by the plant immune system results in PTI-mediated induction of SA-dependent responses (Tsuda *et al.* 2008). In turn, bacteria suppress this response through

the delivery of TTSS effectors (Boller and He 2009; Guo *et al.* 2009) some of which act with coronatin to mediate the JA- and ABA-dependent suppression of the SA pathway (Laurie-Berry*, et al.* 2006; Zabala*, et al.* 2009). The potentiation of SA-inducible defenses upon *Pst* infection in BABA-treated plants was shown to be the results of the BABA-mediated inhibition of Arabiopdis responses to coronatin (Tsai*, et al.* 2011). In addition to this phenomenon, a synergistic effect between the sensitized SA pathway induced by BABA and the PTI-mediated induction of SA-dependent responses could contribute to the priming phenomenon. Thus, investigating BABA-IR in mutants compromised in specific component of PTI could answer this question. Indeed, the Lectin Receptor Kinase-VI.2 that positively regulates PTI was recently shown to be required for priming (Singh *et al.* 2012).

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