Eur J Plant Pathol (2014) 139:707–720
DOI 10.1007/s10658-014-0424-7

Role of two UDP-Glycosyltransferases from the L group of arabidopsis in resistance against pseudomonas syringae

Benoît Boachon · Jordi Gamir · Victoria Pastor · Matthias Erb · John V. Dean · Víctor Flors · Brigitte Mauch-Mani

Accepted: 2 April 2014 / Published online: 13 April 2014

Abstract The role of the salicylic acid (SA) glycosides SA 2-*O*-β-D-glucose (SAG), SA glucose ester (SGE) and the glycosyl transferases *UGT74F1* and *UGT74F2* in the establishment of basal resistance of Arabidopsis against *Pseudomonas syringae* pv *tomato* DC3000 (*Pst*) was investigated. Both mutants altered in the corresponding glycosyl transferases (*ugt74f1* and *ugt74f2*) were affected in their basal resistance against *Pst*. The mutant *ugt74f1* showed enhanced susceptibility, while *ugt74f2* showed enhanced resistance against the same pathogen. Both mutants have to some extent, altered levels of SAG and SGE compared to wild type plants, however, in response to the infection, *ugt74f2* accumulated higher levels of free SA until 24 hpi compared to wild type plants while *ugt74f1* accumulated lower SA levels. These SA levels correlated well with reduced expression in *PR1* and *EDS1* in *ugt74f1*. In contrast, *ugt74f2* has enhanced expression of *Enhanced Disease Susceptibility 1* (*EDS1*) but a strong reduction in the expression of several jasmonate (JA)-dependent genes. Bacterial infection interfered with the expression of *Fatty Acid Desaturase* (*FAD*), *Lipoxygenase2* (*LOX2*), *carboxyl methyltransferase1* (*BSMT1*) and 9-*cis*-epoxycarotenoid dioxygenase (*NCED3*) genes in *ugt74f1*, thus promoting an antagonistic effect with SA-signalling and leading to enhanced bacterial growth. *UGT74F2* might be a target for bacterial effectors since bacterial mutants affected in effector synthesis were impaired in inducing *UGT74F2* expression. These results suggest that *UGT74F2* negatively influences the accumulation of free SA, hence leading to an increased susceptibility due to reduced SA levels and increased expression of the JA and ABA markers *LOX-2*, *FAD* and *NCED-3*.

Keywords Salicylic acid · SA 2-*O*-β-D-glucose · SA glucose ester · Pseudomonas syringae

Electronic supplementary material The online version of this article (doi:10.1007/s10658-014-0424-7) contains supplementary material, which is available to authorized users.

B. Boachon · V. Pastor · B. Mauch-Mani (✉)
Laboratory of Molecular and Cellular Biology, Institute of Botany, University of Neuchâtel,
Rue Emile-Argand 11, Case Postale 158, 2009 Neuchâtel, Switzerland
e-mail: Brigitte.mauch@unine.ch

J. Gamir · V. Flors
Metabolic Integration and Cell Signaling Group, Plant Physiology Section, Department of CAMN, Universitat Jaume I,
Castellón 12071, Spain

M. Erb
Laboratory for Fundamental and Applied Research in Chemical Ecology (FARCE), University of Neuchâtel, Neuchâtel, Switzerland

J. V. Dean
Department of Biological Sciences, DePaul University, Chicago, IL, USA

M. Erb
Root-Herbivore Interactions Group, Max Planck Institute for Chemical Ecology,
Jena, Germany

Introduction

The immune response of a plant following attack by pathogens is regulated by plant hormones. Among the many changes occurring during this expression of basal resistance, is a prominent rise in salicylic acid (SA) levels, especially during interactions with biotrophic pathogens (de Vos et al., 2005).

SA is synthesized from shikimate/chorismate-derived products (about 90 %) essentially through the activity of *ISOCHORISMATE SYNTHASE 1* (*ICS1*) (Garcion et al., 2008; Wildermuth et al., 2001; Wildermuth, 2006). Signaling downstream of SA biosynthesis requires the interaction between *ENHANCED DISEASED RESISTANCE 1* (*EDS1*) and its partner *PHYTOALEXIN DEFICIENT 4* (*PAD4*) to activate SA accumulation (Feys et al., 2001; Wiermer et al., 2005). Subsequent signaling of SA is mostly controlled by *NON-EXPRESSOR of PR PROTEIN 1* (*NPR1*) whose function is regulated by SA-mediated modulation of the cellular redox state (Mou et al., 2003). Cytosolic localization of *NPR1* monomers regulates the SA-mediated repression of jasmonic acid (JA)-responsive genes (Spoel *et al*., 2003). *NPR1* also interacts with TGA transcription factors in the nucleus to regulate the expression of SA-inducible genes such as *PR-1* (Kesarwani et al., 2007; Mou et al., 2003).

The role of SA in plant immune responses depends on a poorly understood interplay between its free and conjugated forms. SA can be metabolized into several conjugates through glycosylation, methylation and conjugation with amino acids (Loake and Grant, 2007; Vlot et al., 2009). The methylated form methylsalicylate (MeSA) is catalyzed from SA by *BSMT1* (Chen et al., 2003). MeSA has been proposed to be the long-distance signal for transduction of systemic acquired resistance (SAR) since it can release free SA in systemic tissue by the activity of *SA BINDING PROTEIN 2* (*SABP2*) (Liu et al., 2010; Park et al., 2007; Vlot et al., 2008). However, MeSA production and *BSMT1* expression are not required for SAR establishment in Arabidopsis (Attaran et al., 2009). Instead, several studies support that MeSA production is involved in the JA-mediated inhibition of the SA pathway (Attaran et al., 2009; Koo et al., 2007; Song et al., 2008; Song et al., 2009). In Arabidopsis, most MeSA produced during compatible and incompatible interactions with *P. syringae* is released to the atmosphere (Attaran et al., 2009).

In regards to glycosylated forms, in tobacco, the bulk of SA produced during pathogen infections or exogenous SA application is primarily metabolized into SA 2-*O*-β-D-glucose (SAG) by a SA glycosyltransferase (SAGT) (Edwards, 1994; Lee and Raskin, 1999; Dean et al., 2005; Song, 2006). SAG accumulates in the vacuole presumably as a hydrolysable source of free SA available for plant defense (Dean et al., 2005). In tobacco, SAG can be converted into SA by a β-glucosidase (Seo et al., 1995). Furthermore, tobacco overexpressing a β-glucosidase accumulates less SAG and elevated SA levels resulting in enhanced resistance against Tobacco Mosaic Virus (TMV) (Yao et al., 2007). In Arabidopsis, in vitro catalytic analysis determined that the glycosyltransferase UGT74F1 can form SAG while UGT74F2 can form both SAG and SGE but with a 10-fold higher specific activity for SGE formation (Lim et al., 2002). The specific activity of *UGT74F1* was 58-fold higher than of *UGT74F2 for* SAG formation. The mutant *ugt74f1* only accumulates SGE in untreated plants and accumulates both SAG and SGE in SA-treated plants due to *UGT74F2* activity (Dean and Delaney, 2008). The mutant *ugt74f2* does not accumulate SGE in both water- and SA-treated plants but still produces SAG in SA-treated plants due to *UGT74F1* activity (Dean and Delaney, 2008).

The biological function of SAG and SGE is not well understood. Some glycosyltransferases from phylogenetic group L have been shown to be relevant for resistance against *Pst* (Langlois-Meurinne et al., 2005). Additionally, overexpression of *UGT74F2* leads to enhanced susceptibility to *Pst* (Song et al., 2008). Previous reports suggest the relevance of glycosylation in resistance against *Pst*. A member of the uridine 5'-disphosphate (UDP)-glycosyltransferase (UGT) (*At1g05680*) confers enhanced resistance against the bacterium. The mutant *ugt74e2* shows augmented systemic immunity during SAR, and increased levels of *PR1*, *PR2* and *PR5* transcripts (Park et al., 2011). *UGT74E2* is a member of the L subclass of UGTs (Ross et al., 2001) and together with *UGT74F2* has been shown to transfer glucose to SA and anthranilate although their biological substrates have not been clearly identified.

Recent findings also describe the SA-related priming activity of several novel chemicals called imprimatins

(Noutoshi et al., 2012). Their priming activity against *Pst* seems to be linked to the inhibition of glycosyltransferase activity. Thus by treating Arabidopsis plants with these compounds free SA increases and the SA-*O*-β-glucoside is reduced. Interestingly, mutants in the *UGT74F1* display enhanced resistance against virulent *Pst DC3000* and avirulent *Pst* avrRmp1. However, only increased levels of SA upon inoculation with the avirulent strain *Pst avrRmp1* have been determined whereas SA and SAG levels upon inoculation with virulent *Pst* were not determined. In addition, the mutant *ugt74f1* used in these experiments showed a surprising up-regulation of *UGT74F1* mRNA (Noutoshi et al., 2012). These investigations reinforce the role of *UGT74F1* in SA glycosylation, however, the role of this transferase in resistance still remains controversial. Interestingly, glycosyltransferases affecting the SA pathway are not restricted to those using SA as a substrate. In fact, a novel role for the *UGT76B1* gene has been discovered (von Saint Paul et al., 2011). By using recombinant systems, it has been demonstrated that this gene encodes an enzyme that uses isoleucic acid (ILA) as a substrate. The absence of this transferase up-regulates the SA pathway and down-regulates JA signalling. In addition, the *ugt76b1* mutants are more resistant to *P. syringae* but more susceptible to *Alternaria brassicicola* (von Saint Paul et al., 2011). Although the relationship between ILA and SA is not clear, it is obvious that glycosylation of both bioactive compounds inactivates them and this has a negative impact on Arabidopsis resistance against *Pst*.

These recent advances provide the impetus for additional studies the role of SA metabolites in plant disease resistance. In this study, the role of both glycosyltranferases *UGT74F1* and *UGT74F2* in Arabidopsis basal resistance against *Pst* was investigated. The transcriptional profile of genes involved upstream and downstream of the SA signaling pathway, SA biosynthesis and SA metabolism upon bacterial infection was examined. Accumulation of SA, SAG and SGE and the emission of MeSA were also monitored. Surprisingly, the mutants showed resistant phenotypes opposite from one another following bacterial infection. They also differed in their response to the bacterial effector Coronatine, suggesting that each has a contrasting function in Arabidopsis basal resistance against *Pst*.

Material and methods

Biological material

Wild-type *Arabidopsis thaliana* accession Col-0 and Ws-0 was obtained from Lehle Seeds (Round Rock, TX). The Col-0 mutants *npr1*, *sid1*, *sid2-1* were kindly provided by C. Nawrath (University of Lausanne, Switzerland). The Ws-0 mutant *ugt74f1* was kindly provided by John V. Dean (DePaul University, USA). The Col-0 mutant *ugt74f2* was a gift from Judith Bender (Brown University, USA). Plants were grown singly in 30 mL Jiffy® peat tablets (Ryomgaard, Denmark), maintained at 20 °C day/18 °C night temperatures with 9 h of light (150 μmol m^{-2} s^{-1}) per 24 h and 70 % RH. The virulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Whalen et al., 1991) was grown overnight 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 each rifampicine and kanamycin.

Plant inoculation and sampling

For the infection, five-week-old plants were inoculated by dipping the leaves in $MgSO_4$ mock suspension or *Pst*, *HrpA* and *COR*$^-$ containing 5×10^7 colony-forming units ml^{-1} in 10 mM $MgSO_4$ 0.01 % v/v Silwet L-77 (Lehle Seeds, Round Rock, TX). After inoculation, plants were grown under 100 % relative humidity until sampling. For the *P. syringae* bioassays, 3 days after inoculation, leaves showing necrotic or water-soaked lesions surrounded by chlorosis were scored and the percentage of leaves presenting disease symptoms was determined. At the indicated time-points leaves were sampled for specific genetic and hormonal analyses, frozen in liquid N_2 and stored at -80ºC.

Quantitative real-time RT-PCR analysis of transcripts and PCR-based quantification of *P. syringae* infection

For quantification of plant gene expression powdered samples from frozen leaf material was homogenized. Total RNA was isolated using the Rneasy plant minikit (Qiagen). 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.

The PCR was performed in a rotor Gene 6000 (Corbett Life Science) and plate samples were prepared with a ROBOT CAS1200 (Corbett Robotics). Two μL of diluted cDNA were amplified using 3 μl of SensiMixPLus SYBR Kit (Quantace) with 250 mM of primers and adjusted to a final volume of 8 μl. Primers are described in the Suplementary Table 1. For the quantification of each transcript, standard curves (number of copies/log Ct) were constructed using serial dilutions of known copy numbers of plasmids (10^8 to 10^1 copies.μl^{-1}). Plasmids for each transcript were transformed with the corresponding PCR product obtained with the same primers used for the real-time PCR and cloned in the pGEM-T easy Vector (Promega). Results of gene aqRT-PCR expression were expressed as copy number of the target gene per total ng of cDNA.

The quantification of *P. syringae* growth and transcripts accumulation was performed by real-time PCR in the same real-time equipment. Diluted cDNA was amplified using the SensiMix plus SYBR Kit (Quantance) in the same conditions described for the plant gene expression. The cycling conditions for *Psoprf* were 95ºC for 10 min followed by 40 cycles at 95ºC for 10 s, 58ºC for 15 s and 72ºC for 20 s, followed by a melting point analysis. Standard curves (Copy number/log Ct) were constructed using plasmids transformed with the respective cDNA or DNA product. Results of the quantification of *P. syringae* growth were expressed as copy numbers of the *Psoprf* gene per copy numbers of the *AtTUB4* gene. For the absolute quantification of transcript accumulation (aqRT-PCR), the quantification of total cDNA of each sample was used for the normalization and performed using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen).

Quantification of SA, SAG and SGE

Frozen material was lyophilized and SA, SAG and SGE were analyzed as is described by Pastor *et al.* 2012. Briefly, 50 mg of dried material was mixed with internal standard and homogenized with a polytron, using an extraction solution containing MeOH/H_2O (10/90, v/v), containing 0.01 % of HCOOH. After centrifugation the supernatant was filtrated through a 0.22 μm cellulose acetate filter. A 20 μl aliquot of this solution was injected into a liquid chromatography coupled to tandem mass spectrometry. Masslynx 4.1 (Waters, Manchester, UK) software was used to process the quantitative data obtained from calibration standards and samples.

Quantification of MeSA emission

Three Arabidopsis plans per condition were placed in glass bottles 24 h post *Pst* infection. A constant airflow of charcoal-filtered, humidified air entered the bottle at a rate of 0.6 l/min. Filters containing 25 mg of the absorbent SuperQ (ARS) were attached to the outlet of the bottle. The system was hermetically closed, thereby forcing all the head space volatiles across the filter. At different time points, the filters were detached and the trapped volatile compounds were eluted with 150 μL $MeCl_2$. Then, 10 μl of a mixture of internal standards (n-octane and nonyl-acetate, 20 ng/mL, Sigma) was added to each sample. All extracts were stored at -80ºC until analyses. Volatiles were identified and quantified using a gas chromatograph (Agilent 6,890 Series GC system G1530A) coupled to a mass spectrometer (Agilent 5,973 Network Mass Selective Detector) that operated in electron impact mode (transfer line 230ºC, source 230ºC, ionization potential 70 eV, scan range 33–280 m/z). A 2 μL 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 40ºC for 3 min and then increased to 100ºC at 8ºC/min and subsequently to 125ºC at 5ºC/min followed by a post run of 5 min at 250ºC. The detected volatiles were identified by comparison of their mass spectra with those of the NIST 05 library and authentic standards of MeSA (Sigma-Aldrich, Switzerland). Quantification of MeSA was carried out by comparing the integrated total ion peaks with that of the internal standard nonyl-acetate. After the sampling period, plants were weighted and the emission of volatiles was determined as the mass of MeSA emitted per mass of fresh weight per hour.

Results

UGT74F1 and *UGT74F2* play a role in basal resistance against *Pseudomonas*

To get a more precise insight into the role of glycosyltransferases involved in the synthesis of SAG and SGE, the mutants *ugt74f1* and *ugt74f2* were inoculated with a virulent strain of *Pst*. Compared to wild type plants, bacterial titers in *ugt74f1* were higher starting at 48 hpi (Fig. 1a). Accordingly, the disease levels expressed as a percentage of infected leaves per plant were higher and the visible symptoms were more pronounced in the mutant (Fig. 1a). On the contrary, *ugt74f2* was more resistant to the infection by *Pst* supporting reduced bacterial growth, showing a lower percentage of diseased leaves and displaying reduced symptoms compared to the wild type (Fig. 1b).

In addition to these observations and in agreement with previous results, *UGT74F2* but not *UGT74F1* was induced in wild type plants challenged with *Pst* in both backgrounds tested (Fig. 2). Interestingly, *UGT74F1*

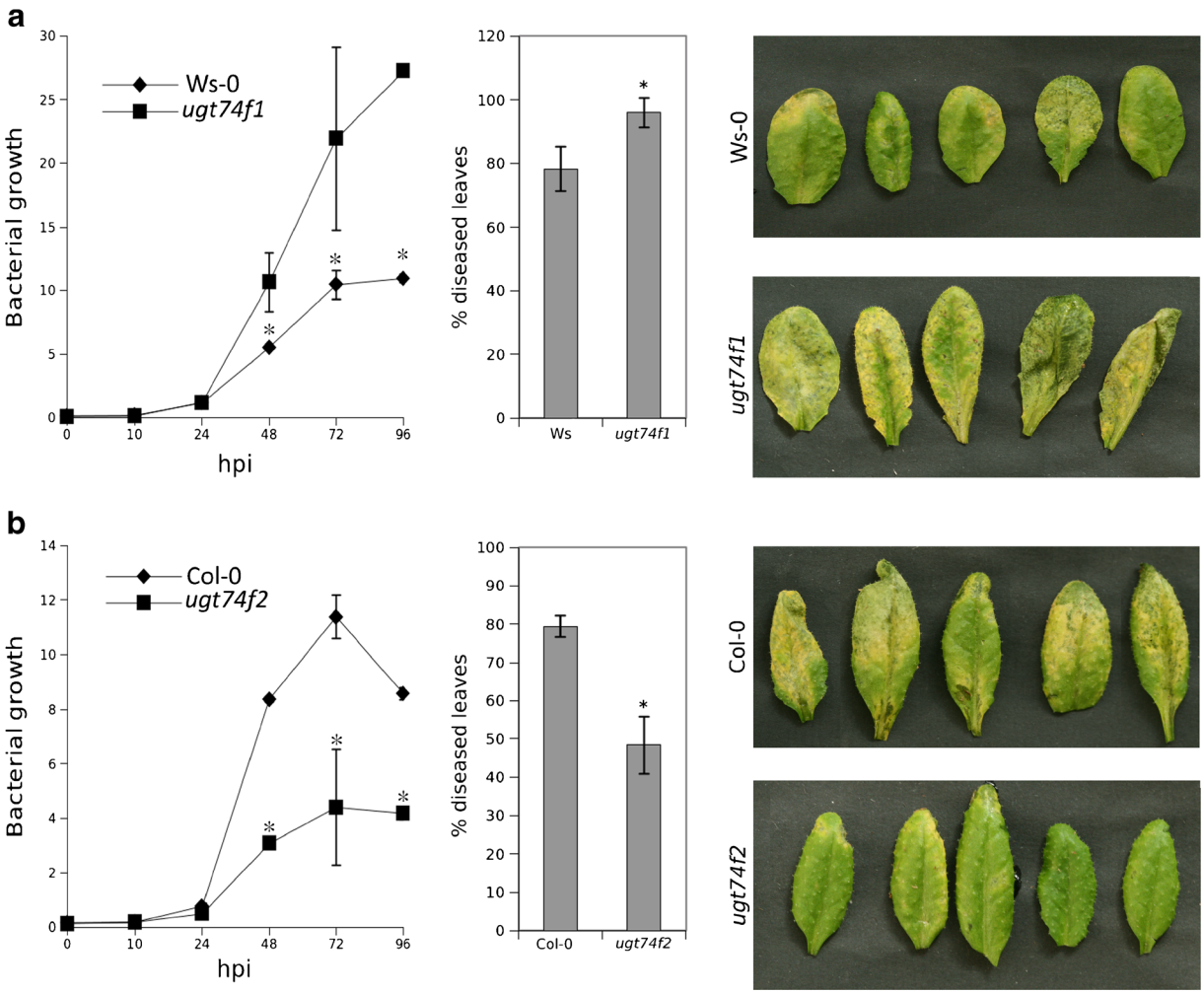


Fig. 1 Basal resistance of Arabidopsis wild type and *ugt74f1* and *ugt74f2* mutants against *Pseudomonas syringae* pv. tomato DC3000. A) Five-week old Col-0, Ws-0, *ugt74f1* and *ugt74f2* plants were challenged with 5.10^7 colony-forming units mL^{-1} of *P. syringae* DC3000. Bacterial growth was measured by real-time PCR at the indicated time-points. Data presented are the means of three technical replicates (±SD) of the ratio of *Psorf* gene copy *number/AtTUB4* gene copy number. Asterisk means statistically significant differences (*T*-test; $p<0.05$). B) Disease rate was measured by calculating the percentage of diseased leaves per plant ($n=20$,±SD). Data are from a representative experiment that was repeated four times with similar results. Asterisk means statistically significant differences (*T*-test; $p<0.05$). Photograph shows a representative picture of disease symptoms in both genetic backgrounds

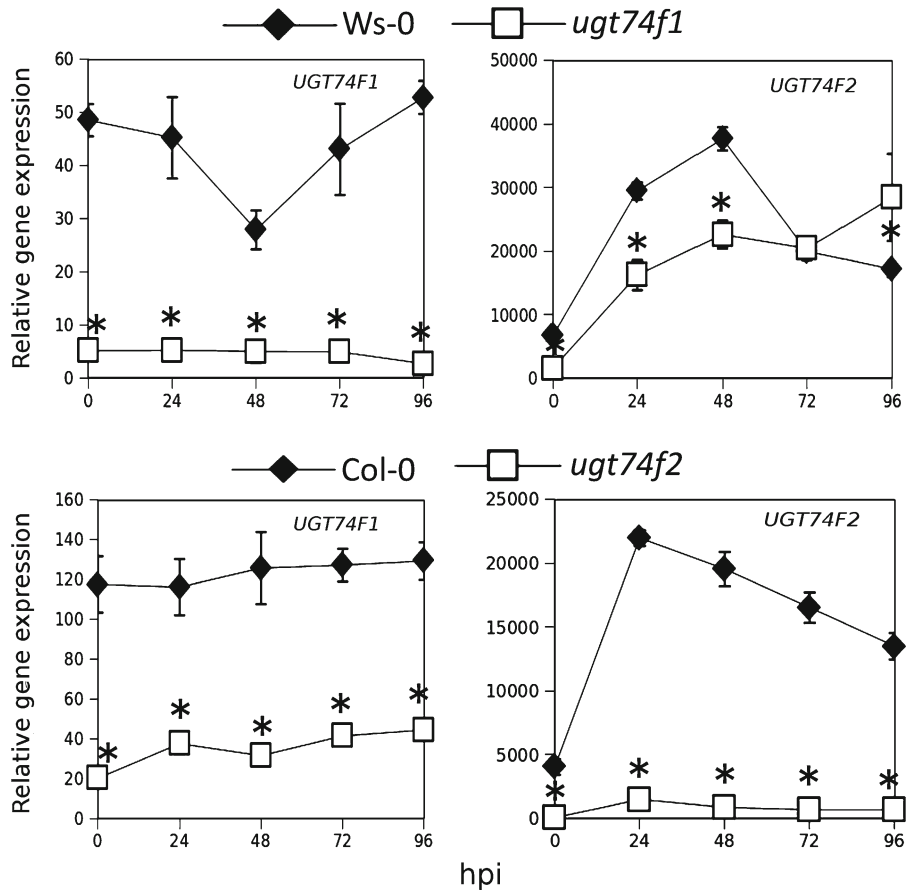


Fig. 2 Profile of glycosyltransferase gene expression upon *P. syringae DC3000* infection. *UGT74F1* and *UGT74F2* were measured at the given time-points by real-time PCR. Data show the mean of three technical replicates (±SD). The experiment was repeated three times with similar results. For inoculation and further experimental details see Fig.1. Asterisk means statistically significant differences within each time-point (*T*-test; $p<0.05$)

and *UGT74F2* displayed a cooperative function upon infection. This cooperative influence was clearly evidenced when *UGT74F2* was disrupted since expression of *UGT74F1* was severely affected in the mutant *ugt74f2* (Fig. 2). Hence, *UGT74F2* seems to contribute negatively to Arabidopsis resistance towards *Pst*.

The hypersusceptible phenotype of *ugt74f1* is due to reduced SA levels and a concomitant enhancement of antagonistic pathways

In order to understand the reasons for the observed phenotype of *ugt74f1*, gene expression of the main disease resistance pathways was assessed in this mutant. As expected, *UGT74F1* expression was absent in the knockout mutant (Fig. 2). Gene expression of SA-related genes *Enhanced Disease Susceptibility1* (*EDS1*) and *Pathogenesis Related-1* (*PR-1*) was clearly reduced while *Isochorismate Syntase1* (*ICS1*) was hardly altered in *ugt74f1* compared to the wild type plants (Fig. 3a). Interestingly, the levels of free SA in *ugt74f1* were reduced at later time points in the infection by *Pst* (Fig. 3b).

According to previous studies, most SAG produced in wild type Arabidopsis is due to the activity of *UGT74F1* while SGE is only formed by UGT74F2 (Dean and Delaney, 2008; Lim et al., 2002). In infected *ugt74f1* plants, the accumulation of SAG is delayed and lower than in wild type plants whereas SGE is hardly affected (Fig. 3b). In *ugt74f1* compared to wild type, the accumulation of Fatty Acid Desaturase (FAD), Lipoxygenase (LOX2) and 9-cis-epoxycarotenoid dioxygenase (NCED3) transcript levels are enhanced (Fig. 3a), pointing to an up-regulation of the oxylipin and the ABA signalling pathway. In contrast, the JA and JA/Et marker genes VSP2 and PDF1.2 were less

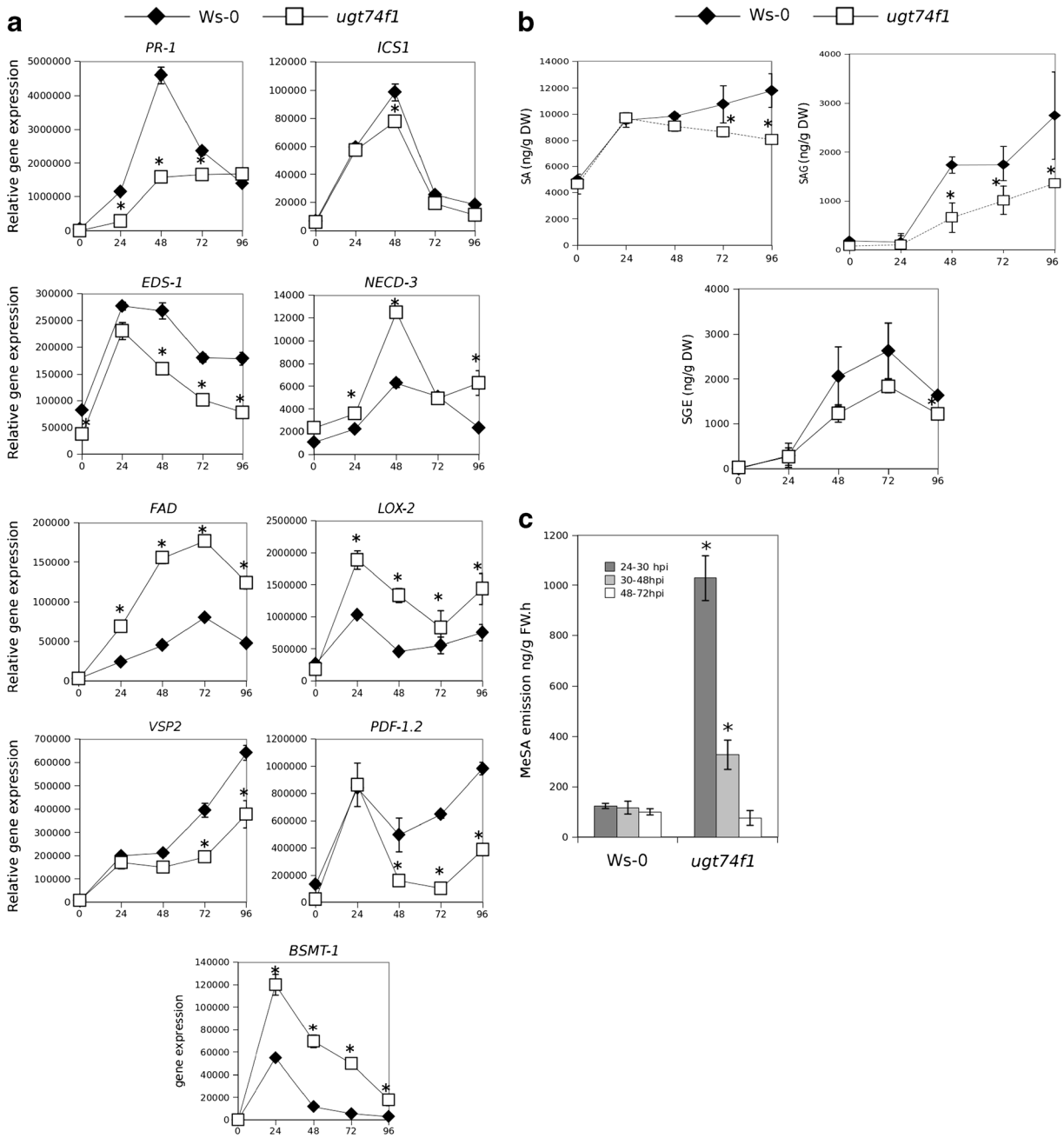


Fig. 3 Profile of plant defence pathways upon *Pst* infection in Ws-0 wild type and *ugt74f1* mutant. A) Expression of genes representing key components of the SA, JA and ABA pathways was measured at the given time-points by real-time PCR. Data show the mean of three technical replicates (±SD). B) The levels of SA, SAG and SGE upon infection were determined by LC-MS. C) The accumulation of MeSA released along the time periods indicated was determined by GC-MS. Gene expression data and MeSA are from representative experiments that were repeated twice with similar results. SA, SAG and SGE data are from a representative experiment that was repeated four times. In the figures A, B and C the asterisk means statistically significant differences within each time-point (*T*-test; $p<0.05$)

expressed in the mutant compared with wild type plants (Fig. 3a). This switch between JA up-stream and JA down-stream genes suggests a diversion in the pathway that may be clearly re-organized towards the accumulation of other oxylipins that differ from JA or its down-stream products. On the other hand, the increase of

BSMT1 gene expression in ugt74f1 correlates with a strong increase in the MeSA emission (Fig. 3c) that may contribute to the observed decrease in free SA levels.

ugt74f2 displays enhanced resistance due to a transient accumulation of free SA and a concomitant negative crosstalk with antagonistic pathways

Although no major changes in the expression of *PR1* and *ICS1* were observed, the expression of *EDS1* was clearly up-regulated in *ugt74f2* (Fig. 4a). Mutant plants displayed a transient increase in free SA until 24 hpi (Fig. 4b). Despite the fact that *UGT74F2* has been reported to be involved in the synthesis of SAG and SGE (Dean and Delaney, 2008; Lim et al., 2002), similar levels of SAG were found in mutant and wild type plants upon infection, while SGE in *ugt74f2* was hardly detected either in the presence or in the absence of infection (Fig. 4b). The expression of *UGT74F2* was almost absent in *ugt74f2* (Fig. 2).

In contrast to the observed enhancement of the JA pathway in *ugt74f1*, expression levels of *LOX2*, *VSP2* and *FAD* were strongly down-regulated in *ugt74f2* after infection (Fig. 4a). *PDF1.2* was expressed higher in the mutant. *NCED3* expression was also down-regulated albeit to a lesser extent. Therefore, the mutation in *UGT74F2* contributes to a shift in the negative crosstalk between SA and JA-ABA signalling pathways towards SA-dependent responses. A likely explanation for the fast SA accumulation in *ugt74f2* is the down-regulation of *BSMT1* and the resulting reduction in MeSA emission (Fig. 4c). Noteworthy the JA levels are higher in *ugt74f1* at 24, 72 and 96 upon infection while they are only increased after 96hpi in the *ugt74f2* mutant (Fig. S1).

To determine whether an alteration in the SA pathway could also affect glycosyltransferase gene expression and glucoside formation. SA mutants and NahG were as expected more susceptible to Pst (Fig. S2a). Neither *UGT74F1* nor *UGT74F2* gene expression was significantly altered following infection with *Pst* in the above-mentioned mutants except in NahG plants (Fig. S2b). As expected, *sid1* and *sid2* mutants affected in SA synthesis and *NahG* in which SA is immediately converted to catechol accumulates neither conjugated nor free forms of SA, although these compounds were clearly not affected in the signaling mutant *npr1* (Fig. S2c).

Functional effector secretion is required for the induction of *UGT74F2*

In order to check whether effector secretion was necessary for the up-regulation of *UGT74F2* upon infection by *Pst*, wild type plants were inoculated with *COR*$^{-}$ and *hrpA* mutants of *Pst*. Bacterial growth was impaired for both mutant strains compared to WT *Pst* (Fig. 5a). This was also reflected in the number of affected leaves (Fig. 5b). The expression of *UGT74F1* was the same following inoculation with wild type and mutant *Pst* strains, respectively (Fig. 5c). It is noteworthy, that the induction of *UGT74F2* expression was strongly induced by the virulent strain of *Pst* while it was reduced in the absence of both the Coronatine and HrA effectors (Fig. 5c). An additional support for the importance of functional effector secretion was found when Col-0 plants where treated with the Pathogen Associated Molecular Pattern (PAMP) Flg22 (Fig. S3). A transient up-regulation of *UGT74F2* was observed at 24 hpt and had reverted to mock levels at 48 hpt. This might suggest that its activation upon infection is dependent on the effector machinery of *Pst* at the beginning of the infection process but not by the elicitor Flg22.

Discussion

In Arabidopsis, about 120 genes encoding UDP-glycosyltranferases have been described. These genes have been classified into 14 groups (Ross et al., 2001). Although the exact function of all these genes has not been yet determined, many of them share similar catalytic properties and appear to be involved in the glycosylation of plant hormones and other substrates (Langlois-Meurinne et al., 2005). The first identified function of a *UGT* was flavonol glycosylation (Dooner and Nelson, 1977). The down-regulation of this gene in tobacco resulted in an enhanced susceptibility towards TMV. In Arabidopsis, *UGT73B3* and *UGT73B5* have been shown to participate in resistance towards *Pst* and this resistance seems to be dependent on SA signalling (Langlois-Meurinne et al., 2005). The *AtSAG1* (*UGT74F2*, *At2g43820*) gene is directly induced upon SA treatments and therefore it could be involved in SA signalling dependent defence (Song, 2006). All three,

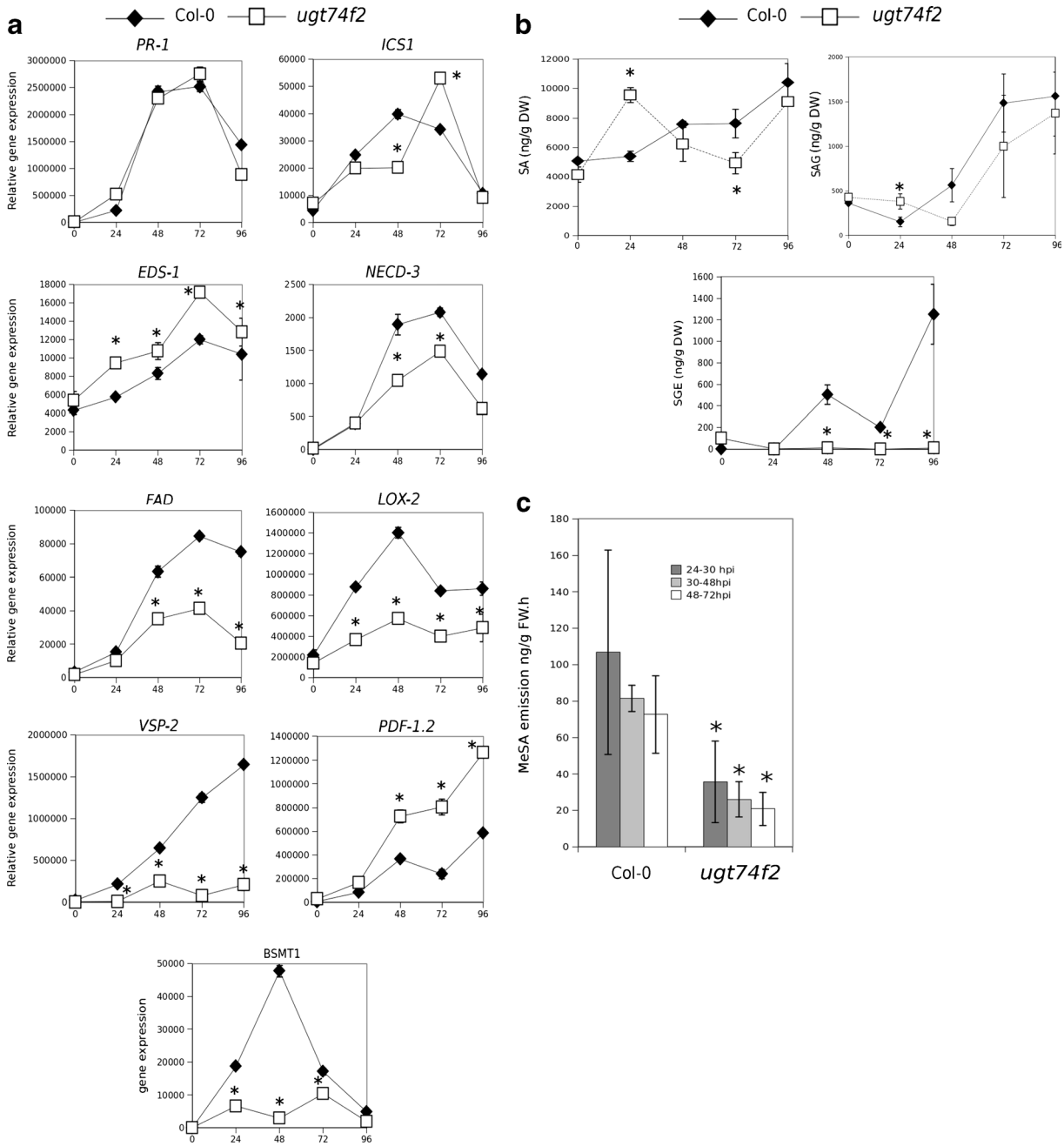
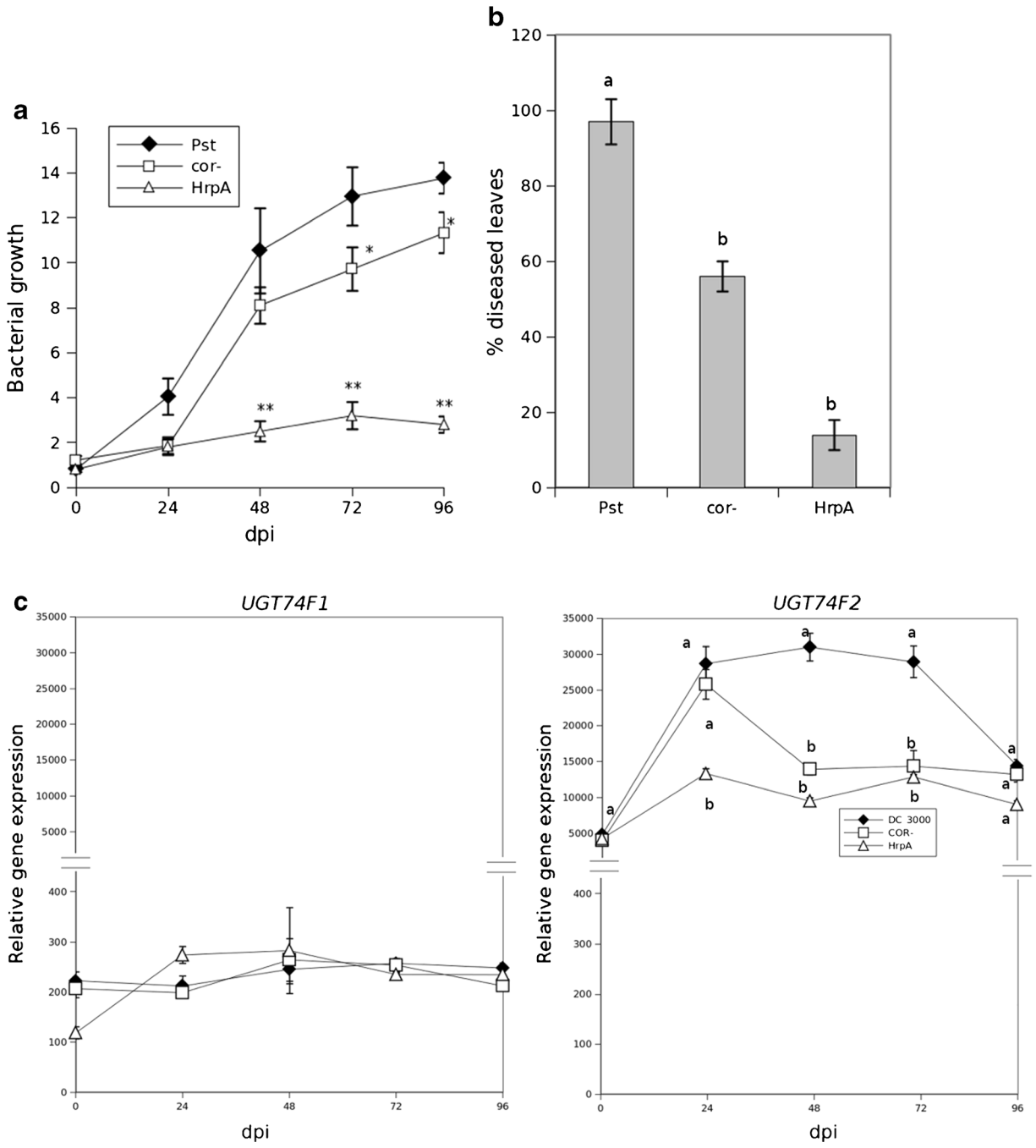


Fig. 4 Profile of plant defence pathways upon *Pst* infection in Col-0 wild type and *ugt74f2* mutant. A) Expression of genes representing key components of the SA, JA and ABA pathways was measured at the given time-points by real-time PCR. Data show the mean of three technical replicates (±SD). B) The levels of SA, SAG and SGE upon infection were determined by LC-MS. C) The accumulation of MeSA released along the time periods indicated was determined by GC-MS. Gene expression data and MeSA are from representative experiments that were repeated twice with similar results. SA, SAG and SGE data are from a representative experiment that was repeated four times. In the figures A, B and C the asterisk means statistically significant differences within each time-point (*T*-test; $p<0.05$)

free and conjugated, forms of SA (SA, SAG and SGE) rise upon infection by *Pst* (Song et al., 2009; Pastor et al., 2012) and also after wounding in a time-dependent manner (Ogawa et al., 2010), clearly pointing



to an interplay between these molecules following biotic challenges. In the results presented here, mutants in two SA-glycosyltransferases display opposite resistance phenotypes against *Pst*.

A mutant in *UGT74F1*, previously reported to be responsible for SAG formation, is hypersusceptible to *Pst*. On the other hand, a mutant in *UGT74F2*, that is supposed to be involved in both SAG and SGE formation, is more resistant to *Pst* (Fig. 1). In contrast, Noutoshi et al. (2012) have reported that *ugt74f1* is more resistant 3 days after inoculation. There may be several reasons for such an apparent contradiction. First, the

◀ **Fig. 5** Basal resistance of Arabidopsis Col-0 against *P. syringae* DC3000 wild type (*Pst*), the coronatine deficient strain (COR-) or with the TTSS deficient strain (HrpA). A) Bacterial growth was measured by real-time PCR at the indicated time-points. Data presented are the means of three technical replicates (±SD) of the ratio of *Psorf* gene copy number/*AtTUB4* gene copy number. Asterisks mean statistical significant differences within time-point (*T*-test, $p<0.05$). B) Disease rate was measured by calculating the percentage of diseased leaves per plant ($n=20$,±SD). Data are from a representative experiment that was repeated four times with similar results. Different letters mean statistical significant differences within treatments (ANOVA; LSD, $p<0.05$). C) Profile of *UGT74F1* and *UGT74F2* gene expression upon *Pst* infection with strains compromised in coronatine production and TTSS. Five week-old plants were challenged with the *P. syrinage* DC3000 wild type (DC3000), the coronatine deficient strain (COR^-) or with the TTSS deficient strain (HrpA) at 5.10^7 c.f.u.ml^{-1}. Gene expression was measured at indicated time-points by real-time PCR. Data represent the mean (±SD) of technical triplicates. Data are from a representative experiment that was repeated twice with similar results. Different letters mean statistical significant differences within treatments at each time-point (ANOVA; LSD, $p<0.05$)

method of inoculation is different. In this study, plants were dip-inoculated while Noutoshi and colleagues syringe-infiltrated the bacteria, thus bypassing the constitutive barriers and stomatal responses to infection. The second and most relevant difference is that the mutant used by Noutoshi et al. (2012) was not impaired in *UGT74F1* expression. In fact, their mutant showed enhanced levels of *UGT74F1* mRNA and therefore was seemingly a gain of function mutant rather than a translational *UGT74F1* impaired mutant. The T-DNA insertional mutant used in the present research is a full knockout with hardly any detectable gene expression.

The evidence presented highlights the relevance of a fine regulation of both genes during defence reactions. In accordance with our results, a line overexpressing *AtSGT1* (*UGT74F2*) was more susceptible to *Pst* and accumulated less free SA, SAG and SGE (Song et al., 2008). Unexpectedly, this line also accumulated more MeSA and MeSA-2-*O*-β-D-glycoside. As shown in Fig. 3, the mutant *ugt74f1* shows less induction of *ICS1* after *Pst* inoculation. Accordingly, after 24 hpi it accumulated less SA in response to the infection compared with Ws-0 and this correlates with a lower *PR1* activation. The mutant *ugt74f2* shows the opposite response since it accumulates free SA until 24hpi. In contrast, Song et al. (2009) showed that a RNAi line of *AtSGT1* (*UGT74F2*) displayed no altered phenotype following infection by *Pst*. These differences in results are probably due to the fact that the mutant used in the present research is a knockout *T-DNA* insertional mutant that completely blocks *UGT74F2* expression as shown in Fig. 2.

A further observation is the altered accumulation of both glucosides in the mutants in response to the bacterial infection. While SAG is mainly affected in *ugt74f1*, SGE is strongly affected in *ugt74f2* (Figs. 3 and 4). Noutoshi et al. (2012) showed that *ugt74f1* accumulated higher levels of SA, but in response to the avirulent strain *Pst-avrRpm1* that was spray-inoculated while the resistant phenotype was obtained following infiltration of the bacterium into the leaves. In addition to glycosyl conjugates, other SA conjugates such as MeSA are also induced after *Pst* and *Pseudomonas maculicola* (*Psm*) infection (Attaran et al., 2009; Song et al., 2008). *UGT74F2* overexpressing plants release much higher levels of MeSA after infection and are more susceptible to the bacteria (Song et al., 2008). Accordingly, as shown here, *ugt74f1* releases more MeSA and displays enhanced activity of *BSMT1* that may contribute to its enhanced susceptibility. On the other hand, the resistant mutant *ugt74f2 s*hows a strongly attenuated expression of *BSMT1* and very low levels of MeSA.

An interesting observation in the *ugt74f1* mutant upon infection is a strong up-regulation of the oxylipin-dependent genes *LOX2*, and *FAD*, probably as a consequence of the host manipulation by bacterial effectors. Both genes are upstream in the LOX-9 regulated oxylipin pathway (Vellosillo et al., 2007). In contrast, *VSP2* and *PDF1.2*, both markers downstream of *COI1* and JA signalling (Fig. 3), are down-regulated. This suggests that the reduction in SA and altered *UGT74F1* function affects the oxylipin pathway and intermediates may be diverted into a branch pathway leading to metabolites that differ from JA and its derivatives. In contrast, the enhanced SA signalling in *ugt74f2* leads to a very low transcription of these JA-dependent genes with the exception of *PDF1.2* (Fig. 4). Since this marker gene is also responsible for ethylene (ET) signalling (de Vos et al., 2005), it is very likely that *ugt74f2* contributes to an enhancement of other ET-dependent responses, otherwise, *VSP2* would be induced in this mutant upon infection and indeed, this is not the case (Fig. 4).

Similar resistance is found when another glycosyltransferase UGT76B1 is absent. The knockout mutant

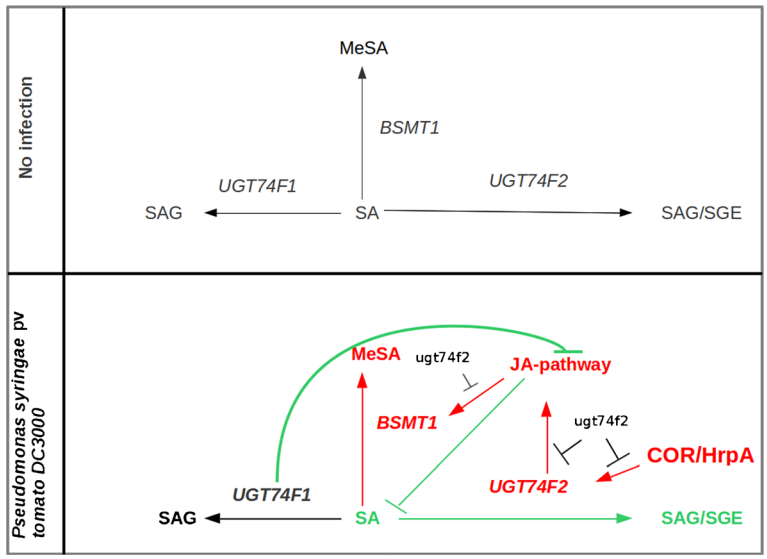


Fig. 6 Model showing the effects of *UGT74F1* and *UGT74F2* during bacterial infection in Arabidopsis. In the absence of infection *UGT74F1* and *UGT74F2* show low levels of basal expression which leads to low amounts of SGE and SAG. Once infection occurs, *UGT74F2* is targeted by bacterial effectors that enhance its expression. Its activation stimulates an up-regulation of a *PDF1.2*-independent branch of JA-signaling and *BSMT1* contributing to increase *Pst* susceptibility. Although *UGT74F1* is not responding to the infection, it plays a key role down-regulating JA-signaling, that is strongly activated in the *ugt74f1* mutant. (Ws) Events found in the Ws-0 background; (Col) events found in the Col-0 background

ugt76b1 presents higher basal levels of SA as well as gene induction in those genes sensibles to SA, while the JA-responsive genes are down-regulated (von Saint Paul et al., 2011). In this case, again, the overexpression of *UGT76B1* leads to an opposite result. Attaran et al. (2009) showed that upon bacterial infection MeSA formation is regulated by the JA pathway. Following infection by virulent *Pst*, the MeSA synthesis depends on the presence of coronatine contributing to the repression of the SA-dependent defences. In the present research it is shown that the induction of *UGT74F2* by the bacteria requires the presence of coronatine and HrpA that contributes to the up-regulation of the JA-pathway in response to the additional UGT74F2. Surprisingly, the loss of function of the *UGT74F2* gene correlates with a reduction in *UFT74F1* expression. On the other hand, although to a lower extent, the mutant *ugt74f1* is also affected in *UGT74F2* expression upon infection, suggesting a cross-regulation between these two glycosyltransferases. The expression levels of both transferases seem not to be significantly affected by mutations in genes in the SA pathway such as *ICS1* or *NPR1*. On the contrary, the scavenging of SA in *NahG* triggers a down-regulation of both UGTs (Fig. S1). Obviously, SA is necessary as a substrate for the generation of SAG and SGE and mutants with disrupted levels of the free hormone are impaired in the accumulation of the glycosides.

In conclusion, *UGT74F1* down-regulates the JA-pathway, therefore, when it is absent in the corresponding mutant, JA signalling is enhanced contributing to increase *BSMT1* expression. This creates an alternative sink for the free form of SA that is convered into MeSA, hence, SA signalling in *ugt74f1* is repressed and it becomes more susceptible. Noteworthy, only *COI1* upstream signals are up-regulated in *ugt74f1*, which suggests that the positive crosstalk between MeSA and oxylipins does not directly involve JA or JA-metabolites but earlier signalling within this pathway. On the other hand, *UGT74F2* is needed for the up-regulation of the JA-pathway in the presence of coronatine and HrpA. Consequently, in the mutant *ugt74f2* the JA-pathway is down-regulated, free SA accumulates and SA-dependent signalling is up-regulated resulting in a resistant phenotype (Fig. 6). As a final remark, it is noteworthy that two genes with 76 % of sequence similarity have opposite effects on the regulation of Arabidopsis resistance against *Pst*. It may be possible that the bacterial effectors are targeting these small differences between the two genes, although this is matter of future research.

Acknowledgments We thank F.J. Uribe-Romeo and Sheng Yang He (Michigan State University, USA) for providing *Pst* strains DC3000 *hrpA* A9 and DC3118 coronatine⁻. We are grateful to P. Saindrenan (IBP, Paris, France) and D. Heintz (IBMP, Strasbourg, France) for providing SGE standards. We thank Pro. Ted C. J. Turlings for acces to the volatile trapping and GS/MS systems. We also thank the SCIC of the Universitat Jaume I for its technical support and the Plan de promoción de la Investigación Universitat Jaume I P1.1B2010-06. Financial support by the Swiss National Science Fundation Grant 31003A_140593 is gratefully acknowledged.

References

Attaran, E., Zeier, T. E., Griebel, T., & Zeier, J. (2009). Methyl salicylate production and jasmonate signaling are not essential for systemic acquired resistance in Arabidopsis. *Plant Cell, 21*, 954–971.

Chen, F., D'Auria, J. C., Tholl, D., Ross, J. R., Gershenzon, J., Noel, J. P., & Pichersky, E. (2003). An Arabidopsis thaliana gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense. *Plant J., 36*, 577–588.

de Vos, M., van Oosten, V. R., van Poecke, R. M. P., van Pelt, J. A., Pozo, M. J., Mueller, M. J., Buchala, A. J., Métraux, J. P., van Loon, J. C., Dicke, M., & Pieterse, C. J. (2005). Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. *Molecular Plant Microbe Interactions, 18*, 923–937.

Dean, J. V., & Delaney, S. P. (2008). Metabolism of salicylic acid in wild-type, ugt74f1 and ugt74f2 glucosyltransferase mutants of Arabidopsis thaliana. *Physiol. Plant., 132*, 417–425.

Dean, J. V., Mohammed, L. A., & Fitzpatrick, T. (2005). The formation, vacuolar localization, and tonoplast transport of salicylic acid glucose conjugates in tobacco cell suspension cultures. *Planta, 221*, 287–296.

Dooner, H. K., & Nelson, O. E. (1977). Genetic control of UDPglucose:flavonol 3-O-glucosyltransferase in the endosperm of maize. *Biochem. Genet., 15*, 509–519.

Edwards, R. (1994). Conjugation and metabolism of salicylic acid in tobacco. *J. Plant Physiol., 143*, 609–614.

Feys, B. J., Moisan, L. J., Newman, M. A., & Parker, J. E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *EMBO J., 20*, 5400–5411.

Garcion, C., Lohmann, A., Lamodiere, E., Catinot, J., Buchala, A., Doermann, P., & Metraux, J. P. (2008). Characterization and biological function of the ISOCHORISMATE SYNTHASE 2 gene of Arabidopsis. *Plant Physiol., 147*, 1279–1287.

Kesarwani, M., Yoo, J. M., & Dong, X. N. (2007). Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. *Plant Physiol., 144*, 336–346.

Koo, Y. J., Kim, M. A., Kim, E. H., Song, J. T., Jung, C., Moon, J. K., Kim, J. H., Seo, H. S., Song, S. I., Kim, J. K., Lee, J. S., Cheong, J. J., & Do Choi, Y. (2007). Overexpression of salicylic acid carboxyl methyltransferase reduces salicylic acid-mediated pathogen resistance in Arabidopsis thaliana. *Plant Mol. Biol., 64*, 1–15.

Langlois-Meurinne, M., Gachon, C. M. M., & Saindrenan, P. (2005). Pathogen responsive expression of glycosyltransferase genes UGT73B3 and UGT73B5 is necessary for resistance to pseudomonas syringae pv tomato in Arabidopsis. *Plant Physiol., 139*, 1890–1901.

Lee, H., & Raskin, I. (1999). Purification, cloning and expression of pathogen-inducible UDP-glucose: salicylic acid glucosyltransferase from tobacco. *J. Biol. Chem., 274*, 36637–36642.

Lim, E. K., Doucet, C. J., Li, Y., Elias, L., Worrall, D., Spencer, S. P., Ross, J., & Bowles, D. J. (2002). The activity of Arabidopsis glycosyltransferases toward salicylic acid, 4-hydroxybenzoic acid, and other benzoates. *J. Biol. Chem., 277*, 586–592.

Liu, P. P., Yang, Y., Pichersky, E., & Klessig, D. F. (2010). Altering expression of benzoic acid/salicylic acid carboxyl methyltransferase 1 compromises systemic acquired resistance and PAMP-triggered immunity in Arabidopsis. *Mol. Plant Microbe Interact., 23*, 82–90.

Loake, G., & Grant, M. (2007). Salicylic acid in plant defence-the players and protagonists. *Curr. Opin. Plant Biol., 10*, 466–472.

Mou, Z., Fan, W. H., & Dong, X. N. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell, 113*, 935–944.

Noutoshi, Y., Okazaki, M., Kida, T., Nishina, Y., Morishita, Y., Ogawa, T., Suzuki, H., Shibata, D., Jikumaru, Y., Hanada, A., Kamiya, Y., & Shirasu, K. (2012). Novel plant immune-priming compounds identified via high-throughput chemical screening target salicylic acid glucosyltransferases in Arabidopsis. *Plant Cell, 24*, 3795–804.

Ogawa, T., Ara, T., Aoki, K., Suzuki, H., & Shibata, D. (2010). Transient increase in salicylic acid and its conjugates after wounding in Arabidopsis leaves. *Plant Biotechnology, 27*, 205–209.

Park, S. W., Kaimoyo, E., Kumar, D., Mosher, S., & Klessig, D. F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science, 318*, 113–116.

Park, H. J., Kwon, C. S., Woo, J. Y., Lee, G. J., Kim, Y. J., & Paek, K. H. (2011). Suppression of UDP-glycosyltransferase-coding Arabidopsis thaliana UGT74E2 gene expression leads to increased resistance to pseudomonas syringae pv. Tomato DC3000 infection. *Plant Pathol. J., 27*, 170–182.

Pastor, V., Vicent, C., Cerezo, M., Mauch-Mani, B., Dean, J., & Flors, V. (2012). Detection, characterization and quantification of salicylic acid conjugates in plant extracts by ESI tandem mass spectrometric techniques. *Plant Physiol. Biochem., 53*, 19–26.

Ross, J., Li, Y., Lim, E., & Bowles, D. J. (2001). Higher plant glycosyltransferases. *Genome Biol., 2*, 30041–30046.

Seo, S., Ishizuka, K., & Ohashi, Y. (1995). Induction of salicylic-acid beta-glucosidase in tobacco-leaves by exogenous salicylic-acid. *Plant Cell Physiol., 36*, 447–453.

Song, J. T. (2006). Induction of a salicylic acid glucosyltransferase, AtSGT1, is an early disease response in Arabidopsis thaliana. *Molecules and Cells, 22*, 233–238.

Song, J. T., Koo, Y. J., Seo, H. S., Kim, M. C., Do Choi, Y., & Kim, J. H. (2008). Overexpression of AtSGT1, an Arabidopsis salicylic acid glucosyltransferase, leads to increased susceptibility to Pseudomonas syringae. *Phytochemistry, 69*, 1128–1134.

Song, J. T., Koo, Y. J., Park, J. B., Seo, Y. J., Cho, Y. J., Seo, H. S., & Choi, Y. D. (2009). The expression patterns of AtBSMT1 and AtSAGT1 encoding a salicylic acid (SA) methyltransferase and a SA glucosyltransferase, respectively, in Arabidopsis plants with altered defense responses. *Molecules and Cells, 28*, 105–109.

Spoel, S. H., Koornneef, A., Claessens, S. M. C., Korzelius, J. P., Van Pelt, J. A., Mueller, M. J., Buchala, A. J., Metraux, J. P., Brown, R., Kazan, K., Van Loon, L. C., Dong, X. N., & Pieterse, C. M. J. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell, 15*, 760–770.

Vellosillo, T., Martínez, M., López, M. A., Vicente, J., Cascón, T., Dolan, L., Hamberg, M., & Castresana, C. (2007). Oxylipins produced by the 9-lipoxygenase pathway in Arabidopsis regulate lateral root development and defense responses through a specific signaling cascade. *Plant Cell, 19*, 831–846.

Vlot, A. C., Liu, P. P., Cameron, R. K., Park, S. W., Yang, Y., Kumar, D., Zhou, F. S., Padukkavidana, T., Gustafsson, C., Pichersky, E., & Klessig, D. F. (2008). Identification of likely orthologs of tobacco salicylic acid-binding protein 2 and their role in systemic acquired resistance in Arabidopsis thaliana. *Plant J., 56*, 445–456.

Vlot, A. C., Dempsey, D. A., & Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annual Revue of Phytopathology, 47*, 177–206.

Von Saint Paul, V., Zhang, W., Kanawati, B., Geist, B., Faus-Kessler, T., Schmitt-Kopplin, P., & Schäfner, A. R. (2011). The Arabidopsis glucosyltransferase UGT76B1 conjugates isoleucic acid and modulates plant defense and senescence. *Plant Cell, 23*, 4124–4145.

Wiermer, M., Feys, B. J., & Parker, J. E. (2005). Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol., 8*, 383–389.

Wildermuth, M. C. (2006). Variations on a theme: synthesis and modification of plant benzoic acids. *Curr. Opin. Plant Biol., 9*, 288–296.

Wildermuth, M. C., Dewdney, J., Wu, G., & Ausubel, F. M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature, 414*, 562–565.

Yao, J., Huot, B., Foune, C., Doddapaneni, H., & Enyedi, A. (2007). Expression of a beta-glucosidase gene results in increased accumulation of salicylic acid in transgenic Nicotiana tabacum cv. Xanthi-nc NN genotype. *Plant Cell Rep., 26*, 291–301.