Local and systemic resistance in *Arabidopsis thaliana* in response to *Pseudomonas syringae*: impact of light and phytosterols

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Content

1. Summary

Inoculation with plant pathogens induces a diverse range of plant responses which potentially contribute to disease resistance or susceptibility. Plant responses occuring in consequence of pathogen infection include activation of classical defence pathways and changes in metabolic activity. The main defence route against hemibiotrophic bacterial pathogens such as *Pseudomonas syringae* is based on the phytohormone salicylic acid (SA). SA-mediated responses are strictly regulated and have also been shown to depend on external factors, e.g. the presence of light. A major goal of this work was to provide a better understanding of the light dependency of plant defence responses mediated through SA. Therefore, I studied the defence behaviour of *Arabidopsis thaliana* plants after inoculation with an avirulent strain of *P. syringae* pv. *maculicola* (*Psm avrRpm1*) at different daytimes. I found that inoculation with *Psm avrRpm1* in the morning, when light is initially available for a prolonged time period, produces higher levels of SA and earlier increases in expression of the defence marker gene *PR-1* (*PATHOGENESIS RELATED-1*) compared to inoculations in the evening or in the night. These results indicate that the observed dependency of plant defence responses upon inoculation time during a day/night cycle is related to the initial light availability after infection. Moreover, the plants circadian rhythm has a minor influence on pathogen-induced SA accumulation.

To specify the light dependency of plant defences at the molecular level, the defence behaviour of different mutants affected in light perception was investigated. The blue light receptor double mutants *cryptochrome1cryptochrome2* (*cry1cry2*) and *phototropin1-phototropin2* (*phot1phot2*) were not impaired in inducing local and systemic defence responses. Moreover, the red light double mutant *phytochromeAphytochromeB* (*phyAphyB*) exhibited wild-type like defences at the site of *Psm avrRpm1* inoculation. These results indicate that there is no crosstalk between photoreceptor signalling and defence pathways leading to resistance at inoculation sites. In contrast, I found that the establishment of systemic acquired resistance (SAR) requires a functional phytochrome signal pathway, because SAR, as estimated by a bacterial growth assay of pathogen-pre-treated plants, did not develop in *phyAphyB* double mutants. Additionally, unlike wild-type plants, *phyAphyB* mutants failed to systemically elevate SA levels and expression of *PR-1* after a localized pathogen inoculation. Thus, the previously described failure of plants to express SAR under dark conditions (Zeier et al., 2004) can now be explained by a requirement of phytochrome signalling for SAR establishment.

The second part of the project focussed on the influence of plant sterols on plant resistance. I analyzed leaf lipid composition and found that accumulation of the phytosterol stigmasterol in leaves and in isolated (plasma) membranes is a significant plant metabolic

process occurring upon pathogen infection. Arabidopsis leaves induced this reaction after inoculation with pathogenic and non-pathogenic *P. syringae* strains, but also in response to infections with the necrotrophic fungus *Botrytis cinerea* or the biotrophic powdery mildew fungus *Golovinomyces cichoracearum*. Pathogen-induced stigmasterol is synthesized from β-sitosterol via C22-desaturation by the cytochrome P450 enzyme CYP710A1. Gene expression of *CYP710A1* is strongly induced after inoculation with pathogens. Moreover, Arabidopsis *cyp710A1* mutant lines impaired in pathogen-inducible expression of the C22-desaturase and concomitant stigmasterol accumulation are more resistant to both avirulent and virulent *P. syringae* strains than wild-type plants, indicating that induced sterol desaturation in the wild-type favours pathogen multiplication and plant susceptibility. An increase of the stigma-/ β-sitosterol ratio was also found when Arabidopsis leaves were inoculated with type III-deficient or non-adapted *P. syringae* strains, after treatment of leaves with flagellin or lipopolysaccharide preparations, and after application of reactive oxygen species (ROS) generating substance mixtures such as xanthine/xanthine oxidase. This indicates that recognition of pathogen-associated molecular patterns and subsequent ROS formation provokes the β-sitosterol desaturation. Several classical defence pathways do not influence sterol desaturation because mutants impaired in SA, jasmonic acid (JA), or ethylene signalling still induce stigmasterol formation in a wild-type-like manner. Additionally, the *cyp710A1* lines exhibit conventional SA, JA, and camalexin biosynthesis after *P. syringae* inoculation. The elevated contents of stigmasterol after pathogen attack do not influence SA-mediated defence signalling but attenuate the pathogen-induced expression of the defence regulator *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*). In summary, this data suggests that *P. syringae* interacts with an FMO1-associated resistance pathway through PAMP exposure, ROS generation and C22-sterol desaturation to favor its own multiplication in leaves and promote plant susceptibility.

2. Zusammenfassung

Eine Infektion mit Pathogenen veranlasst Pflanzen zur Aktivierung zahlreicher Abwehrreaktionen, welche entscheidend dazu beitragen können, ob die Pflanze anfällig ist und erkrankt oder eine erfolgreiche Resistenz ausbilden kann. Die Abwehr gegen hemibiotrophe bakterielle Pathogene basiert vor allem auf der verstärkten Bildung des Pflanzenhormons Salicylsäure (SA) und der Aktivierung SA-vermittelter Abwehrreaktionen. Beides ist nicht nur intern genau reguliert, sondern auch von externen Faktoren beeinflusst. So trägt zum Beispiel die Verfügbarkeit von Licht wesentlich zum Ausmaß und zum Erfolg dieser Abwehrreaktionen bei. Ein Ziel dieser Arbeit ist es, zu einem besseren Verständnis des Einflusses von Licht auf die Pathogenabwehr beizutragen. Hierzu wurde das Abwehrverhalten von *Arabidopsis thaliana* nach Inokulation mit dem avirulenten Bakterienstamm *Pseudomonas syringae* pv. *maculicola* (*Psm avrRpm1*) zu verschiedenen Tageszeiten untersucht. Bei einer Pathogenbehandlung am Morgen, wenn Licht anschließend für eine längere Phase verfügbar ist, akkumuliert die Pflanze höhere Gehalte an SA und exprimiert das SA-regulierte Abwehrgen *PR-1* (*PATHOGENESIS RELATED-1*) früher als bei Inokulationen am Abend oder in der Nacht. Die Ergebnisse dieser Arbeit zeigen auch, dass die beobachteten Lichtabhängigkeiten unter experimentellen, aber natürlich angepassten Tag- und Nachtbedingungen in Zusammenhang mit der Lichtverfügbarkeit nach erfolgter Infektion stehen und keine Konsequenz des circadianen Rhythmus sind.

Um der Lichtabhängigkeit dieser Reaktionen genauer auf den Grund zu gehen, wurde das Abwehrverhalten von Photorezeptor-Mutanten untersucht. Hierzu wurden die Doppelmutanten der Blaulichtrezeptoren Cryptochrom 1 und Cryptochrom 2 (*cry1cry2*) sowie Phototropin 1 und Phototropin 2 (*phot1phot2*) verwendet. Beide Mutantenlinien waren in der Ausbildung der Resistenz am Infektionsort und in der systemisch erworbenen Resistenz (SAR) nicht beeinträchtigt. Ebenso zeigte die Rotlicht-Doppelmutante von Phytochrom A und B (*phyAphyB*) nur geringfügige Einschränkungen in ihrer Resistenz am Infektionsort. Die Ausbildung der SAR erfordert jedoch funktionelle Phytochrom A- und Phytochrom B-Photorezeptoren, denn es zeigte sich, dass typische SAR-Reaktionen in der *phyAphyB* Doppelmutante ausbleiben. So führte eine lokale Pathogenbehandlung nicht zu einer gesteigerten systemischen Resistenz und zu einer Akkumulation von SA oder einer Expression von *PR-1* im systemischen Gewebe. Während die Lichtabhängigkeit der lokalen Abwehrreaktionen also nicht durch die Photorezeptorsignalwege beeinflusst wird, kann das Ausbleiben der SAR-Reaktionen im Dunkeln mit inaktiven Phytochromen erklärt werden. Ein von Phytochromen ausgelöstes Signal, welches die Lichtverfügbarkeit unterstreicht, ist also für die Ausbildung der SAR erforderlich.

Das zweite Projekt dieser Arbeit stellt die Lipidzusammensetzung pathogen-infizierter Blätter in den Mittelpunkt. Bei der Analyse pflanzlicher Sterole zeigte sich, dass das C22-ungesättigte Sterol Stigmasterol in Blättern und in daraus isolierten Plasmamembranen nach Pathogenbehandlungen akkumuliert. Dies konnte für die Interaktionen von Arabidopsis mit pathogenen und nicht-pathogenen *P. syringae*-Stämmen, mit dem nekrotrophen Pilz *Botrytis cinerea* und mit dem biotrophen Mehltaupilz *Golovinomyces cichoracearum* gezeigt werden. Die pathogen-induzierte Synthese von Stigmasterol aus β-Sitosterol erfolgt über eine Desaturierung durch das Cytochrom P450-Enzym CYP710A1, dessen Genexpression in Blättern durch den Kontakt mit Pathogenen aktiviert wird. Entsprechende Mutanten (*cyp710A1*) mit ausbleibender induzierter Genexpression und fehlender Stigmasterol-akkumulation nach Pathogenbehandlung zeigten eine erhöhte Resistenz gegenüber avirulenten und virulenten *P. syringae*-Stämmen, nicht jedoch gegenüber einem *Botrytis cinerea*-Isolat. Die pathogen-induzierte Steroldesaturierung ist also ein Prozess, der in Wildtyp-Pflanzen die Vermehrung bakterieller Pathogene mit hemibiotropher Lebensweise fördert und damit die Anfälligkeit der Pflanze erhöht. Der Anstieg an Stigmasterol wird durch die Erkennung von bakteriellen PAMPs (pathogen-associated molecular patterns) vermittelt, da eine Stigmasterolbildung nach Blattinfiltrationen mit Flagellin- und Lipopolysaccharidlösungen beobachtet werden konnte. Zudem wurde die C22-Desaturierung von β-Sitosterol auch durch *P. syringae*-Stämme, die einen Defekt im Typ-III-Sekretionssystem aufweisen oder nicht adaptiert für ein Wachstum in Arabidopsis-Blättern sind, ausgelöst. Ebenso lösten Behandlungen, welche die Bildung von reaktiven Sauerstoffspezies initiierten, die Stigmasterolbiosynthese aus. Klassische Abwehrantworten und Signalwege haben jedoch keinen Einfluss auf die Steroldesaturierung und werden auch nicht durch sie verändert: Weder zeigten die untersuchten *cyp710A1*-Linien veränderte Gehalte an SA, Jasmonsäure (JA) und Camalexin, noch war die Stigmasterolakkumulation in Mutanten des SA-, JA- oder des Ethylensignallweges positiv oder negativ beeinträchtigt. Erhöhte Stigmasterolwerte haben jedoch eine abschwächende Wirkung auf die Expression der Flavin-abhängigen Monooxygenase *FMO1*, die als ein zentraler Regulator von basalen und systemischen Abwehrreaktionen beschrieben ist. Diese Ergebnisse können im folgenden Modell zusammengefasst werden: *P. syringae* bewirkt durch die Exposition der eigenen PAMPs eine kontiniuierliche Bildung von ROS in der Pflanze, die eine Erhöhung der Stigmasterol-/β-Sitosterol-Verhältnisse in Blättern und Blattmembranen zur Folge hat. Dies führt zur Abschwächung eines FMO1-assoziierten Resistenzsignalwegs und fördert dadurch die pflanzliche Anfälligkeit gegenüber dem angreifenden Bakterium.

3. Introduction

3.1 Plant defence against pathogens

Plants, like all other organisms, do not live on their own. They are surrounded by many other organisms as other plants, animals, and microbes with which they interact and compete. Even the surface of plants is densely populated by a multitude of microbes. It is estimated that a typical leaf contains up to 10^6-10^7 bacteria per square centimetre and one gram of leaf material can reach up to 10^7 colonies of fungi and yeasts (Leveau, 2006). The coexistence is not always friendly or neutral, but it exposes the plants to an intense competition for environmental resources and turns out to be a real threat for the survival of plants if they are interacting, for instance, with herbivores or pathogens. Hence, plants are in the need to develop numerous mechanisms and barriers to defend themselves. This thesis focuses on the defence of plants against pathogens. Pathogens are mostly disease causing micro-organisms and include viruses, bacteria, fungi, oomycetes, protozoa, but also some invertebrates. Within the framework of this work, the defence of the model plant *Arabidopsis thaliana* (L.) Heynh (Arabidopsis) towards microbial pathogens was investigated. It is important to remind that even though plants are surrounded and challenged by an enormous number of microbes with possible pathogenic character, most plants appear healthy and the formation of disease is rather the exception. On one hand, the potential pathogen needs appropriate environmental conditions to infect the plant and cause disease. On the other hand, plants defend themselves with preformed barriers and induced responses that pathogenic intruders need to overcome. Thus, resistance and not susceptibility is the outcome of most plant-pathogen interactions.

3.1.1 Non-host resistance

Plant non-host resistance is the most frequent outcome of an interaction between a microbial pathogen and a plant. It is defined as the resistance of an entire plant species to a non-adapted microbe. The plant is a non-host and the microbe a corresponding non-host pathogen. The non-host pathogen is not able to multiply on a plant's surface or in the plant apoplast and thereby, can not overcome initial layers of plant defence and thus fails to infect the plant (Thordal-Christensen, 2003; Lipka et al., 2008). For example, the fungus *Blumeria graminis* forma specialis (f.sp.) *hordei* (*Bgh*), a barley pathogen causing powdery mildew, can not infect wheat (Lipka et al., 2008). Inoculations of Arabidopsis leaves with the non-adapted *Pseudomonas syringae* pathovar (pv.) *glycinea* (*Psg*) and *Pseudomonas syringae* pv. *phaseolicola* (*Psp*), representing both non-host pathogens for Arabidopsis, are

characterized by a rapid reduction in bacterial number (Mishina and Zeier, 2007a). In nature, *P. syringae* needs an appropriate environment to establish large surface populations before migrating into the plant apoplast through open stomata or wounds and subsequent infecting of the plant (Hirano and Upper, 2000). Generally, non-host resistance is the interaction of two non-adapted partners and the result of preformed and induced defence of the plant.

3.1.2 Preformed defence

The first layer of plant defence that putative pathogens need to overcome are preformed barriers, including physical and chemical components which provide protection against a wide range of pathogens in a non-specific way. The plant surface and, in particular, the waxy plant cuticle represents such a physical barrier. However, the plant cuticle is not only a strong wall against attacking pathogens. The composition of leaf surface lipids also seems to play an essential role in early recognition processes and fungal pre-penetration pathogenesis (Tsuba et al., 2002; Gniwotta et al., 2005; Zabka et al., 2008). For instance, hexacosanol, a C-26-aldehyde, strongly induces appressorium formation of *Bgh* (Tsuba et al., 2002).

Phytoanticipins are examples of preformed chemical defence components of low molecular weight. They are present in plant cells already before an infection with pathogens takes place, and either possess inhibitory and antimicrobial properties themselves or act as direct precursors for active compounds (Van Etten et al., 1994). Phytoanticipins are known from many groups of secondary metabolites: saponins, phenolics, cyanogenic glycosides, sulphur-containing indole derivatives, and terpenes (Osbourn, 1999). Probably the most familiar substitutes of phytoanticipins are the saponins avenacin and tomatine. Saponins are composed of at least one hydrophilic glycoside residue and of a lipophilic triterpenoid part. Their ability to produce soap-like foaming is a common feature and also the eponym of the group. Avenacin A1, a triterpenoid saponin of the roots of oat plants but not of wheat and barley, mediates resistance against the root-infecting fungus *Gaeumannomyces graminis* var. *tritici*, which is highly sensitive to avenacin A1 and thereby, is able to infect wheat and barley but not oat. *G. graminis* var. *avenae*, in contrast, carries the saponine-detoxifying enzyme avenacinase enabling the infection of oat roots. A mutant oat line unable to produce avenacin A1 is susceptible to *G. graminis* var. *tritici* indicating a typical example of non-host resistance based on the function and presence of a phytoanticipin (Papadopoulou et al., 1999). α-tomatine, another saponin, accumulates in tomato and has antimicrobial activities against many fungi by connecting to 3β-hydroxy sterols of fungal membranes. Thereby, it causes membrane pores and leakage of the cellular content. In comparison to non-adapted pathogens, tomato pathogens can tolerate specific amounts of α-tomatine. *Fusarium oxysporum* f. sp. *lycopersici*, a soilborne fungus inducing vascular wilt disease on tomato

plants, produces the tomatinase enzyme Tom1, which degrades α-tomatine to less toxic components and is required for full virulence (Pareja-Jaime, 2008).

A further group of phytoanticipins are the glucosinolates. Glucosinolates are sulphur- and nitrogen-containing secondary plant metabolites and are mainly famous for their occurrence in cruciferous species (Fahey et al., 2001). Upon wounding of a plant, they are converted into toxic compounds by myrosinase enzymes. The particular role of glucosinolates in preformed and induced defence against pathogens is only emerging, and their function as defensive compounds in herbivory is much better understood. Nevertheless, composition of glucosinolates and their respective myrosinase-induced degradation products might affect non-host resistance to bacteria. Two glucosinolate mutant lines, *gsm1-1* (lacking alkyl glucosinolates with butyl-, pentyl-, and hexyl core groups) and TU3 (failing levels of glucosinolates with heptyl and octyl core groups) show altered bacterial growth after infection with the non-adapted *Psg* and *Psp* (Mishina and Zeier, 2007a). Recently, Bednarek and colleagues (2009) identified a glucosinolate pathway mediating antifungal defence responses. The Arabidopsis gene *CYP81F2* contributes to the activation of this pathway and is triggered by PEN2, which represents an atypical myrosinase. PEN2, however, is also described in the next chapter as an essential component of basal resistance.

3.1.3 Induced basal defence

A prerequisite for the plant to induce defence response is the recognition of microbes and putative pathogens. Therefore, plants detect conserved microbial molecules called pathogen- (or microbe-) associated molecular patterns (PAMPs/MAMPs) as well as damage-associated molecular patterns (DAMPs; Boller and Felix, 2009). DAMPs are endogenous elicitors of the plant and are produced in consequence of the damage that is caused by plant-colonizing pathogens. However, resistance as a consequence of PAMP-induced defence responses is designated as PAMP-triggered immunity (PTI). PAMPs are usually epitopic structures within molecules and are essential structural components for the microbe. They are present in many microbial species, and are, while not existing in the potential host, perceived by a broad spectrum of host species. Some typical bacterial PAMPs are lipopolysaccharides (LPS), peptidoglycans (PGNs), flagellin, and the elongation factor Tu (EF-Tu). LPS are found in the outer membrane of Gram-negative bacteria, and plant cells predominantly recognise the conserved lipid A but also the core oligosaccharide and the O-antigen structure of LPS. PGNs constitute the major cell wall components of Gram-positive bacteria, and plant cells perceive the sugar structure but not the protein part of the molecule. The third extracellular bacterial PAMP recognised by many plant species including Arabidopsis is the conserved N-terminal domain of flagellin (a 22-amino acid stretch called flg22), which is the principal substituent of the flagellum. The bacterial cold shock protein and EF-Tu are examples of

intracellular PAMPs. *Solanaceae* and *Brassicaceae* are able to identify the core 22-amino acid (csp22) of the RNA-binding domain-1 of cold shock protein and the N-acetylated N-terminal 18 amino acids of EF-Tu (elf18), respectively. It is worth to mention that LPS, PGN and flagellin are also indispensable elicitors of the animal immune system. Examples for fungal PAMPs are the cell wall components β-glucan, ergosterol, and chitin. The first identified PAMP, Pep13, represents a peptide sequence of a cell wall transglutaminase from the oomycete *Phytophtera sojae*. Recently, the oomycete cellulose-binding lectin was also shown to function as a PAMP in Arabidopsis and tobacco (Schwessinger and Zipfel, 2008).

How do plants recognise all these different PAMPs? For this, plants posses pattern recognition receptors (PRR) consisting mostly of receptor-like kinases (RLKs) and receptor-like proteins (RLPs). The recognition of flagellin is based on its direct binding to the plant leucine-rich repeat receptor kinase (LRR-RK) FLS2 (FLAGELLIN SENSING 2). Functional homologues of FLS2 have been identified in Arabidopsis, tomato, tobacco, and rice. EF-Tu binds to a LRR-RK named EFR. In particular, FLS2 was intensively studied in the last years. Flg22 binds to the LRRs 9-15 of the extracellular FLS2 domain inducing FLS2 endocyctosis from the plasma membrane. Prior to that, the LRR-RK BAK1 (brassinosteroid insensitive 1-associated receptor kinase 1) dimerizes with FLS2 and thereby regulates flg22-induced responses. Generally, BAK1 seems to be a regulatory adapter protein of many LRR-RLKs (Zipfel, 2009).

PAMP treatment and recognition of non-host pathogens induces rapid responses (seconds to minutes) in the plant cell: e.g. ion-flux across the plasma membrane, accumulation of intracellular Ca^{2+}, oxidative burst, MAPK activation, and receptor endocytosis (Schwessinger and Zipfel, 2008). Furthermore, non-host pathogens and PAMPs contribute to an activation of the salicylic acid defence pathway including SA accumulation and PR (pathogenesis-related) protein expression, and presumably trigger cell wall fortification events such as lignification (Mishina and Zeier, 2007). Further responses include ethylene biosynthesis, stomatal closure and callose deposition (Schwessinger and Zipfel, 2008).

Downstream of PAMP perception, mitogen-activated protein kinases (MAPK) are activated, followed by the activation of WRKY transcription factors. For instance, flg22 perception triggers MPK3, MPK4 and MPK6 activation. In Arabidopsis, MPK3 and MPK6 are required for biosynthesis of the phytoalexin camalexin which is critical for resistance against necrotrophic fungi. Hence, the mutants *mpk3* and *mpk6* produce less camalexin and are more susceptible to *Botrytis cinerea* (Pitzschke et al., 2009). MPK3 further functions in stomatal closure in response to pathogen defence and MPK4 activates a pathway for negative regulation of defence responses (Pitzschke et al., 2009). The WRKY transcription factors translate the activation of MAPKs in altered gene expression. WRKYs contain at least

one WRKY domain, a highly conserved DNA-binding region with the peptide sequence WRKYGQK and a zinc finger motif (Pandey and Somssich, 2009).

Two main responses of PTI are stomatal closure and cell wall fortifications. Melotto et al. (2006) demonstrated that pathogens induce stomatal closure to restrict bacterial invasion and that this response is PAMP-dependent and can also be triggered by application of flg22 and LPS. A PAMP-induced structural barrier is the formation of papillae designating local cell wall fortifications at sites of pathogen contact and consisting predominantly of callose, phenolics, and hydroxylproline-rich glycoproteins (Hauck et al., 2003). Callose deposition is strongly activated in the non-host interaction of Arabidopsis and *Psp* and an essential response of plant non-host resistance. Generally, papillae formation is mainly studied in response to powdery mildews. The *MLO* of barley is a negative regulator of papilla formation and *mlo*-deficient barley confers a papilla-conferring resistance to the classical host *Bgh* (Freialdenhoven et al., 1996). In Arabidopsis, papilla formation is responsible for 80 % of the arrested penetration attempts of germinated sporeling of the non-host *Bgh*. In this context, three Arabidopsis mutants with impaired penetration (*pen*) resistance were identified: *pen1*, *pen2*, and *pen3*. Invasion rates of the non-adapted powdery mildew are strongly enhanced in these mutants. By contrast, non-host resistance to bacteria is not affected, but the expression of all three *PEN* genes can be activated by bacterial flagellin. PEN1 is a plasma-membrane-anchored syntaxin with putative functions in endocytosis and exocytosis. In plasma membranes, PEN1 relocates and accumulates at fungal penetration sites (Lipka et al., 2008). *PEN2* encodes a family 1 glycoside hydrolase (F1GH) and an atypical myrosinase altering glucosinolate metabolism. Thereby, PEN2 contributes to pathogen-triggered callose deposition. This is mainly based and dependent on the pathogen-induced and PEN2-triggered accumulation of 4-methoxyindol-3-ylmethylglucosinolate. The *pen2* mutant is deficient in resistance against a broad spectrum of fungi (Clay et al., 2009; Bednarek et al., 2009). *PEN3* encodes the ATP binding cassette (ABC) transporter PDR8 (pleiotropic drug resistance 8) which is described to transport cadmium out of the cell, and contributes to flg22-triggered callose accumulation (Stein et al., 2006; Clay et al., 2009; Kim et al. 2007).

Generally, plant basal resistance does not only contribute to restrict the growth of non-host pathogens but rather influence the severity of disease induced by adapted pathogens (Lipka et al., 2008).

3.1.4 Susceptibility requires suppression of basal resistance

For successful infection and disease development, pathogenic microbes need to overcome preformed and induced defence barriers and responses. If they do so, the interaction is compatible and the pathogens are called virulent, because they cause disease and the plant is susceptible. In plant-bacteria interaction, successful infection correlates with the setup of a

functional type III secretion system (TTSS) and colonization of the plant apoplast. The TTSS is a proteinaceous structure similar to a syringe or a pore that delivers a multitude of type III effector proteins directly into the host cytoplasm. Because many of these TTSS effectors are contributing to virulence, establishing a functional TTSS is often decisive for pathogenicity. The TTSS is encoded by *hrp* (*hypersensitive response and pathogenicity*) genes. Bacterial strains deficient in one of the *hrp* genes show a loss of virulence. The translocated TTSS effectors mainly cause disease and susceptibility by interfering and suppressing the plant's induced basal defence responses (da Cunha et al., 2007; Grant et al., 2006; Nomura et al., 2005). For instance, the TTSS effector AvrPto of the *P. syringae* pv. *tomato* (*Pst*) strain DC3000 anticipates pathogen-induced callose deposition and papilla formation in contrast to inducing TTSS-deficient strains (Hauck et al., 2003). Transgenic Arabidopsis plants expressing bacterial AvrPto and AvrRpt2 show reduced resistance to TTSS-deficient *P. syringae* strains, indicating resistance suppressing properties of both effector proteins (Hauck et al., 2003; Chen Z et al., 2004). AvrRpt2, a cysteine protease, and AvrRpm1 inhibit PAMP-induced defence by interacting with RIN4, a negative regulator of basal defence (Kim et al., 2005). A list of many identified TTSS effectors of *P. syringae* and their putative function in virulence is provided in Table 1.

Table 1 Selection of TTSS effectors of *P. syringae* and their proposed virulence function and biochemical activity. Adapted from da Cunha et al. (2007) and Grant et al. (2006).

TTSS effectors (previous name)	**Pathovar**	**Biochemical activity**	**Proposed virulence action**
AvrB1 (AvrB)	glycinea	Serine/threonine kinase	Suppresses basal defense Induces JA-responsive genes
AvrB2 (AvrPphC)	phaseolicola	Unknown	Suppresses R-gene mediated cell death
AvrD1 (AvrD)	Many pathovars	Syringolide synthase	Unkown
AvrE1 (AvrE)	Many pathovars	Unknown	Suppresses basal defence Induces disease symptoms on susceptible host
AvrPto1 (AvrRto)	tomato	Unknown	Manipulates ethylene biosynthesis Suppresses basal defences Inhibits flagellin induced NHO1 expression Suppresses cell death in non-host interactions Suppresses papillae formation
AvrRpm1	maculicola	Unknown	Suppresses basal defense Suppresses R-gene induced cell death
AvrRpt2	maculicola	Staphopain cysteine protease	Suppresses basal defence and papilla formation Induces JA-responsive genes Suppresses RPM1-induced HR SA-independent virulence function
HopA1 (HopPsyA)	syringae	Unknown	Induces JA-responsive genes

HopAA1-1 (HopPtoA1)	maculicola	Unknown	Inhibits flagellin induced *NHO1* expression
HopAB1 (VirPphA)	phaseolicola	Unknown	Suppresses cell death
HopAB2 (AvrPtoB)	tomato	Ubiquitin E3 ligase s	Alter ethylene reponses Suppresses cell death Suppresses HopPsyA-dependent HR Suppresses basal defence
HopAF1 (HolPtoN)	tomato	Unknown	Inhibits flagellin induced *NHO1* expression
HopAI1 (HopPtoAI)	tomato	Phosphothreonine lyase	Inhibits MAPK-signalling Inhibits flagellin-induced
HopAM1-1 (AvrPpiB1)	tomato	Unknown	Suppresses HopPsyA-dependent HR
HopAO1 (HopPtoD2)	tomato	Protein tyrosine kinase	Suppresses cell death in non-host plant Induces JA-responsive genes
HopAR1 (AvrPphB)	phaseolicola	Papain-like cysteine protease	Induces JA-responsive genes Causes degradation of PBS1
HopC1 (AvrPpiC2)	pisi	Unknown	Inhibits flagellin induced *NHO1* expression
HopC1 (HopPtoC, AvrPpiC)	tomato	Papain-like cysteine protease	Inhibits flagellin-induced *NHO1* expression
HopD1 (HopPtoD1)	tomato	Unknown	Induces JA-responsive genes Suppresses HopPsyA-dependent HR
HopE1 (HopPtoE)	tomato	Unknown	Suppresses HopPsyA-dependent HR
HopF1 (AvrPphF)	phaseolicola	Unknown	Suppresses R-gene mediated cell death
HopF2 (HopPtoF)	tomato	Unknown	Suppresses HopPsyA-dependent HR Inhibits flagellin induced *NHO1* expression
HopG1 (HopPtoG)	tomato	Unknown	Suppresses basal defence
HopK (HopPtoK)	tomato	Unknown	Induces JA-responsive genes Suppresses HopPsyA-dependent HR
HopM1 (HopPtoM1)	tomato	Unkown	Suppresses basal defense Manipulates vesicle trafficking Induces disease symptoms on susceptible host Suppresses cell death in non-host plant
HopN1 (HopPtoN)	tomato	Papain-like cysteine protease	Suppresses cell death in non-host plant
HopS1 (HolPtoZ)	tomato	Unknown	Inhibits flagellin induced *NHO1* expression
HopT1-1 (HolPtoU1)	tomato	Unknown	Inhibits flagellin induced *NHO1* expression
HopT1-2 (HolPtoU2)	tomato	Unknown	Inhibits flagellin induced *NHO1* expression
HopX1 (AvrPphE)	Many pathovars	Cysteine protease	Induces JA-responsive genes Suppresses HopPsyA-dependent HR
HopZ2 (AvrPpiG1)	pisi	YopJ-like cysteine protease	Targets host SUMOylated proteins

Additional to TTSS effectors, pathogenic bacteria also use phytotoxins to mediate virulence. Many *P. syringae* strains produce the phytotoxin coronatin (COR). COR mimics methyl jasmonate (MeJA), an activator of jasmonic acid (JA) signalling that mediates plant defence against herbivores and necrotrophic pathogens. COR activates JA signalling counteracting salicylic acid (SA)-based plant defence, which initiates resistance against *P. syringae* (Brooks et al., 2005; Nomura et al., 2005; Attaran et al., 2009).

Furthermore, pathogenic fungi use effectors to increase the susceptibility of their host plants. For example, *Bgh* produces the effectors AVRa10 and AVRk1. How these effectors are transferred into the host is still unknown, but transient expression of the effector genes in plants showed enhanced infection and thereby, enhanced susceptibility of the plant (Ridout et al., 2006; Kamoun, 2007). A whole set of virulence effectors of *Botrytis cinerea* was listed by Choquer et al. (2007). Furthermore, phytotoxins are also an essential part of the fungal virulence strategy. Botrydial, for instance, is a phytotoxin produced by *Botrytis cinerea* and induces chlorosis and cell collapse of infected tissue supporting fungal penetration and colonization (Choquer et al., 2007). Different ways of fungal phytotoxin-mediated virulence were reviewed recently by Möbius and Hertweck (2009).

3.1.5 Gene-for-gene resistance

As mentioned above, bacteria use the TTSS to deliver effector proteins into the host's apoplast or cells. There, they contribute to disease formation and increase the plant susceptibility. However, the infected plants are not always helplessly surrendered to the action of these effectors. Using resistance (R)-proteins, the plant can recognize pathogenic effectors [then called avirulence (avr) proteins] leading to gene-for-gene resistance, which is also designated as specific resistance or as effector-triggered immunity (ETI). The most characteristic feature of gene-for-gene-resistance is the hypersensitive response (HR), an induced programmed cell death of infected plant cells resulting in dry necrotic lesions, being aimed to restrict pathogen growth and to protect healthy surrounding cells. The gene-for-gene resistance was described by Flor et al. (1971) and is based on fitting pathogenic *avr*-genes and plant *R*-genes. This type of interaction is incompatible, and the pathogens are called avirulent, because the recognition anticipates the virulence of the pathogen. In the *P. syringae*-Arabidopsis interaction, some essential avr-proteins are avrRpm1, avrRpt2, avrB, avrRps4 and avrPphB, which are recognised by the plant R-proteins RPM1, RPS2, RPM1, RPS4 and RPS5, respectively (Kim et al., 2008). R-proteins are predominantly receptors containing a central nucleotide binding site (NBS) followed by leucine-rich repeats (LRR) at the C-terminal end. NBS-LRR R-proteins can be grouped into two categories according to the structure of their N-terminal site, having either a coiled-coiled (CC) domain (CC-NBS-LRR) or a TIR-domain, which bears similarities to the Toll receptor of Drosophila and the

interleukin (IL-1) receptor of mammals (TIR-NBS-LRR). How do Avr-proteins and R-proteins interact? One possibility is a direct interaction and binding. Yeast two-hybrid assays demonstrated that the recognition of flax rust fungus AvrL567 proteins by L5, L6 und L7 R-proteins of flax is based on direct Avr/R-interactions (Dodds et al., 2006). As an alternative to a direct interaction, the “guard hypothesis” provides another possibility and model of recognition. Here, R-proteins do not recognise avr-proteins themselves but perceive perturbations induced by the interaction of the avr-protein with its host target (Bent and Mackey, 2007; Kim et al., 2008). For instance, the R-proteins RPM1 and RPS2 function as “guards” corresponding to this model, and include the RPM1-interacting protein 4 (RIN4) as host target. AvrRpm1 and AvrB directly interact with RIN4 and induce its phosphorylation. Phosphorylation-caused conformational changes of RIN4 activate RPM1 (Mackey et al., 2002). By contrast, avrRpt2 mobilises its corresponding R-protein RPS2 through cleavage of RIN4. The followed disappearance of avrRpt2 enables RPS2 activity (Mackey et al., 2003; Axtell and Stakawicz, 2003; Kim et al., 2009). Similarly, the protease avrPphB cleaves the protein kinase PBS1, whose degradation triggers PBS5 (Shao et al., 2003).

Plant recognition of a pathogenic effector leads to a fast activation of many defence responses that restrict high multiplication of the pathogen and disease formation. One of the first responses after inoculation with avirulent bacteria is the oxidative burst, designating the massive production of reactive oxygen species (ROS) such as superoxide and H_2O_2 at local infection sites. The oxidative burst is preceded by alterations of ion fluxes over the plasma membrane. For instance, fungal elicitors induce ion fluxes which in turn trigger production of superoxide in parsley cells (Jabs et al., 1997). While inoculation with a virulent pathogen results in only one transient and mild oxidative burst, inoculation with avirulent pathogens induces a second and strong phase of ROS production between 3 and 6 h after infection (Lamb and Dixon, 1997). Apoplastic ROS are predominantly produced by plasma membrane NADPH oxidases. In Arabidopsis, the NADPH oxidases RbohD and RbohF (respiratory burst oxidases homologues) mediate apoplastic ROS production in response to pathogen defence (Torres et al., 2006). Besides exerting direct antimicrobial activity, ROS function indirectly in triggering genes related to plant defence and thereby, act as important signals inducing hypersensitive cell death (Lamb and Dixon, 1997).

3.1.6 Induced metabolic defence pathways

An essential strategy contributing to defence signalling is based on the activation of the salicylic acid (SA)-dependent pathway. SA (2-hydroxybenzoic acid) is a phenolic phytohormone carrying a carboxyl and a hydroxyl group. Two different biosynthesis pathways are known for the production of SA, both starting with chorismate as the basic product. The first one uses the phenylpropanoid pathway and requires the cytosolic key

enzyme phenylalanine ammonium lyase (PAL), which is induced after pathogen treatment. In the second one, chorismate is converted to isochorismate by the chloroplast-located isochorismate synthase, with subsequent conversion to SA, possibly by isochorismate pyruvate lyase (Wildermuth, 2006). The pathogen-induced SA accumulation predominantly underlies this second pathway. The Arabidopsis *sid2* (*salicylic acid induction-deficient 2*) mutant lacking a functional isochorismate synthase (ICS1) is strongly impaired in the pathogen-induced production of SA and exhibits enhanced susceptibility to the biotroph fungus *Perenospora parasitica* and different virulent and avirulent *P. syringae* strains (Wildermuth et al., 2001; Nawrath and Métraux, 1999). Another salicylic acid-lacking mutant, *eds5* (*enhanced disease sudeptibility5*, previously named *sid1*), encodes a MATE family transporter probably involved in transporting SA biosynthesis intermediates or SA itsself (Nawrath and Métraux,1999; Nawrath et al., 2002).

Which events are known to occur upstream of the activation of SA-biosynthesis and are induced after recognition of TTSS effectors or bacterial PAMPs? Upon activation of R-proteins of the TIR-NBS-LRR group, the lipase-like protein EDS1 is a central player and interacts in complex either with PAD4 (PHYTALEXIN-DEFICIENT4) or SAG101 (SENESCENCE-ASSOCIATED GENE101) to promote SA biosynthesis. This includes positive feedback regulation by SA and is involved in specific, basal and non-host resistance (Vlot et al., 2009). Furthermore, an Arabidopsis mutant failing *PAD4* expression is deficient in the production of the phytoalexin camalexin (Glazebrook and Ausubel, 1994). Phytoalexins are low-molecular-weight compounds that accumulate in plants in response to stress caused by infection. They possess antimicrobial activity and function in resistance against specific sets of pathogens (Kuc, 1995).

NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE-1) is also reported to be a player downstream of SA and represents a putative glycosylphosphatitylinositol (GPI)-anchored plasma membrane protein that interacts with RIN4 (Vlot et al., 2009). In contrast to EDS1, NDR1 regulates resistance responses in connection with activated R-proteins of the CC-NBS-LRR group, such as RPM1, RPS2, or RPS5 (Vlot et al., 2009). However, a mutant lacking NDR1 is not impaired in SA-production (Attaran et al., 2009).

How does SA exert its defensive function? The phenolic substance possesses antimicrobial activitiy and accumulates in plant intercellular spaces where it can directly influence bacterial growth. Growth-inhibitional effects of SA were found after its application into *P. syringae* cultures (Cameron and Zaton, 2004). Furthermore, SA contributes to defence and resistance by activation of defence-related genes and by boosting the oxidative burst and the hypersensitive response. Even exogenous application of SA and its synthetic analogs INA (2,6-dichloro-isonicotinic acid) and BTH (Benzo-[1,2,3]-thiadiazole-7-carbothioic acid S-methyl ester), both acting downstream of SA, promote SA-dependent signalling and

defence activation leading to enhanced resistance against many pathogens (Sticher et al., 1997).

Another pathogen-induced signal pathway triggers biosynthesis of jasmonates. Jasmonates are oxilipin-derived lipids and include the bioactive compounds 12-oxophytodienoic acid (OPDA), the phytohormone JA and the volatile methyljasmonate (MeJA). Pathogen-treated plants produce elevated levels of JA and MeJA, and the exogenous application of both compounds results in expression of defence-related genes, confirming a role in defence primarily against herbivores but also against certain types of pathogens. For instance, the biosynthesis of JA is induced after recognition of the bacterial effector protein avrRpm1 (Kachroo and Kachroo, 2009). The active form of JA is JA-isoleucine (JA-Ile). The JA-amino synthethase JAR1 (JASMONATE RESISTANT1) is responsible for conjugation of JA to several amino acids. The *jar1* mutant demonstrates the relevance of JA signalling to pathogen defence by increased susceptibility to necrotrophic pathogens as *Botrytis cinerea* and *Pythium irregulare* (Kachroo and Kachroo, 2009). Similar results were obtained from studies with the *coi-1* (*coronatine insensitive 1*) mutant which is insensitive to exogenous JA application and exhibits enhanced susceptibility to herbivores and necrotrophic pathogens (Kachroo and Kachroo, 2009). By contrast, *coi1* showes increased resistance to (hemi)biotrophic pathogens such as *P. syringae*. This indicates that the JA-based defence pathway predominantly affects resistance against necrothrophs (Kachroo and Kachroo, 2009). COI1 was recently described to be the receptor not only for the bacterial phytotoxin coronatine but primarily for the JA-moiety of JA-Ile (Yan, 2009).

The activation and function of the gaseous phytohormone ethylene is synergistically to JA. Five integrale membrane proteins (ETR1, ETR2, EIN4, ERS1, ERS2) sharing similarities with bacterial two-component histidine-kinases are part of the ethylene-receptor family (Stephanova and Ecker, 2000). Ethylene insensitivity in soybean mutants lacking functions e.g. of ETR1 show very ambivalent and variable response to pathogens: enhanced susceptibility to the virulent pathogens *Septoria glycines* but a beneficial or neutral effect towards virulent *P. syringae* pv. *glycinea* and *Phytophtora sojae* or to avirulent pathogens (Hoffman et al., 1999). Ethylene and JA in combination enable the expression of some defence-associated genes.

Next to SA, JA and ethylene, other phytohormones such as abscisic acid, auxin, gibberellic acid and cytokinins are discussed to contribute to plant defence, but their role is less well studied. As their relevance to this work is minor, their impact to plant resistance responses is not illustrated here. A review by Bari and Jones (2009) provides the newest results in this context.

Another pathogen-induced response is the production of so-called pathogenesis-related (PR) proteins. The expression of *PR* genes is often a result of triggered SA-, JA- or

ethylene-dependent defence pathways. PR-proteins are classified in at least 17 different families based on their primary structure (Table 2). Many PR protein families possess direct antimicrobial activities: proteins of the PR3, PR4 and PR-11 families work as chitinases, whereas the PR-2 family represent β-1,3-glucanases. By contrast, the microbial targets of many PR-families are still unkown (Sels et al., 2008).

How is *PR* gene expression activated? *PR-1* gene expression is predominantly triggered by the SA-pathway and, exogenous application of SA is sufficient to induce *PR-1* expression (Nawrath and Métraux, 1999). NPR-1 (NON-EXPRESSOR OF PR GENES1) is a central mediator of SA signalling and PR gene expression. The corresponding mutant impaired in *NPR-1* expression does not respond to SA application by PR gene expression, but accumulates SA after pathogen infection. NPR1 represents an ankyrin-repeat domain with possible properties for protein-protein interactions. Depending on SA levels, NPR1 is located in the cytoplasma in an oligomeric form (low SA levels) or NPR dissociates into monomers (increasing SA levels) translocating to nucleus where it interacts with TGA-transcription factors, especially the TGAs 2, 5 and 6, which are required for activation of *PR-1* (Mou et al. 2003; Fan and Dong, 2002). By contrast, expression of *PR-2* and *PR-5* does not necessarily depend on SA (Nawrath and Métraux, 1999). PR-3, PR-4 and the defensin PDF1.2 are connected with JA signalling and are induced after application of methyl jasmonate (Thomma et al., 1998).

Table 2 Classified families of PR proteins and their described properties. Adapted from Sels et al. (2008).

Family	Type member	Properties	Proposed microbial target
PR-1	Tobacco PR-1a	Antifungal	Unknown
PR-2	Tobacco PR-2	β-1,3-Glucanase	β-1,3-Glucan
PR-3	Tobacco P,Q	Chitinase	Chitin
PR-4	Tobacco R	Chitinase	Chitin
PR-5	Tobacco S	Thaumatin-like	Membrane
PR-6	Tobacco Inhibitor I	Proteinase inhibitor	No in vitro antimicrobial activity reported
PR-7	Tomato P69	Endoproteinase	No in vitro antimicrobial activity reported
PR-8	Cucumber chitinase	Chitinase	Chitin
PR-9	Tobacco lignin-forming peroxidase	Peroxidase	No in vitro antimicrobial activity reported
PR-10	Parsley PR1	Ribonuclease-like	No in vitro antimicrobial activity reported
PR-11	Tobacco class V chitinase	Chitinase	Chitin
PR-12	Radish Rs-AFP3	Defensin	Membrane
PR-13	*Arabidopsis* THI2.1	Thionin	Membrane
PR-14	Barley LTP4	Lipid-transfer protein	Membrane
PR-15	Barley OxOa	Oxalate oxidase	No in vitro antimicrobial activity reported
PR-16	Barley OxOLP	Oxalate oxidase-like	No in vitro antimicrobial activity reported
PR-17	Tobacco PRp27	Unkown	No in vitro antimicrobial activity reported

3.1.7 Systemic acquired resistance (SAR)

Plant resistance is not only triggered locally at infection sites but also systemically in distant, uninfected leaves and tissues. This form of induced resistance is called systemic acquired resistance (SAR) and confers a long lasting protection against a multitude of pathogens belonging to classes of viruses, bacteria, oomycetes and fungi. The enhanced resistance is attended and characterized by systemic increase in SA and expression of *PR* genes, especially *PR-1*, *PR-2* and *PR-5* (Durrant and Dong, 2004; Sticher et al., 1997; Uknes et al., 1992). SAR can be subdivided into three phases: first, initiation and release of SAR signal(s) in and from infected tissues, second, transport of the signal(s), and third, signal perception and establishment of SAR in distant leaves. For many years, initiation of SAR was considered to require a necrotizing primary infection due to HR within incompatible interactions or in consequence of disease symptoms as a result of successful compatible interactions (Durrant and Dong, 2004). Recently, Mishina and Zeier (2007b) demonstrated that SAR is also triggered without necrotic lesion formation but by infection with non-pathogenic bacteria. Furthermore, treatment with PAMPs, such as flagellin or lipopolysaccharides, induces SAR. This indicates a strong contribution of PTI to the activation of systemic defence responses. The independency of HR to the establishment of SAR could be confirmed by Liu and colleagues (2010) showing that also in the incompatible specific interaction of ETI, SAR is activated in the absence of cell death. Another indispensable component of SAR is an induced and functional SA-pathway. Exogenous application of SA or its analogs BTH and INA triggers SAR. This is corroborated by the findings that the mutants *sid1* and *sid2*, which are impaired in SA biosynthesis, as well as the transgenic NahG plants expressing a bacterial enzyme, which degrades SA to catechol, are not able to establish SAR (Nawrath and Métraux, 1999; Gaffney et al., 1993). Vernooji et al. (1994) performed tobacco experiments with wildtype scions grafted on transgenic root stocks expressing the NahG gene. The approach demonstrated that active SA is not nessarily needed in the infected leaves for triggering SAR but in the systemic leaves. This indicates simarily that SA is not the mobile signal for SAR and its generation is independent of larger amounts of SA. Besides, reciprocal grafts showed that SAR activation in the distant tissues requires SA accumulation.

A critical step for SAR is the generation of the putative phloem mobile signal. After the exclusion of SA, further candidates for the mobile SAR signal are its glycosidic (SAG) and methylated (MeSA) derivatives. Whereas the current literature does not proof the SAR signalling role of SAG, MeSA's function as a long distance signal is controversially discussed. Park et al. (2007) investigated SAR in the interaction of tobacco with tobacco mosaic virus, and based on their results, they proclaim the following model for MeSA as the mobile signal: In infected leaves, accumulating SA is converted to MeSA, which travels

through the phloem to distant leaves. There, the esterase activity of the SA-binding protein SABP2 reconverts the arriving MeSA into SA, causing the establishment of SAR. Also in Arabidopsis, SABP2 orthologues were found (Vlot et al., 2008). Investigation of T-DNA knock out lines of some of these methyl esterases resulted in an impaired SAR activation prompting the authors to suggest that MeSA is the unique and conserved mobile signal for SAR in many plant species. By contrast, the study of Arabidopsis mutants impaired in MeSA production, which is caused by the pathogen-inducible SA methyl transferase BSMT1, disproved the above-mentioned model, because *bsmt1* T-DNA knockout mutants completely lacking pathogen-induced MeSA production are still fully capable to establish SAR (Attaran et al., 2009). Furthermore, the same study demonstrates that inoculated Arabidopsis leaves emit 97% of the volatile and pathogen-induced MeSA into the air, doubting a role for MeSA as a good and efficient phloem-mobile signal. The results of Attaran et al. (2009) rather suggest that systemic SA accumulation is predominantly a result of de-novo synthesis via ICS1.

Another group of candidates for mobile SAR signals are lipid-based molecules. For instance, a functional *DIR1* (*DEFECTIVE IN INDUCED RESISTANCE1*) gene is required for activation of SAR. *Dir1* knock-out mutants are defective in SAR, and, as activity assays with petiole exudates demonstrated, they do not release a SAR activating signal from petioles of inoculated leaves (Maldonado et al., 2002). DIR1 encodes an extracellular and non-specific lipid transfer protein and is discussed to act as a chaperone for a lipid-derived molecule which could be the SAR signal. Support for lipid-derived long distance signals also comes from the studies of the mutants *pad4* and *eds1*, which are both defective in lipase-like proteins and are impaired in the establishment of SAR (Durrant and Dong, 2004). Additionally, SAR is compromised in further mutants defective in lipid metabolism: *sfd1* (*suppressor of fatty acid desaturase deficiency1*) with deficiency in a dihydroxyacetone phosphate reductase (Nandi et al., 2004), *sfd2*, *fad7* (*fatty acid desaturase 7*) and *mgd1* (*monogalactosyldiacylglycerol synthase1*; Chaturvedi et al., 2008). Recently, the C9-dicarboxylic acid azelaic acid was proposed to accumulate in response to pathogens in the phloem and to prime plants for systemic accumulation of SA (Jung et al., 2009). Mutation of the gene *AZI1* (*AZELAIC ACID INDUCED1*), whose expression is induced by azelaic acid, implicates a lack of SAR induction. Contradictorily discussed is the function of oxylipin-derived compounds as mobile SAR signals. A study from Truman et al. (2007) proposes JA or a related oxylipin as the mobile signal. They demonstrate that JA accumulates in infected and distant leaves as well as in the phloem, and that mutants impaired in jasmonate synthesis and response such as *opr3* and *jin1* do not trigger a SAR response. On the other hand, Chaturvedi et al. (2008) contradict these results as they could not induce SAR by exogenous application of JA and MeJA, and they found that petiole exudates of mutants not-

containing the putative signal(s) could not be reactivated by addition of jasmonates. Furthermore, Attaran and colleagues (2009) observed a SAR response in the JA pathway and signalling mutants *dde2*, *coi1, jar1*, *opr3*, and *jin*, refuting a role for JA signalling during SAR. Hence, the consideration of JA as the mobile SAR signal remains disputable. Besides lipids, peptides have been implicated in SAR activation. In response to pathogen infection, the apoplastic aspartat protease CDR1 (CONSTITUTIVE DISEASE RESISTANCE1) is thought to generate a mobile peptide required for SAR (Xia et al., 2004).

In distant leaves, the SAR signal must be perceived and translated into responses leading to SA accumulation and *PR*-gene expression. The acyl carrier protein, ACP4, is proposed to be involved in the perception of the SAR signal (Xia et al., 2009). Petiole exudates assays demonstrated that *acp4* plants are capable to produce the mobile signal but are defective in triggering SAR in distant leaves. After perception of the signal, ROS are produced in systemic leaves and it is possible that so-called microbursts contribute to the establishment of SAR (Alvarez et al., 1998). Mishina and Zeier (2006) suggest a signal amplification loop in the systemic leaves involving ROS, NDR1, FMO1, SA, and NPR1, fortifying the perceived signal for mediating SAR. The flavin-dependent monooxygenase1 (FMO1) is an essential and critical component for SAR activation in the systemic tissue. The knock-out mutant *fmo1* is completely defective in SAR activation, and remains active and capable for local resistance mediated by NDR1-, but not EDS1- and PAD4-mediated defence signalling at inoculation sites (Mishina and Zeier, 2006; Bartsch et al., 2006).

Another systemic resistance represents induced systemic resistance (ISR), which describes induced systemic responses as a consequence of root colonisation by plant growth-promoting rhizoshere bacteria (Van Loon et al., 1998; Pieterse et al., 1998). ISR also results in increased systemic resistance to pathogen infection but proceeds independently of SA signalling and increased *PR* gene expression. As ISR is not of interest for this thesis, a detailed description is neglected here.

3.2 The used model pathosystems

3.2.1 The pathosystem *Arabidopsis thaliana - Pseudomonas syringae*

Today, the *Arabidopsis thaliana* - *Pseudomonas syringae* pathosystem is often used to study resistance and susceptibility responses of plants. As *Arabidopsis thaliana* has become a well-established model for genetic and molecular studies, also plant pathologists wanted to benefit from this model. Pathogenic interaction partners of *Arabidopsis thaliana* were not known until 1991, when two groups characterised different *P. syringae* strains as possible disease causing pathogens on Arabidopsis in laboratory settings (Dong et al., 1991; Whalen et al., 1991). Depending on the *Pseudomonas syringae* strain or the Arabidopsis accession

used within this model system, three different outcomes of plant-pathogen interactions can be studied: non-host resistance, incompatible interactions based on gene-for-gene resistance, and compatible interactions underlying basal resistance and resulting in susceptibility.

3.2.1.1 *Arabidopsis thaliana* as model plant

Arabidopsis thaliana L. Heynh (Arabidopsis), also called thale cress, is a small spring annual plant belonging to the mustard family (*Brassicaceae*) with naturally habitats in Europe, Asia and northwestern Africa. Arabidopsis is usually growing to 30 cm tall with leaves forming a rosette at the base of the plant and small flowers at the end of the stem. Its habit is typical to all *Brassicaceae*. The history of Arabidopsis research started 1943 when Friedrich Laibach presented first results describing the plant as a possible genetic model. Already 1907, he published the number of the five counted chromosomes of Arabidopsis. The first International Arabidopsis Conference was held 1965 in Göttingen. In the 1970s and 1980s, some findings promoted the breakthrough of Arabidopsis research (Meyerowitz, 2001). One key step was undertaken in 1986, when Lloyd and colleagues published their results of a first T-DNA-mediated Arabidopsis transformation (Lloyd et al., 1986).

What are the advantages for using Arabidopsis as a model plant? Although it does not have agronomic significance, it is part of a plant family including many crop plants and cultivated species, e.g. cabbage or rape. It is a small plant with high seed productivity and a rapid life cycle of only six to eight weeks, which offers easy, fast, and frequent cultivation in the laboratories. The entire genome was sequenced in 2000 by The Arabidopsis Genome Initiative (AGI). It provides easy use for transformation with *Agrobacterium tumefaciens*, and allows the application of forward and reverse genetic approaches to investigate the approximately 30000 genes. Thereby, a large number of mutants are available. Also many natural accessions of Arabidopsis exist. In most laboratory settings, the ecotype Columbia (Col-0) is used, which is the sequenced Arabidopsis accession and the background of the majority of mutants.

3.2.1.2 *Pseudomonas syringae* as a model pathogen

Pseudomonas syringae is a gram-negative and strictly aerobe rod-shaped bacterium with polar flagella. To distinguish between bacteria within the same species but with different pathogenic abilities, the epithet pathovar (pv.) is used. In 1899, the first pathovar of *P. syringae* was isolated from a diseased lilac (*Syringa vulgaris*) and gives the whole species the name which linked to the host the bacterium was first found. Later, this pathovar was

designated as *P. syringae* pv. *syringae*, and more than 40 different pathovars are described until now (Hirano and Upper, 2000).

P. syringae is a phytopathogen with a biotrophic or hemibiotrohic lifestyle. The bacterium infects the host through wounds or open stomata and multiplies in the intercellular spaces. Most of the life cycle occurs in the absence of host cell death. Not until late stages of pathogenicity, host cells die and the infected tissues appear necrotic (Glazebrook, 2005). For instance, *P. syringae* pv. *tomato* causes the bacterial speck disease on tomato plants which has economical importance throughout the world (Agrios, 2005).

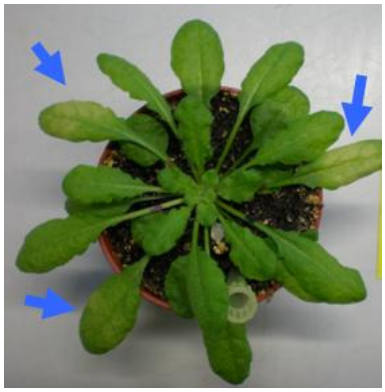

Figure 1. Disease symptoms of Arabidopsis leaves infected with *Psm*

In the 1980s, several *P. syringae* strains of the pathovars *tomato*, *maculicola*, *pisi*, and *atropurpurea* were discovered to infect the model plant Arabidopsis (Crute et al., 1994). As mentioned above, three different levels of pathogenic interaction can be studied in Arabidopsis depending on the used *P. syringae* strain. The compatible interaction which occurs after infection with virulent pathogens that overcome basal resistance, can be investigated with the strains *P. syringae* pv. *tomato* DC3000 (*Pst*) and *P.syringae* pv. *maculicola* ES4326 (*Psm*). The discovery of bacterial avirulence genes like *avrRpt2* and *avrRpm1* and the plant resistance genes *RPS2* and *RPM1*, respectively, facilitated within this pathosystem to study the so-called gene-for-gene resistance which is an incompatible interaction (Dong et al., 1991; Whalen et al., 1991; Dangl et al., 1992). *P. syringae* pv. *glycinea* (*Psg*) and *P. syringae* pv. *phaseolicola* (*Psp*) are not able to multiply and cause disease in Arabidopsis, and thereby enable to study non-host resistance (Lu et al., 2001; Ham et al., 2007; Mishina and Zeier, 2007a). The genomes of strains like *P. syringae* pv. *tomato* DC3000, pv. *syringae* B278a, and pv. *phaseolicola* 1448A are completely sequenced, which is a helpful tool for using *P. syringae* as a model pathogen.

3.2.2 The pathosystem *Arabidopsis thaliana - Botrytis cinerea*

As the model plant Arabidopsis was described already, I will focus on the fungus *Botrytis cinerea* in this chapter. *Botrytis cinerea* is a necrotrophic pathogen killing host cells at early stages in infection and triggering intensive tissue damage (Glazebrook, 2005). The fungus belongs to the phylum of the ascomycetes, causes gray mold on many fruits and vegetables and is also responsible for the noble rot on graves. *Botrytis cinerea* is described to infect more than 200 host plants including the model plant *Arabidopsis thaliana*. Penetration of the host surface, killing of host tissue with primary lesion formation, tissue maceration with lesion expansion, and finally sporulation are essential stages of the pathogenesis of the fungus. In

contrast to biotrophs, the necrotroph *Botrytis cinerea* is able to benefit also from dead tissues as food source. The host cell death is induced by phytotoxic metabolites, oxidative burst, oxalic acid, and host-selective toxins (van Kan, 2006; Choquer et al., 2007). An important part of the virulence strategy of necrotrophic pathogens is the degradation of the plant cell wall. Therefore, *Botrytis cinerea* possesses at least 6 different polygalaturonases for hydrolyzation of de-esterified homogalaturonans representing a main component of cell wall pectin. Furthermore, it harbors pectate lyases, pectin methyl-esterases and endo-β-1,4-xylaneses, such as xyn11A. On the plant side, JA- and ethylene-dependent pathways form a basic level of defence against necrotrophic pathogens including the formation of the antimicrobial chitinase PR-3, the hevein-like protein PR-4, and a plant defensin PDF1.2 (Thomma et al., 1998). Furthermore, Arabidopsis plants produce the phytoalexin camalexin (3-thiazol-2'-yl-indole) in response to microbial pathogens as *P. syringae* and *Cladosporium cucumericum* (Tsuji et al., 1992). Studies with the *pad3-1* mutant, showing highly reduced levels of pathogen-induced camalexin, revealed that camalexin does not contribute to plant protection against (hemi)biotrophic bacterial pathogens (Glazebrook and Ausubel, 1994). In contrast, *pad3-1* is more susceptible for necrotrophic pathogens, e.g. the ascomycete *Alternaria brassicicola*, indicating a role for camalexin in necrotrophic interactions (Thomma et al. 1999). Conflicting results were obtained for the defence against *Botrytis cinerea*. Kliebenstein and colleagues (2005) depicted that different isolates of *Botrytis cinerea* show differently pronounced sensitivity to camalexin.

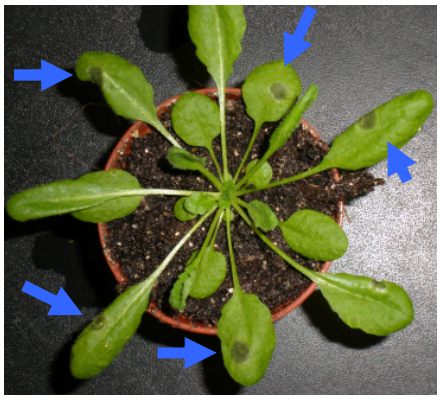

Figure 2. *Botrytis cinerea*-droplet infections on Arabidopsis leaves

3.3 Light and defence

Host plants and pathogens are not the only players that are decisive for the outcome and the amount of plant defence and resistance. Environmental factors also take pivotal roles in this interplay. In particular, light quantity and quality has a strong influence on the plant's metabolism, growth and development. Light enables photosynthesis, which is the primary source of carbohydrates and energy in plants, and furthermore, it has high potential to regulate and control the expression of a multitude of genes (Casal and Yanovsky, 2005). Many pathways in plants are described to be light-dependent and light-regulated. For instance, the phenylpropanoid pathway is triggered and regulated by light. Light-grown Arabidopsis roots strongly accumulate different metabolites derived from this pathway and express enhanced levels of phenylpropanoid genes (Hemm et al., 2004). Similarly, light

exposure of soybean cotyledons boosts phenylpropanoid derivates as well (Graham and Graham, 1996). Within this pathway, light is a key regulator of lignin biosynthesis (Rogers et al., 2005).

In the last years, many publications were released describing direct effects of light on plant defence responses. First of all, the formation of the hypersensitive response is connected to the presence of light. Arabidopsis protoplasts treated with the fungal toxin fumosin B1 develop a higher degree of programmed cell death in the light than in darkness (Asai et al., 2000). A double mutant of *acd11* (*accelerated cell death*) and NahG only exhibits hypersensitive cell death after treatment with BTH when the plants are exposed to light (Brodersen et al., 2002). The *lsd1* (*lesion simulating disease 1*) mutant shows constitutive cell death phenotype but only under long day conditions (> 16 h photoperiod; Mateo et al., 2004). After an infection with *Psm avrRpm1*, Arabidopsis plants develop a more pronounced HR in the light than in the dark (Zeier, 2004). The light dependency of the HR in the interaction of Arabidopsis (ecotype accession Landsberg erecta) and *Psm avrRpt2* correlates with functional phytochrome signalling (Genoud et al., 2002). By contrast, the light-dependent HR of Arabidopsis ecotype Dijon-17 and induced by Turnip crinkle virus is not connected with functional phytochrome receptors (Chandra-Shekara et al., 2006). Next to HR, also the SA pathway requires the presence of light. *Psm avrRpm1*-infected and dark-kept Arabidopsis plants only accumulate low SA contents and do not express the SA-dependent defence gene *PR-1* (Zeier et al., 2004). Moreover, endogenous application of SA in the dark does not restore *PR-1* expression (Genoud et al., 2002). Thus, there is at least a double dependency of light for a functional SA defence pathway including both SA biosynthesis and SA perception. The requirement of light for the activation of the SA signalling pathway is not the only connection to the light-dependent nature of HR. Functional chloroplasts are also needed for the HR, indicating a relation between the redox status of the plastids and plant immunity (Genoud et al., 2002). In nature, high light exposure of plants produces excess excitation energy resulting in ROS accumulation, and this is regulated partially by variations of the redox status. Altering the redox status of the plastoquinone pool, which is an essential component of the photosynthetic electron transport chain, influences plant defence. Reduction of the plastoquinone pool by the electron transport inhibitor 2,5-dibromo-6-isoprobyl-3-methyl-1,4-benzoquinone increases programmed cell death, H_2O_2 production and induction of ethylene in Arabidopsis, and results in increased resistance to virulent *Pst DC3000* (Mühlenbrock et al., 2008). By contrast, oxidation of the plastoquinone pool by treatment with DCMU before high light exposure does not alter susceptibility towards this bacterial strain (Mühlenbrock et al., 2008). How light affects the production of pathogen-induced ROS is not fully cleared, but intensively discussed (Roberts and Paul, 2006). The elicitor cryptogein induces cell death in tobacco cells via light-dependent ROS production in

the light but in a light-independent manner in the dark (Montillet et al., 2005). The NADPH-oxidases responsible for pathogen-induced ROS accumulation are located in the plasma membrane and not in the chloroplasts, indicating that light is not demanded in this case (Apel and Hirt, 2004). Furthermore, Arabidopsis plants produce similar levels of H_2O_2 and identical expression of the ROS-associated gluthatione-S-transferase in the dark and in the light after pathogen treatment (Zeier et al., 2004). On the other hand, chloroplasts generate light-dependent ROS involved in hypersensitive response and mediated by mitogen-activated protein kinases in tobacco plants (Liu et al., 2007). However, several inducible plant defence responses are not at all light-dependent in Arabidopsis, for instance accumulation of the phytoalexin camalexin and the plant hormone jasmonic acid (Zeier et al., 2004).

In addition to local resistance responses, SAR establishment requires the presence of light. Arabidopsis plants kept in the dark during the SAR induction period do not accumulate SA and express *PR-1* at the systemic level after a local infection with *Psm avrRpm1,* and the establishment of SAR is totally abolished (Zeier et al., 2004).

Generally, light is also discussed to be a regulator of defence-associated genes (Roden and Ingle, 2009). It was recently shown that the promoter element $FORC^A$ is expressed in Arabidopsis after infection with fungal and oomycete pathogens as well as after light treatments (Evrard et al., 2009). $FORC^A$ is a light-responsive and conserved element that is modulated by the length of the light period.

The perception of light by specific plant photoreceptors and the thereby induced light signalling events at least partially underly the interplay of the light factor with defence signalling pathways. The plant photoreceptor system is described in the next chapter.

3.5 Plant photoreceptors

Plants use at least three different classes of photoreceptors to perceive light signals and to monitor changes in the light environment: one class of red light receptors called the phytochromes (PHY), and two classes of blue light receptors involving the cryptochromes (CRY) and the phototropins (PHOT). In addition, there is at least one putative but not yet identified receptor for UV-B light (Chen M et al., 2004, Gyula et al., 2003). In the last years, a new class of blue light photoreceptors was described comprising Zeitlupe (ZTL), LOV Kelch Protein 2 (LKP2), and Flavin-binding Kelch F-box1 (FKF1), which modulates the circadian clock and the photoperiod-dependent flowering process (Demarsy and Fankhauser, 2009).

3.5.1 The phytochromes

The phytochromes were first discovered in the 1950s and are the only known class of plant red light receptors. The model plant Arabidopsis contains five distinct phytochrome

members: phytochrome A (PHYA) to phytochrome E (PHYE). PHYB, PHYD and PHYE are the most recently evolved members, and they share higher amino acid similarities than the more ancient PHYA and PHYC (Goosey et al., 1997). The PHYs are controlling physiological responses for seed germination and shade avoidance (Chen M et al., 2004). Other PHY-mediated responses such as seedling development and floral induction are also associated with the CRYs. Within the PHYs, PHYA is responsible for de-etoilation in far-red light and floral induction. By contrast, PHYB mediates de-etoilation in the red light and, assisted by PHYD and PHYE, also shade avoidance. PHYC supports PHYA in the regulation of floral induction and de-etoilation processes. Seed germination is regulated by PHYA, PHYB, and PHYE. Generally, PHYC, PHYD and PHYE perform their physiological roles in combination with either PHYA or PHYB, and many functions are redundant (Smith, 2000; Schepens et al., 2004; Hennig et al., 2002).

The PHYs absorb red and far-red light (600-800 nm) by a covalently attached bilin chromophore, a linear tetrapyrrol. In the absence of light, the holoprotein is synthesised in the stable, red light absorbing conformation (Pr). Absorption of red light leads to a conformational change converting Pr into the far-red light absorbing Pfr state. Similarly, Pfr absorbs far-red light and is subsequently reconverted to the Pr conformation. Under normal light conditions, an equilibrium between Pr and Pfr is achieved. The absorption maxima for Pr and Pfr are near 670 nm and 730 nm, respectively. While the amino terminal domain of all plant PHYs contains the photosensory system, the carboxy-terminal region possesses two PAS (Per/Arnt/Sim)-like domains and a sequence with similarities to bacterial two-component histidine kinases, which is responsible for dimerization. PHYs are kinases and have auto-phosphorylation and phosphorylation properties. The inactive Pr form is mostly located in the cytoplasm, whereas the phosphorylated Pfr dimer migrates into the nucleus. There, Pfr activates in complex with the phytochrome interacting factor 3 (PIF3) the expression of phytochrome-regulated genes (Sharrock, 2008; Bae and Choi, 2008; Smith, 2000; Gyula et al., 2003).

3.5.2 The cryptochromes

Cryptochromes are blue light receptors known in plants and animals. They absorb light in the range of approximately 320-530 nm. Until today, three cryptochromes (CRY) are described in Arabidopsis: CRY1, CRY2, and CRY3. The existence of blue light receptors was assumed for many years and many blue light-dependent functions were known, when in 1993, HY4 (later named CRY1) was the first blue light receptor to be identified (Ahmad and Cashmore, 1993). CRY1 functions in anthocyanin production and chalcone synthase gene expression. CRY1 and CRY2 redundantly control hypocotyl elongation, flowering time, and entrainment of the circadian lock. Besides, CRY2 is involved in de-etiolation responses, and in

combination with the phototropins, the CRYs regulate flowering time. The identification of a functional role of CRY3 is still lacking (Li and Yang, 2007).

The structures of CRYs are related to those of photolyases, but CRYs do not possess DNA photolyase activity. In particular, the N-terminal domain of all CRYs presents a photolyase homology region (PHR) which binds a flavin adenine dinucleotide (FAD) chromophore and a pterin or a deazaflavin as an additional light-harvesting chromophore. The carboxy-terminal domains of CRY1 and CRY2 possess more variable motifs designated as DAS-regions. The structure of CRY3 is quite different and lacks a carboxy-terminal DAS-motif but contains an amino-terminal transient peptide sequence enabling translocation into chloroplasts and mitochondria. CRY3 is more related to a recently identified cryptochrome from cyanobacteria, CRY-DASH (DASH for Drosophila, Arabidopsis, Synechocystis, Homo; Li and Yang, 2007; Chen M et al., 2004).

While *CRY1* and *CRY2* RNA levels are expressed in a circadian rhythm with low amplitudes, protein levels of CRY1 are stable and not affected by light. In contrast, CRY2 is degraded in the presence of light. CRY2 is constitutively present in the nucleus, whereas the localization of CRY1 is light-dependent: nuclear in the dark, cytoplasmic in the light. In accordance with its structure, CRY3 can be found in mitochondria as well as in chloroplasts. The activation of CRY1 and CRY2 is dependent on blue light perception and electron transfer followed by conformational changes and phosphorylation at the carboxy terminal domain, which is connected with the E3-ubiquitin ligase COP1. The light-activated conformational change of the CRYs thereby induces alterations of the structure of COP1, which releases another COP1-bound factor HY5. HY5 is a transcription factor binding to light-regulated promoter elements. In the dark, COP1 degrades HY5 and other transcription factors (Wang et al., 2001; Chen M et al. 2004; Li and Yang, 2007).

3.5.3 The phototropins

A second class of blue light receptors are the phototropins. Phototropins (PHOTs) are ubiquitinous in higher plants. Arabidopsis contains the two phototropins PHOT1 and PHOT2, originally designated NPH1 (NON-PHOTOTROPIC HYPOCOTHYL 1) and NPL1 (NPH1-LIKE), respectively. Like the CRYs, the PHOTs mediate responses activated by UV-A and blue light (320-500nm). They mainly modulate photosynthetic efficiency and within that, their physiological functions comprise the regulation of phototropism, activation of stomatal opening, and movement control of chloroplasts in response to different light conditions. Moreover, they contribute to cotyledon and leaf expansion in Arabidopsis. Most responses of PHOT1 and PHOT2 are regulated redundantly. Both are responsible for hypocotyl phototropism in high light, but under low light conditions, only PHOT1 mediates this response. By contrast, PHOT2 on its own moderates the avoidance movement of

choroplasts in Arabidopsis. The structure of both phototropins contains a serine/threonine kinase at the carboxy-terminal domain and two LOV (light, oxygen, voltage) domains at the amino-terminal part. The LOV domains LOV1 and LOV2 are the photosensory system of the receptors. In darkness, they non-covalently bind flavin mononucleotide as cofactor and chromophor. Blue light activates a covalent but reversible binding of the flavin mononucleotides to the LOV domains, which induces conformational changes, autophosphorylation, and kinase activity. PHOT1 and PHOT2 are associated with the plasma membrane, but they do not possess a transmembrane domaine and the mechanism of membrane binding is still unknown. Upon blue light activation, PHOT1 is released into the cytoplasm and PHOT2 heads to the Golgi apparatus. Downstream signalling of the PHOTs is still an open field to investigate. Studies indicate early branching for different PHOT-mediated responses, and the involvement of NPH3 that directly interacts with PHOT1 (Chen M et al., 2004; Kong et al., 2006; Christie, 2007; Demarsy and Frankhauser, 2009).

Recently, a new class of blue light receptors also containing LOV domains was described. Zeitlupe (ZTL), LOV Kelch Protein 2 (LKP2), and Flavin-binding Kelch F-box1 (FKF1) are members of this family and they function in regulation and modulation of the circadian clock and photoperiod-dependent flowering. Similar to the PHOTs, the amino-terminal LOV domains of these receptors bind flavin mononucleotide. Their structure further contains an F-box and six Kelch repeats. F-box proteins are often components of ubiquitin E3 ligases indicating functions in light-regulated protein degradation. Kelch-repeat domains forming β-propeller structure are typically involved in protein-protein interactions. Contrasting to the PHOTs, the light activated-status of the LOV domains of the members belonging this family is not reversible. This might explain why ZTL, LPK2 and FKF1 mediate slow responses compared to the faster ones of the PHOTs (Christie, 2007; Demarsy and Frankhauser, 2009).

3.5.4 Putative Photoreceptors

Up to now, thirteen photoreceptors have been characterized in Arabidopsis (see 1.5.1-1.5.3). There are indications that at least two more photoreceptors exist in plants: an UV-B receptor and a green light receptor.

Often, UV-B is regarded to have harmful influence on animals and plants. However, plants are adapted to certain amount of UV-B radiation. Moreover, UV-B regulates many essential responses in plants. For instance, it reduces the extension of growth and the expansion of leaves. It facilitates branching and induces the production of secondary metabolites, e.g. the UV-protective flavonoids. UV-B-dependent responses also alter the expression of many genes, including the key flavonoid biosynthesis gene *CHALCONE SYNTHASE* (*CHS*). By contrast, a specific receptor for UV-B perception is not yet found. A

mutant called *UV light insensitive 3* (*uli3*) shows reduced expression levels of some UV-B-dependent genes, but seems to be rather involved in UV-B signalling than in perception. A strong candidate is UVR8 (UV-B-specific regulator 8), which is involved in many UV-B responses, and after activation, migrates into the nucleus to influence gene expression similar to the PHYA. However, the existence of chromophor binding to this protein could not yet be confirmed and is only based on speculation (Jenkins, 2009).

The main part of green light is reflected by plants, but plants also perceive it. Green light is discussed to affect vegetative growth, organ growth and stature, stomatal opening and chloroplast gene expression. Plants can sense green light via PHYs and CRYs, but in addition, an independent green light receptor is assumed. The nature of this unidentified green light receptor is unknown. Zeaxanthin is one possible candidate for mediating green light responses independent from CRYs and PHYs (Folta and Maruhnich, 2007).

3.6 Phytosterols

Sterols are essential for all eukaryotes. The most prominent sterol is certainly cholesterol, the main sterol of mammal's membranes. Cholesterol was also the first known sterol giving all other sterols the name. As major part of human gallstones it was named after the Greek

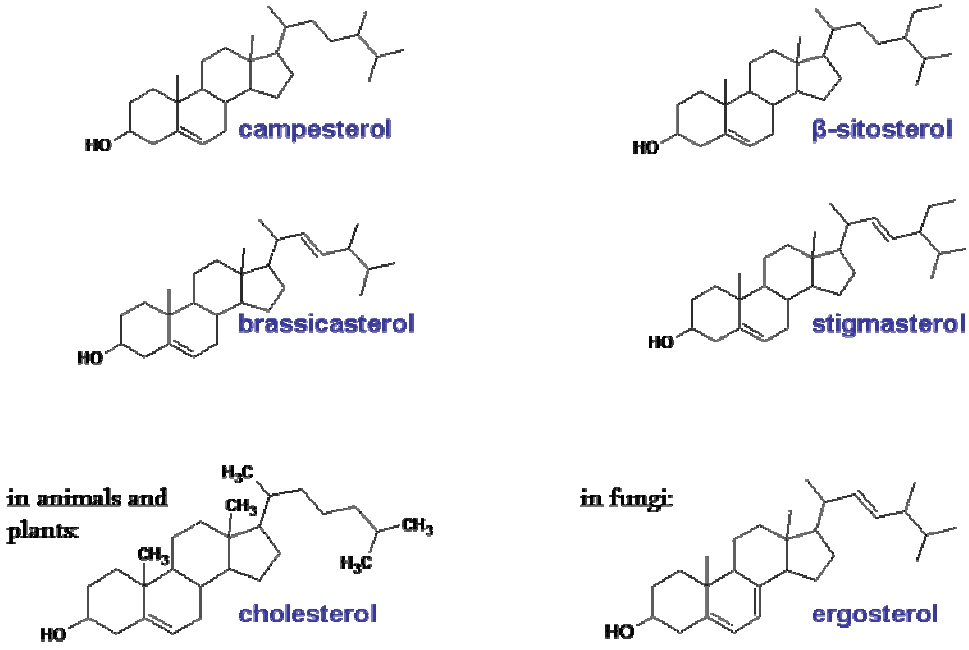

Figure 3. Chemical structures of main sterols in plants, mammals and fungi

chole (=bile) and *stereos* (=solid). How are sterols defined? David Nes proposed the following definition: 'any chiral tetracyclic isopentenoid which may be formed by cyclization of squalene oxide … and retains a polar group at C-3 (hydroxyl or keto), an all-trans-anti stereochemistry in the ring system and a side chain 20R-configuration' (Goad and Akihisa, 1997). Cholesterol and ergosterol, respectively, are the predominant sterols of mammals and of fungi. By contrast, plants posses a more diverse mixture of plant sterols (phytosterols), including the main components β-sitosterol, campesterol, stigmasterol and cholesterol (Hartmann, 1998). In generally, plant tissues contain an average quantity of 1-3 mg of sterols per gram dry weight (DW; Schaller, 2004). The quantity of sterols also depends on the developmental status of the plant. For instance, mature tobacco leaves have higher sterol contents than immature leaves which is principally caused by an increase of stigmasterol (Grunwald, 1975). A boost of stigmasterol is also described in ripening tomato fruits (Whitaker and Gapper, 2008). Besides numerous minor sterols, the sterol mixture of Arabidopsis accession C-24 contains up to 64% of the predominant β-sitosterol, 11% of 24-methyl cholesterol, 6% of stigmasterol, 3% of isofucosterol, and 2% of brassicasterol, with a total sterol content of 2478 µg/g DW (Schaeffer et al., 2001). Sterol profiles of the moss *Physcomitrella patens* contrast to those of higher plants: stigmasterol (40 µg/g FW) and campesterol (40 µg/g FW) are the predominant sterols of *P. patens*, whereas β-sitosterol occurs in lower amounts of (16 µg/g FW; Morikawa et al., 2009).

The complex mixture of phytosterols raises questions that are still not completely answered: Why do plants have a complex mixture of sterols instead of one unique main sterol? Are there functional varieties between the different phytosterols? Before the function of phytosterols will be discussed, an outline of their biosyntheses will be provided.

3.6.1 Biosynthesis

Sterols consist of isoprene units, which are built through the cytoplasmic mevalonate pathway. The condensation of three molecules of acetyl-CoA by a thiolase and by hydroxymethylglutaryl-CoA synthase results in 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in mammals and fungi. In plants, this step only needs the HMG-CoA synthase. Next, the HMG-CoA-reductase (HMGR) converts HMG-CoA to mevalonate. This is the first committed step that regulates the amount of sterols. In mammals, an inhibition of HMGR strongly reduces the biosynthesis of cholesterol. Cholesterol itself uses a feedback loop to regulate HMGR expression, and thereby its own biosynthesis at the transcriptional and post-translational level (Goldstein and Brown, 1990). HMGR functions in regulating the amount of sterols also in plants. Transgenic Tobacco plants overexpressing *HMGR1* from *Hevea brasiliensis* show an overproduction of free sterols (Schaller et al, 1995). In contrast, a loss of function of HMGR1 in Arabidopsis results in reduced sterol levels showing a β-sitosterol level

of 93,3 µg/100mg DW in the mutant compared to 200 µg/100mg DW in the wild-type (Suzuki et al., 2004).

Mevalonate formation by HMGR is followed by a double phosphorylation and a decarboxylation converting mevalonate to isopentenyldiphosphat (IPP) and its isomer dimethylallyldiphosphate (DMAPP). IPP and DMAPP from the mevalonate pathway serve as basic units for the biosynthesis of sterols. Furthermore, IPP and DMAPP can be synthesized via a non-mevalonate pathway in the plastids using pyruvate and glyceraldehyd 3-phosphat as substrates (MEP pathway; Kuzuyama, 2002). IPP and DMAPP are then conjugated to form geranyl diphosphate and the addition of another IPP results in the 15-carbon farnesyl diphosphate. The head-to-head condensation of two farnesyl diphosphates forms the triterpene squalen, which is subsequently oxidized to 2,3-oxidosqualene by the enzyme squalene epoxidase (Phillips et al., 2006; Rasberry et al. 2007). In plants, 2,3-oxidosqualene is converted by cycloartenol synthase to cycloartenol, the first cyclic intermediate of phytosterol biosynthesis. By contrast, the first phytosterol intermediate in nonphotosynthetic

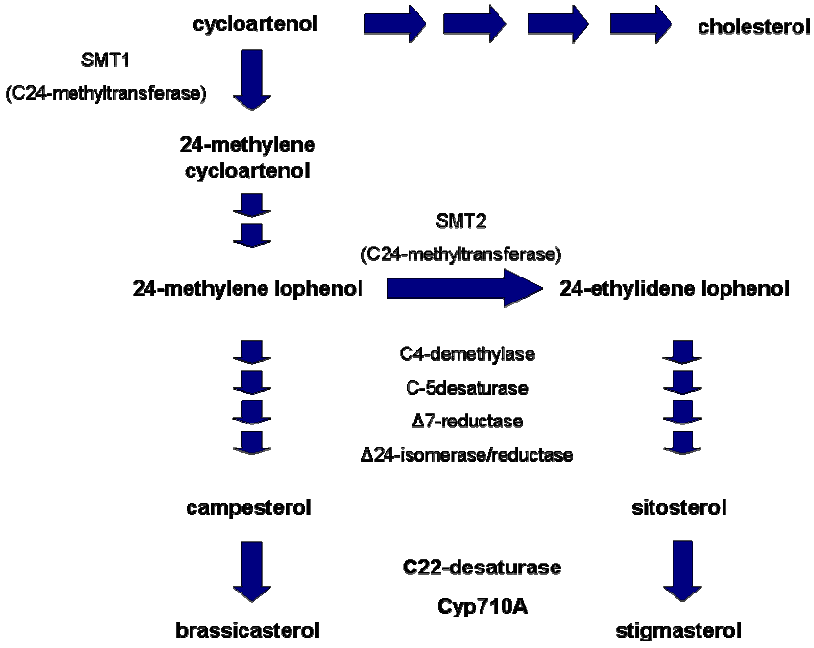

Figure 4. Simplified model of plant phytosterol biosynthesis

eukaryotes like mammals and fungi is lanosterol, which is formed by lanosterol synthase. Recently, a lanosterol synthase LAS1 was also identified in Arabidopsis and shown to contribute to the amount of sterols and steroids (Benveniste, 2004; Babiychuk et al., 2008; Suzuki et al., 2006; Ohyama et al., 2009).

The first branching of the phytosterol pathway depends on the activity of sterol methyltransferase 1 (SMT1). SMT1 methylates cycloartenol at the C24 by means of S-adenosylmethionine, resulting in 24-methylene cycloartenol as a precursor for all 24-methyl and 24-ethyl sterols. Unmethylated cycloartenol feeds into the cholesterol biosynthesis

pathway. The function of SMT1 in regulating the ratio of cholesterol to 24-methyl/24-ethyl sterols is described. *smt1* mutants show enhanced cholesterol levels and decreased amounts of sitosterol (Diener et al., 2000).

Sterol methyltransferase 2;1 (SMT2;1) is the next branching step that adds another methyl group to built the 24-ethyl sterols and modulates the ratio of the 24-methyl sterol campesterol to the 24-ethyl sterol sitosterol (Schaeffer et al., 2001). Four further enzymatic steps are needed to form campesterol and β-sitosterol from 24-methylene lophenol and 24-ethylidene lophenol, respectively. C22-desaturation by the cytochrome P450 member 710A (CYP710A) can produce stigmasterol from β-sitosterol and brassicasterol from campesterol (Morikawa et al. 2007; Arnquist et al., 2008). Brassicasterol is a phytosterol unique to the plant family of Brassicaceae. The biosynthesis of sterols is located in the membranes of the endoplasmatic reticulum (ER) but for some final steps a possible involvement of the plasma membrane cannot be excluded (Hartmann, 1998).

3.6.2 Functions

The predominant function of sterols is to regulate membrane fluidity and permeability (Hartmann, 1998). In vitro approaches with soybean phosphatidylcholine bilayers show that sitosterol is negatively regulating membrane permeability to water and possess high membrane ordering powers. The only sterol described not to have both abilities is stigmasterol, which only differs from β-sitosterol by an additional double bond at the C-22 position. The functional role of stigmasterol remains to be investigated (Schuler et al., 1991). Furthermore, the modulation of the membrane sterol composition is discussed and described to affect properties of membrane bound proteins, such as enzymes, channels, or receptors. For instance, the influence of different phytosterols to activate a plasma membrane H^+-ATPase purified from corn roots was studied in vitro. While β-sitosterol had inhibitory effects, cholesterol stimulated the protone pump. Stigmasterol, however, showed a dual behaviour: activating the pump at low concentrations, and inhibiting the H^+-ATPase at higher concentrations (Grandmougin-Ferjani et al., 1997). Generally, how the sterols influence the activity of this proton pump remains to be clarified. On one hand, there might be a direct interaction with the enzyme, on the other hand, the sterol composition could alter the physical properties of the membrane and thereby induce conformational changes of the enzyme.

In 1997, Simons and Ikonen reviewed another function of sterols in cell membranes. Within mammalian cell membranes, domains enriched in sphingolipids and cholesterol are associated with specific proteins and organized as platforms with putative functions in membrane trafficking or membrane signalling. These domains can be isolated using specific detergents and are therefore called detergent-resistant membranes (DRM) or lipid rafts.

These DRMs are proposed to exist also in plants where they are not only enriched in cholesterol but also in all other phytosterols (Laloi et al., 2007; Mongrand et al., 2004).

A further role of sterols is associated with the auxin-regulated and clathrin-dependent endocytosis. Auxin signalling mutants show reduced sterol contents, whereas mutants of sterol biosynthesis are impaired in auxin-regulated endocytosis. Similar results were obtained in wild-type plants after treatment with the sterol biosynthetic inhibitor fenpropimorph (Pan et al., 2009; reviewed in Boutté and Grebe, 2009).

Additionally, phytosterols function as precursors of other metabolites, e.g. the brassinosteroids (BR). The first identified BR was brassinolide, which had been isolated from the pollen of rape (*Brassica napus*) in 1979 (Grove et al., 1979). Recently, 65 free BRs and five BR conjugates have been characterised in plants and their metabolism is intensively studied (Bajguz, 2007). BRs are polyhydroxylated sterol derivates and plant hormones with similarities to animal and insect steroids. They seem to function in many physiological responses: cell division and elongation in stems and roots, photomorphogenesis, reproductive development, leaf senescence, and stress responses. Sterol precursor of many BRs such as brassinolide is campesterol, and many BR biosynthetic mutants are described (Clouse and Sasse, 1998).

Furthermore, sterols are also progenitors of many secondary metabolites: e.g. cardenolides, glykoalkaloids, pregnane derivates, and saponins, which operate as phytoanticipins in resistance against fungal pathogens.

It is obvious that sterols fulfil many important functions in plants, many of them are not fully understood. Sterol mutants of Arabidopsis often show a dwarfed phenotype. Exogenous BR application can not fully restore this impairment in growth indicating further unknown and essential roles for phytosterols themselves (Benaviste, 2004).

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4. Aim of the work

Plant defence against microbial pathogens is based on the activation of many different signalling pathways and on the accumulation of a diverse range of metabolites. Some main events occurring upon microbial infection are already described and identified, but we can assume that many components and mechanisms influencing the outcome of plant-pathogen interactions are still unkown and remain to be elucidated.

The first aim of this project was to provide a better understanding of the light dependency of plant defence responses (chapter 5, Griebel and Zeier, 2008). Previous studies have used rather artificial dark treatments for this purpose, and did not consider the light variations occurring naturally in day/night cycles. Therefore, I investigated the defence behaviour of Arabidopsis plants challenged with the bacterial pathogen *P. syingae* at different daytimes within a fixed day/night cycle. The observed findings clearly demonstrate that activation of the SA-associated defence pathway is strongly promoted in the presence of light. Light might affect the plant's defence system through photosynthesis, by influencing the plants' metabolism, or through impact of light-induced signalling events on defence responses. In the current work, I particularly studied the possible influence of photoreceptor-triggered light signalling events on plant defence responses. Therefore, local and systemic defence responses of Arabidopsis photoreceptor mutants impaired in phytochrome-, cryptochrome, or phototropin-mediated photoperception were investigated upon pathogen inoculation.

The second goal of this work was to identify further molecular events that are induced in Arabidopsis after pathogen contact, and to determine their impact on plant defence and resistance. Particularly, I analysed whether the sterol composition of Arabidopsis leaves would change after inoculation with biotrophic, hemibiotrphic, or necrotrophic pathogen (chapter 6, Griebel and Zeier, 2010). In this context, I found that the phytosterol stigmasterol accumulates to high levels in pathogen-treated Arabidopsis leaves. The production of stigmasterol is mediated through the desaturation of β-sitosterol by the cytochrome P-450 enzyme CYP710A1. On this basis, my aims were to examine whether the pathogen-induced up-regulation of the corresponding gene would correlate with the accumulation of stigmasterol, and whether the pathogen-induced alterations in the stigmasterol/β-sitosterol ratios would affect plant resistance or susceptibility. Further goals were to unravel the regulatory events leading to stigmasterol production, and to analyse the biological compartments of leaves in which stigmasterol is integrated after its pathogen-induced synthesis.

5. Light regulation and daytime dependency of inducible plant defences in Arabidopsis: phytochrome signalling controls systemic acquired resistance rather than local defence

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Formulation of results: 30%

5.1 Summary

We have examined molecular and physiological principles underlying the light-dependency of defence activation in Arabidopsis plants challenged with the bacterial pathogen *Pseudomonas syringae*. Within a fixed light/dark cycle, plant defence responses and disease resistance significantly depend on the time of day when pathogen contact takes place. Morning and midday inoculations result in higher salicylic acid (SA) accumulation, faster expression of pathogenesis-related (PR) genes, and a more pronounced hypersensitive response than inoculations in the evening or at night. Rather than to the plants' circadian rhythm, this increased plant defence capability upon day inoculations is attributable to the availability of a prolonged light period during the early plant-pathogen interaction. Moreover, pathogen responses of Arabidopsis double mutants affected in light perception, i.e. *cryptochrome1cryptochrome2* (*cry1cry2*), *phototropin1phototropin2* (*phot1phot2*), and *phytochromeAphytochromeB (phyAphyB)* were assessed. Induction of defence responses by either avirulent or virulent *P. syringae* at inoculation sites is relatively robust in leaves of photoreceptor mutants, indicating little cross-talk between local defence and light signalling. In addition, the blue-light receptor mutants *cry1cry2* and *phot1phot2* are both capable to establish a full systemic acquired resistance (SAR) response. Induction of SAR and SA-dependent systemic defence reactions, however, are compromised in *phyAphyB* mutants. Phytochrome regulation of SAR involves the essential SAR component FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1). Our findings highlight the importance of phytochrome photoperception during systemic rather than local resistance induction. The phytochrome system seems to accommodate the supply of light energy to the energetically costly increase in whole plant resistance.

5.2 Introduction

To successfully adapt to a changing environment, plants must simultaneously perceive and appropriately respond to a variety of different biotic and abiotic stimuli. Upon attempted infection by microbial pathogens, plants induce a multitude of defence responses to combat the attacking intruders (Dangl and Jones, 2001). At infection sites, these responses often include rapid production of reactive oxygen species (ROS), biosynthesis of low-molecular-weight defence signals such as salicylic acid (SA) and jasmonic acid (JA), accumulation of phytoalexins, increased expression of pathogenesis-related (PR) proteins, and hypersensitive cell death (hypersensitive response, HR). A localized contact of leaf tissue with pathogenic or non-pathogenic microbes can further lead to systemic acquired resistance (SAR), a state of enhanced, broad-spectrum resistance at the whole plant level that protects against subsequent pathogen attack (Durrant and Dong, 2004; Mishina and Zeier, 2007).

Plant SA levels rise systemically during SAR, and this increase is required for induced expression of SA-dependent *PR* genes and systemic enhancement of disease resistance (Ryals et al., 1996; Métraux, 2002).

Inducible plant defences and resistance against pathogens can be affected by changing environmental conditions (Colhoun, 1973). Light is the major external factor influencing plant growth and development, and an appropriate light environment is also required for the establishment of a complete set of resistance responses in several plant-pathogen interactions (Roberts and Paul, 2006). In tobacco, rice and *Arabidopsis thaliana* (Arabidopsis), HR-associated programmed cell death triggered by bacterial and viral pathogens is light-dependent (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Chandra-Shekara et al., 2006). Similarly, the constitutive cell death phenotype of Arabidopsis *acd11* and *lsd1* mutants is only evident when light of a certain quantity or duration is present (Brodersen et al., 2002; Mateo et al., 2004). Pathogen-induced activation of phenylpropanoid biosynthesis is another major defence pathway controlled by light. Deposition of lignin-like polymers in *Xanthomonas oryza*-treated rice leaves decrease when light is absent during the first hours after inoculation (Guo et al., 1993). Moreover, Arabidopsis plants inoculated in darkness with an avirulent strain of *Pseudomonas syringae* are not able to substantially accumulate the phenolic metabolite SA and fail to induce expression of the key phenylpropanoid pathway enzyme phenylalanine ammonia lyase (PAL; Zeier et al, 2004). Light is not only required for SA biosynthesis, but also controls SA perception, because treatment of Arabidopsis leaves with exogenous SA in dim light or in the dark results in strongly reduced expression of the SA-induced defence gene *PR-1* (Genoud et al., 2002). Both impaired production and perception of SA therefore account for the observation that *PR-1* expression in *P. syringae*-treated Arabidopsis leaves is completely suppressed in dark-situated plants (Zeier et al., 2004).

The HR and SA-associated defences are effective means to restrict invasion of biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). Thus, light-controlled activation of these responses can explain why resistance of plants to many bacterial and viral pathogens is attenuated in the dark (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Chandra-Shekara et al., 2006). It is noteworthy, however, that several inducible plant defences occurring at sites of pathogen inoculation do not require the presence of light. In Arabidopsis, these responses include biosynthesis of the phytoalexin camalexin, accumulation of the oxylipin-derived signal jasmonic acid, and expression of the ROS-associated glutathione-S-transferase *GST1* (Zeier et al., 2004). Similarly, in tomato, activation of lipoxygenase and lipid peroxidation are not light-dependent (Peever and Higgins, 1989). Induction of resistance at the whole plant level during SAR and associated systemic elevation of SA levels and *PR-1* gene expression in Arabidopsis, by contrast,

strictly depend on the presence of a light period during the first two days after pathogen contact (Zeier et al., 2004).

The molecular mechanisms by which responses to light and biotic stress interact are only poorly understood (Roberts and Paul, 2006). Through photosynthesis, light can directly provide energy, reduction equivalents, and metabolic precursors for the production of defence metabolites. Light also acts as a signal to regulate many aspects of plant growth, development and physiology. Regulatory light signals are perceived and transduced into cellular responses by different photoreceptors families: the cryptochromes and phototropins, which both absorb UV-A and blue light, the phytochromes, which sense red/far-red light, and as yet unidentified UV-B receptors (Gyula et al., 2003). Whether and how specific light-induced signalling pathways interact with defence pathways has only scarcely been investigated. Genoud et al. (2002) have demonstrated cross-talk between phytochrome signalling and both SA-perception and HR development in Arabidopsis upon inoculation with avirulent *P. syringae*. The light-dependent HR triggered by turnip crinkle virus and resistance to viral infection, on the other hand, proved to be phytochrome-independent (Chandra-Shekara et al., 2006).

In the present work, we study the principles underlying light-dependency of inducible plant defences in the Arabidopsis-*P. syringae* model interaction at the molecular level. Our data indicate that light regulation of defence responses manifests itself not only during artificial dark treatments but is also relevant within naturally occurring light/dark cycles. Further, employing Arabidopsis photoreceptor double mutants, we show that inducible defence responses at inoculation sites are not or only moderately altered when cryptochrome, phototropin or phytochrome photoperception is impaired. SAR, by contrast, strongly depends on phytochrome photoperception, and can be established without functional cryptochrome or phototropin signalling pathways.

5.3 Results

5.3.1 Plant defences and resistance depend on the daytime of inoculation

To study the influence of light on inducible plant defences and disease resistance, we previously compared resistance responses of Arabidopsis Col-0 plants situated in conventional 9 h light / 15 h dark photoperiodic conditions with those of plants transferred to continuous darkness before pathogen inoculation. The HR-inducing bacterial strain *Pseudomonas syringae* pv. *maculicola* ES4326 carrying the avirulence gene *avrRpm1* (*Psm*

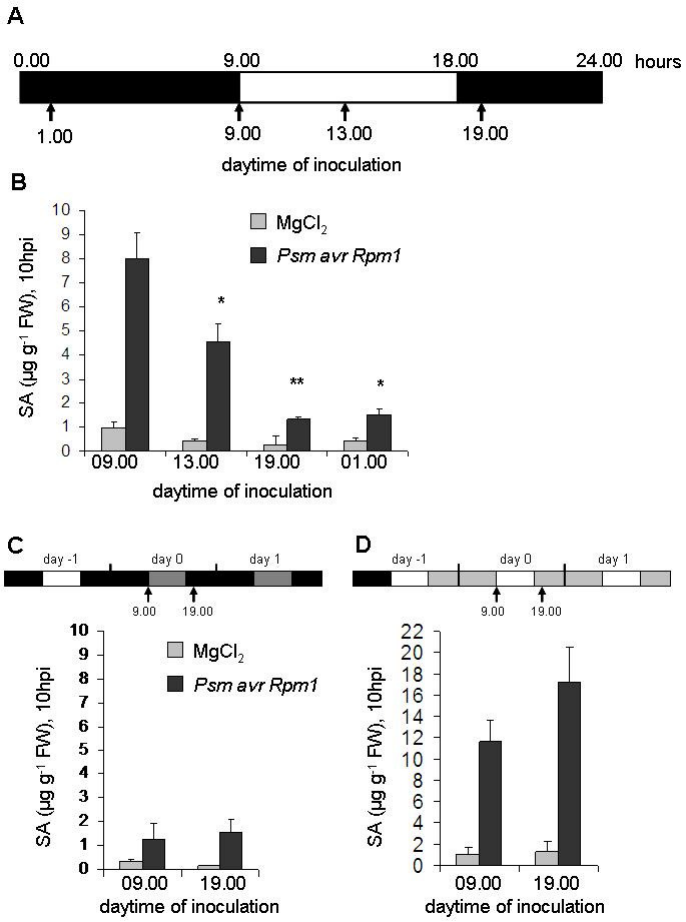

Figure 1. Salicylic acid accumulation in Arabidopsis depends on the daytime of pathogen inoculation. A, Daytimes of *Psm avrRpm1*-inoculation and light/dark regime in the plant growth chamber. Black and white boxes correspond to dark and light periods, respectively, during a normal growth chamber day. Arrows and bottom numbers indicate the four different inoculation times. B, Salicylic acid accumulation in Arabidopsis Col-0 leaves at 10 hours after inoculation with *Psm avrRpm1* (OD = 0.005) following the experimental setup described in A. Control samples were infiltrated with 10 mM $MgCl_2$. Values of free and glycosidic SA were added to yield total SA levels. Bars represent mean values (± SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisks denote values with statistically significant differences to the 09.00h-value (*: $P < 0.05$, ** $P < 0.001$; Student's t-test). Light bars: $MgCl_2$-treatment, dark bars: *Psm avrRpm1*-inoculation. C, Accumulation of total SA in continuous darkness after a 09.00 h- and a 19.00 h-inoculation. The top illustration indicates light regime and inoculation times during three consecutive days around the beginning of the experiment (day 0). Until "day -2", normal light/dark-cycles (depicted in A) were applied. Dark grey boxes correspond to dark phases with subjective day character. D, Accumulation of total SA in continuous light after a 09.00 h- and a 19.00 h-inoculation. The top illustration is according to C except that light grey boxes indicate light periods with subjective night character.

avrRpm1) was used in these experiments. In summary, we observed that induction of a specific subset of plant defence responses, which includes SA-associated responses and the HR, depends on the presence of light after pathogen inoculation (Zeier et al., 2004). To examine whether light regulation of defence reactions is relevant not only during artificial darkening experiments but also within a light/dark cycle that naturally occurs during the course of a day, we inoculated Col-0 plants at defined daytimes with *Psm avrRpm1*, i.e. in the morning (09.00 hours), at midday (13.00 hours), in the evening (19.00 hours), and in the night (01.00 hours), and scored resistance responses at constant times after each treatment. As in previous experiments (Zeier et al., 2004), the applied day/night cycle in the growth chamber consisted of a 9 h light period (PFD = 70 µmol m^{-2} s^{-1}) starting from 9.00 hours until 18.00 hours, and a dark period during the remaining daytime (Fig. 1A).

In Col-0 leaves, biosynthesis of salicylic acid is induced during the first 4-8 hours after pressure infiltration of *Psm avrRpm1* suspensions (Mishina et al., 2008). When applying bacteria at different daytimes, we found that the amount of total (sum of free and glucosidic) SA produced within the first 10 hours post inoculation (hpi) strongly depends on the inoculation daytime, with SA accumulating to 8.0 µg g^{-1} fresh weight (FW), 4.5 µg g^{-1} FW, 1.3 µg g^{-1} FW, or 1.5 µg g^{-1} FW after morning, midday, evening and night inoculations, respectively (Fig. 1B). The differences in leaf SA accumulation between morning, midday and evening/night inoculations were statistically significant ($P < 0.02$), and the trend for total SA depicted in Fig. 1B was similarly observed for the levels of both free and glucosidic SA (data not shown). The amounts of SA produced during the first 10 hpi thus correlated with the number of light hours (9 h for morning, 5 h for midday, 0 h for evening, 2 h for night inoculations, respectively) during this early infection period.

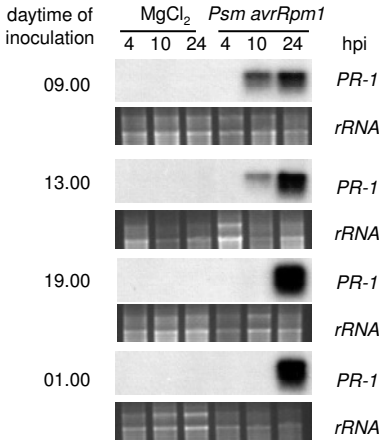

Figure 2. Expression of defence genes is dependent on inoculation daytime. *PR-1* expression in leaves inoculated with *Psm avrRpm1* (OD = 0.005) at different daytimes were assessed by Northern blot analysis. Plants were kept in the light/dark regime depicted in Fig. 1A. Control samples were treated with 10mM $MgCl_2$. Samples were taken at 4 h, 10 h, and 24 h post inoculation (hpi).

Because pathogen defence has previously been linked with the circadian rhythm (Sauerbrunn and Schlaich, 2004), we examined a possible contribution of the circadian clock to the observed daytime effect on SA accumulation. Conventionally grown plants were therefore placed into continuous darkness from dusk of day -1 (the day before the pathogen

experiment was started), and leaves were inoculated with *Psm avrRpm1* the following day (day 0) at 9.00 hours or at 19.00 hours (Fig. 1C). In both cases, lower SA levels comparable with those accumulating in leaves of evening inoculated plants experiencing the normal light/dark-cycle (Fig. 1B) were detected at 10 hpi, suggesting that the contribution of the circadian rhythm to the daytime effect is negligible, and indicating that the differences in SA accumulation observed during the daytime experiment (Fig. 1B) essentially resulted from distinctive lengths of the light period during the early plant-pathogen interaction. Conversely, we also placed plants into continuous light from dawn of day -1 onwards, treated leaves with *Psm avrRpm1* at 9.00 hours or at 19.00 hours of day 0, and scored SA accumulation at 10 hpi (Fig. 1D). High SA levels (11.7 µg g^{-1} FW), which exceeded the 9.00 hours value (8.0 µg g^{-1} FW) from the normal daytime experiment (Fig. 1B), accumulated after the 9.00 hours-inoculation at continuous light. Although circadian clock-regulation of SA production would imply a lower SA value for the 19.00 hours-inoculation under continuous light, we detected an even higher mean value of 17.2 µg g^{-1} FW than for the 9.00 hours-treatment. This again emphasizes that the circadian clock does not regulate pathogen-induced SA production. Although differences between both daytimes under continuous light were statistically not different ($P = 0.07$), the tendencies observed in Figs. 1B and 1D might suggest that the duration of the light period just before bacterial inoculation has an influence on the amount of accumulating SA.

We next assessed whether expression of the SA-inducible defence gene *PR-1* and HR cell death, two responses that had previously been shown to be light-regulated (Genoud et al., 2002; Zeier et al., 2004), would also depend on inoculation daytime. Whereas a morning or midday treatment of Col-0 leaves with *Psm avrRpm1* induced a distinct *PR-1* expression already at 10 hours after pathogen contact, evening or night inoculation did not result in induction of the defence gene at 10 hpi (Fig. 2). Thus, like SA accumulation, early expression of *PR-1* depends on the presence of a light period immediately after pathogen inoculation. Later, at 24 hpi, *PR-1* was strongly expressed under each of the experimental conditions. The hypersensitive cell death response induced by *Psm avrRpm1* in Col-0 leaves results in necrotic, semi-translucent lesions (Delledonne et al., 1998). When scoring macroscopic HR development 5 days after bacterial treatment, we found that tissue necrosis developed most prominently after morning inoculations, and that macroscopic lesion intensity gradually decreased in the order morning, midday, evening and night inoculation, respectively (Fig. 3A). Finally, we assessed whether the stronger defence capacity following morning compared with evening inoculations would express itself in a higher plant resistance towards *Psm avrRpm1* by scoring bacterial growth in leaves at 3 days post inoculation (dpi) for each case. Plants inoculated at 9.00 hours indeed were able to restrict bacterial growth more efficiently than plants inoculated at 19.00 hours, with a statistically significant, 3-fold

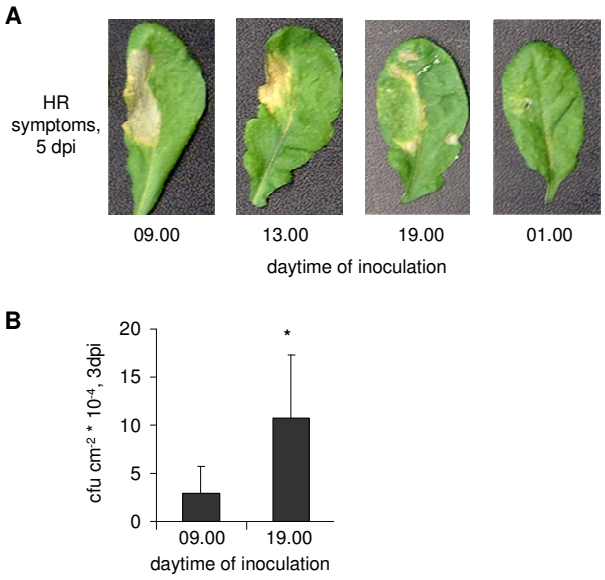

Figure 3. HR symptoms and disease resistance are dependent on inoculation daytime. A, Macroscopic HR symptoms of leaves five days post inoculation (dpi) after treatment with *Psm avrRpm1* (OD = 0.005) at different daytimes, as described in Fig. 1A. B, Bacterial growth quantification in Col-0 leaves three days after *Psm avrRpm*-inoculation (OD = 0.002) at either 09.00 h or 19.00 h. Bars represent mean values (± SD) of colony forming units (cfu) per square centimetre from at least 5 parallel samples, each sample consisting of three leaf disks. Asterisk denotes statistically significant differences between 09.00 h- and 19.00 h-inoculations (*: $P < 0.006$; Student's t-test). To ensure the uniformity of infiltrations, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for both inoculation times (data not shown).

lower multiplication of bacteria at 3 dpi (Fig. 3B). Together, these data demonstrate that, like SA accumulation and *PR-1* expression, HR lesion development and disease resistance in Arabidopsis leaves are markedly influenced by the daytime of *P. syringae* inoculation, and are positively correlated with the length of the light period during the early plant-pathogen interaction.

To exclude that the observed differences in defence responses and resistance result from bacterial rather than plant performance, we used batches of bacteria originating from the same overnight culture for each daytime inoculation. We attempted to minimize relative ageing effects of bacterial batches by growing the overnight culture already five days before the pathogen experiments were initiated, and stored purified batches at 4°C before use. Moreover, permutation of the experimental starting point (e.g. comparing the inoculation series 9.00, 13.00, 19.00, 01.00 hours with the series 19.00, 01.00, 9.00, 13.00 hours) had no influence on the relative tendencies of defence responses (Figs. 1-3), indicating that light-mediated differences in plant performance were causative for the observed defence outcomes.

5.3.2 Photoreceptor signalling only moderately affects induction of Arabidopsis defences at sites of *Psm* (± *avrRpm1*) inoculation

Light could influence defence responses through photosynthetic means or by cross-talk of photoreceptor-mediated light signalling with plant defence signalling. Light signalling is mediated by the blue/UV-A-absorbing cryptochromes and phototropins, and the red and far-red light-absorbing phytochromes (Gyula et al., 2003). To test whether light perception by

these photoreceptors is required for *P. syringae*-induced defence responses and disease resistance, we examined the interactions of the following Arabidopsis double mutants impaired in either cryptochrome, phototropin, or phytochrome photoperception, with an avirulent (*Psm avrRpm1*) or a virulent strain (*Psm*) of *P. syringae* pv. *maculicola*: *cry1cry2* (*cry1-304 cry2-1*; Mockler et al., 1999), *phot1phot2* (*phot1-5 phot2-1*; Sakai et al., 2001), and *phyAphyB* (*phyA-211 phyB-9*; Cerdán and Chory, 2003). Common genetic background for all examined mutants is accession Columbia (Col; Col-0 for *cry1cry2* and *phyAphyB*, Col-3 for *phot1phot2*), implicating that each line harbours the resistance gene Rpm1 whose product recognizes the bacterial avirulence protein AvrRpm1. This recognition event is causative for the *Psm avrRpm1*-induced HR and early SA accumulation in wild-type Columbia (Bisgrove et al., 1994; Mishina et al., 2008).

At sites of *Psm avrRpm1* inoculation, loss of UV/blue light perception by cryptochrome or phototropin in *cry1cry2* and *phot1phot2*, respectively, did not impede plants to mount light-dependent defence responses (Figs. 4, 5). Whereas leaves of the *phot1phot2* double mutant and the corresponding Col-3 wild-type showed similar levels of total SA at 10 hpi, leaves of *cry1cry2* actually accumulated SA to significantly (P = 0.04) higher levels than Col-0 wild-type leaves (Fig. 4A). Trypan blue staining at 24 hpi of leaves inoculated with the avirulent pathogen revealed that both UV/blue light receptor mutants were able to execute a wild-type like hypersensitive cell death response (Fig. 4B). Moreover, pathogen-induced expression of the light-dependent defence genes *PR-1* and *PAL1* occurred independently of either a functional cryptochrome or phototropin pathway (Fig. 5). Assessment of H_2O_2 production at inoculation sites through staining of leaves with 3,3'-diaminobenzidine (DAB;

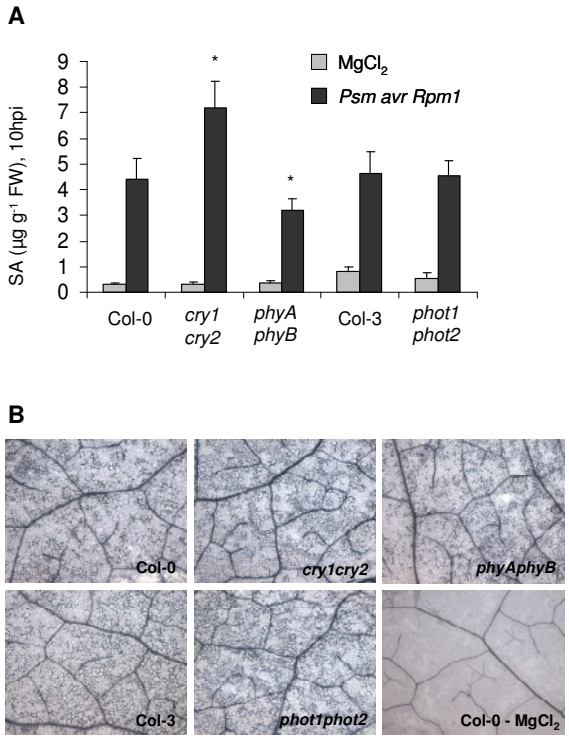

Figure 4. Salicylic acid accumulation and HR development in leaves of Col-0, Col-3, *cry1cry2*, *phot1phot2* and *phyAphyB* plants treated with *Psm avrRpm1* (OD = 0.005). Inoculations were performed at 10.00 h within the light/dark regime depicted in Fig. 1A. A, Total SA levels in leaves 10 h after *Psm avrRpm1* or $MgCl_2$-treatment. Bars represent mean values (± SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisk denotes value with statistically significant difference to the values of the respective wild-type (*: P < 0.05; Student's t-test). Light bars: $MgCl_2$-treatment, dark bars: *Psm avrRpm1* inoculation. B, Microscopic HR lesions of representative leaf samples at 24 h after inoculation with *Psm avrRpm1*, as assessed by Trypan blue staining. For all lines under investigation, inoculated leaf areas harbour patches of blue-stained, dead cells clearly delimited from surrounding healthy (unstained) tissue (magnification 100-fold). For comparison, the staining outcome of an $MgCl_2$-treated Col-0 leaf is depicted (bottom right). Similar staining results were obtained for $MgCl_2$-treated leaves of the remainder lines (not shown).

data not shown), and expression patterns of the ROS-inducible *GST1* gene further indicated that the oxidative burst is not affected in *cry1cry2* or *phot1phot2* (Fig. 5). Likewise, *Psm avrRpm1*-induced accumulation of jasmonic acid and camalexin also occurred to similar levels in *cry1cry2*, *phot1phot1*, and the respective wild-type leaves (data not shown).

Although phytochrome photoperception has been previously implicated with SA signalling (Genoud et al., 2002), *phyAphyB* plants appreciably induced SA biosynthesis and expression of the SA-responsive *PR-1* gene in *Psm avrRpm1*-inoculated leaves. Compared with the Col-0 wild-type, however, accumulation of both free and glucosidic SA were modestly reduced in *phyAphyB* (Fig. 4A), and *PR-1* expression was marginally delayed (Fig. 5C). After trypan blue staining of *Psm avrRpm1*-infiltrated leaves, we observed distinct blue-stained patches of dead cells in both *phyApyhB* and in Col-0 (Fig. 4B), indicating that *phyAphyB* plants are able to mount a wild-type-like HR. DAB staining, metabolite determination and gene expression analyses further revealed that *phyAphyB* leaves induce an oxidative burst, JA biosynthesis, camalexin accumulation, and expression patterns of *GST1* and *PAL1* that are similar to the respective responses in Col-0 leaves (Fig. 5C and data not shown).

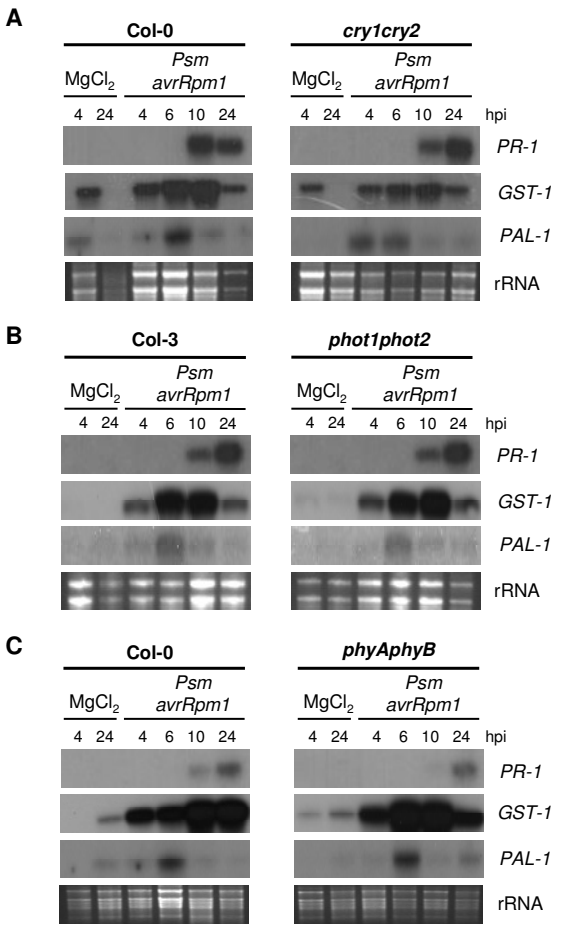

Figure 5. Expression of defence-related genes in leaves of wild-type Columbia and mutants impaired in light perception at sites of *Psm avrRpm1* (OD = 0.005) inoculation, as assessed by Northern blot analysis. Control samples were treated with 10 mM $MgCl_2$. Numbers indicate hours post inoculation (hpi). A, Comparison Col-0 – *cry1cry2*. B, Comparison Col-3 – *phot1phot2*. C, Comparison Col-0 – *phyAphyB*.

When comparing resistance towards the avirulent *Psm avrRpm1* strain in terms of bacterial multiplication at 3 dpi, we did not detect statistically significant differences between wild-type and photoreceptor mutant plants (Fig. 6A). In compatible interactions with the disease-causing, virulent *Psm* strain, bacterial growth differences between Col-0 and *phyAphyB* were more pronounced, and a significant, 3-fold higher multiplication of *Psm* in leaves of *phyAphyB* was detected compared with Col-0 leaves. In contrast to this moderate attenuation of basal resistance in the phytochrome mutants, no *Psm*-growth differences in the UV/blue-light receptor mutants and wild-type plants existed (Fig. 6B). Taken together,

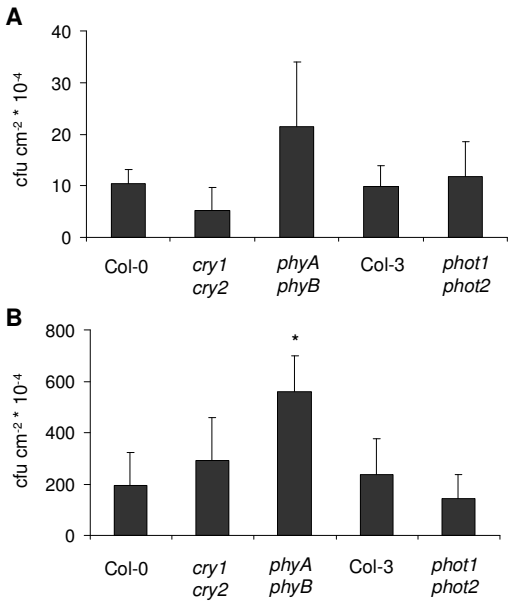

Figure 6. Specific and basal disease resistance of wild-type and photoreceptor mutant plants. Bacterial growth quantification of A, *Psm avrRpm1* (OD = 0.002)- and B, *Psm* (OD = 0.002)-inoculated leaves of wild-type and photoreceptor mutants three days after inoculation. Bars represent mean values (± SD) of colony forming units (cfu) per square centimetre from at least five parallel samples, each sample consisting of three leaf disks. Asterisk denotes value with statistically significant differences to the value of the respective wild-type (*: $P < 0.05$; Student's t-test). To ensure the uniformity of infiltrations, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for leaves of different lines (data not shown).

these data suggest a marginal cross-talk between phytochrome-mediated light signalling and defence signalling at sites of pathogen attack, and indicate an even lesser influence of the cryptochrome and phototropin pathways on local defence and resistance.

5.3.3 SAR requires functional phytochrome photoperception but is independent of cryptochrome and phototropin signalling

Because our previous studies indicate an absolute requirement of light for biological induction of SAR (Zeier et al., 2004), we tested whether this light dependency would be mediated by photoreceptors. To examine a potential pathogen-induced enhancement of systemic resistance, three lower rosette leaves (here designated as "primary leaves") of a given plant were either infiltrated with 10 mM $MgCl_2$ in a control treatment, or inoculated with a suspension of *Psm* (OD 0.01) for SAR induction (Mishina and Zeier, 2007). Two days later, three upper, previously non-treated leaves ("systemic leaves") were either collected and analysed for SA content and *PR* gene expression, or they were subject to a subsequent challenge infection with lower inoculi of *Psm* (OD 0.002). SAR was directly assessed by scoring bacterial growth in systemic leaves three days after the challenge infection.

Compared to $MgCl_2$-inoculated controls, *Psm*-pre-treated Col-0, *cry1cry2*, Col-3, and *phot1phot2* plants significantly enhanced their resistance towards challenge infections by factors ranging from 6 to 14 (Fig. 7A). SA contents of systemic leaves were considerably elevated in these lines after *Psm*-infection of primary leaves (Fig. 7B). Moreover, expression levels of the SAR genes *PR-1*, a typical SA-inducible defence gene, and of *PR-2*, whose up-regulation is SA-independent (Nawrath and Métraux, 1999), were both elevated in systemic leaves after *Psm*-treatment (Fig. 8A, C). Thus, SAR developed in both Col lines as well as in

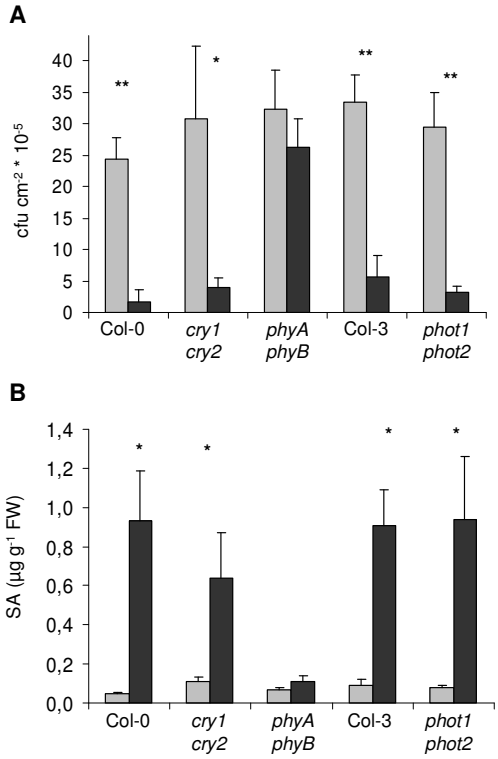

Figure 7. Systemic acquired resistance is compromised in *phyAphyB*. A, Bacterial growth quantification to directly assess SAR. Wild-type and photoreceptor mutant plants were pre-treated with 10 mM $MgCl_2$ or *Psm* (OD = 0.01) in three primary leaves (primary treatment), and two days later, three systemic leaves located directly above the primary leaves were inoculated with *Psm* (OD = 0.002). Bacterial growth in systemic leaves was assessed three days (3 dpi) after the secondary inoculation. Bars represent mean values (± SD) of colony forming units (cfu) per square centimetre from at least five parallel samples consisting each of 3 leaf disks. Asterisks denote statistically significant differences in systemic growth between *Psm*- and $MgCl_2$-pre-treated plants of a particular line (*: $P < 0.05$, ** $P < 0.001$; Student's t-test). B, Systemic accumulation of free salicylic acid. Primary treatments were performed as described in A. Untreated, upper leaves were harvested two days later for SA analysis. Bars represent mean values (± SD) of three independent samples, each sample consisting of six leaves from two different plants. Asterisks denote pathogen treatment with statistically significant differences to the respective $MgCl_2$ control (*: $P < 0.05$; Student's t-test). Light bars: $MgCl_2$-pre-treatment, dark bars: *Psm* pre-inoculation.

the *cry1cry2* and *phot1phot2* receptor mutants. By contrast, the *phyAphyB* mutant completely failed to enhance whole plant resistance in response to a primary *Psm*-infection (Fig. 7A), and systemic levels of SA did not significantly increase upon the normally SAR-inducing bacterial treatment (Fig. 7B). In addition, the SA-marker gene *PR-1* was not up-regulated in systemic leaves of *Psm*-pre-infected *phyAphyB* mutants (Fig. 8B). These data demonstrate that a functional phytochrome pathway is required for biological induction of SAR and systemic elevation of SA-associated defences. Interestingly, *phyAphyB* mutant plants are not fully compromised in mounting systemic defence reactions, because they still proved capable to increase systemic expression of the SA-independent *PR-2* gene upon *Psm*-inoculation (Fig. 8B).

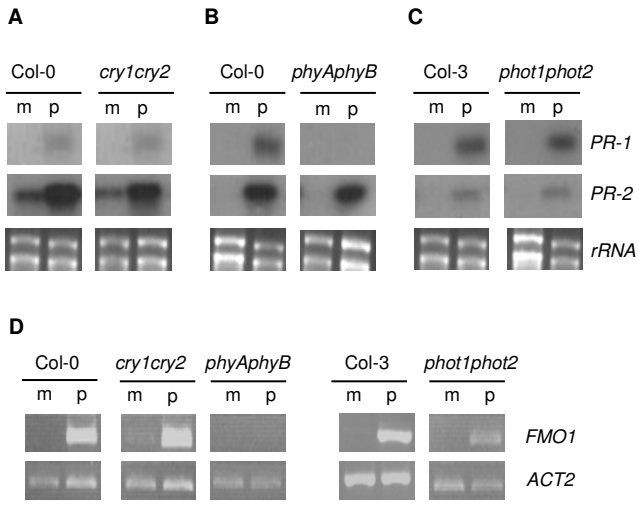

Figure 8. Systemic expression of defence related genes in wild-type and photoreceptor mutant plants. Primary leaves were treated as described in legend of Fig. 7. Untreated, distant leaves were harvested for analysis. A, B, and C, Systemic expression of *PR-1* and *PR-2*, as assessed by northern blot analysis. D, Expression of the *FLAVIN-DEPENDENT MONOOXYGENASE 1* (*FMO1)* gene, as assessed by RT-PCR. m: $MgCl_2$-pre-treatment, p: *Psm*-pre-treatment.

We have previously shown that the flavin-dependent monooxygenase FMO1 is an essential component for *P. syringae*-induced SAR in Arabidopsis (Mishina and Zeier, 2006). *FMO1* is up-regulated in both inoculated and systemic leaves, and *fmo1* mutant plants, although capable to mount defence reactions at inoculation sites, completely lack induction of SAR and systemic defence responses. Notably, all SAR-defective defence mutants investigated so far fail to up-regulate *FMO1* in distant (but not necessarily in inoculated) leaves, indicating that systemic expression of *FMO1* is a prerequisite for the SAR-induced state. We examined expression of *FMO1* in non-inoculated leaves of *Psm*-treated wild-type and photoreceptor mutant plants. Whereas the Col wild-type lines and the SAR-competent *cry1cry2* and *phot1phot2* plants increased expression of *FMO1* in systemic leaves two days post *Psm*-treatment, the SAR-defective *phyAphyB* mutants did not (Fig. 8B). These findings support our previous hypothesis that FMO1 is required in systemic leaves for SAR to be realized, and indicates that phytochrome-mediated light signalling is required upstream of FMO1 during SAR establishment.

5.4 Discussion

5.4.1 Daytime dependency of resistance responses

In the present manuscript, we show that, within a fixed light/dark cycle, resistance responses of Arabidopsis plants towards the incompatible *P. syringae* strain *Psm avrRpm1* depend on the time of the day when pathogen contact takes place. Within the light/dark cycle, the length of the light period during the early plant-pathogen interaction correlates with the magnitude of SA production, *PR-1* accumulation, and macroscopic HR lesion development (Figs. 1-3). Moreover, a stronger activation of defences observed after morning in comparison with evening inoculations entails a higher degree of resistance against *Psm avrRpm1* (Fig. 3B).

The plant circadian clock runs with a period close to 24 hours and controls several aspects of plant biochemistry and physiology. One of the consequences of circadian control is that stimuli of equal strength applied at different times of the day can lead to different intensities of a particular plant response, a phenomenon designated as gating (Hotta et al., 2007). It would thus be conceivable that the observed daytime-dependent differences in *P. syringae*-induced plant defences result from the circadian rhythm. On the basis that some genes implicated in plant defence follow a circadian expression pattern, a link between defence and circadian signalling has been established previously (Sauerbrunn and Schlaich, 2004). Examples for such genes are Arabidopsis *PCC1* (*Pathogen and Circadian Controlled 1*) and *PAL1* (Sauerbrunn and Schlaich, 2004; Rogers et al., 2005). The plant circadian clock maintains a relatively constant period, even in the absence of environmental cues such as light (Hotta et al., 2007). To discriminate between circadian control and light effects, we have

therefore conducted the daytime experiment both in continuous darkness and in continuous light (Figs. 1C, D). In contrast to the light/dark cycle-situation, the 19.00 hours-inoculation did not result in diminished SA production when compared with the 9.00 hours-inoculation under continuous light or darkness. This indicates that the circadian rhythm does not account for the daytime-dependent differences in plant defence activation under light/dark cycle-conditions.

The correlation between the magnitude of defence activation and the number of available light hours after *P. syringae* inoculation rather suggests that the daytime-dependency of defence responses in Arabidopsis is based on the direct influence of light on inducible plant defences (Zeier et al., 2004; Roberts and Paul, 2006). A light period of a certain length after pathogen contact has been reported as a prerequisite for optimal defence also in other pathosystems. In the interaction between an incompatible *Xanthomonas oryzae* strain and rice, for instance, a minimum of 8 h of light after bacterial inoculation was required for proper development of HR cell death, lignin deposition at inoculation sites, and effective restriction of bacterial multiplication (Guo et al., 1993). Similarly, in the incompatible interaction of Arabidopsis accession Di-17 and turnip crinkle virus, an HR and strong *PR-1* gene expression failed to occur when the initial light period after infection was less than 6 h (Chandra-Shekara et al., 2006). Together, these data suggest that light availability is important particularly during the early phases of plant defence activation. The absence of light during the early plant-pathogen interaction upon evening or night inoculations negatively affects development of the HR at later stages of the interaction, because the HR is determined during the first few hours after pathogen attack following specific recognition of avirulence factors (Fig. 3A). Responses like SA accumulation or *PR-1* gene expression, by contrast, are more continuously activated after recognition of both specific and general elicitors, and their magnitude at later infection stages is independent of the inoculation daytime (Fig. 2). However, the absence of light during the early interaction period entails a delayed and thus less efficient SA-associated defence mobilisation (Figs. 1B, 2).

Inoculation daytime and light conditions do influence plant defences and the outcome of a particular plant-pathogen interaction under laboratory conditions. To obtain reproducible results, researches should therefore aim to start comparative experiments at a fixed daytime rather than in a randomized fashion. A more effective activation of inducible plant defences under light influence could be relevant also in naturally occurring plant-pathogen interactions. An attenuated plant defence capacity at night might influence the infection strategy of pathogens, i.e. favour an attack during the dark hours. There is evidence that germination of spores from certain pathogenic fungi is inhibited by light, and plants are probably subject to an overall greater pathogen challenge at night than during the day (Roberts and Paul, 2006). For pathogenic bacteria, however, besides a light-dependent effectiveness of plant defences,

a number of other factors can contribute to determine the timing of pathogen attack and the final outcome of a particular plant-pathogen interaction in natural habitats (Colhoun, 1973). These include the necessity for bacteria to enter through open stomata, temperature influences on bacterial virulence, and humidity effects (Underwood et al., 2007; van Dijk et al., 1999).

5.4.2 Cross-talk of photoreceptor signalling and plant defence

A light-dependent nature of distinct plant defence responses has been established by several laboratories (Lozano and Sequeira, 1970; Guo et al., 1993; Genoud et al., 2002; Zeier et al., 2004; Bechtold et al., 2005; Chandra-Shekara et al., 2006). In *Psm avrRpm1*-inoculated Col-0 leaves, we have observed that SA accumulation, expression of *PAL1* and *PR-1*, as well as HR cell death are compromised in continuous darkness, whereas camalexin production, JA accumulation, and expression of *GST1* are not negatively affected. Moreover, local resistance against the avirulent *Psm avrRpm1* strain is partly, and SAR fully abrogated in darkened plants (Zeier et al., 2004). Two general mechanisms are conceivable by which light can regulate plant defence responses: 1) through photosynthesis and its consequences for energy status, reduction equivalents, and biochemical activity related with defence metabolism, or 2) through cross-talk of photoreceptor signalling with components of plant defence activation.

In the present work, we have addressed the latter issue by examining a possible requirement of light signalling pathways initiated by one of the three characterized photoreceptor systems, cryptochrome, phytochrome and phototropin (Gyula et al., 2003), for the establishment local and systemic resistance responses. Each photoreceptor double mutant used for these studies lacks physiological responses that are characteristically mediated by the respective light perception system. Seedlings of the *cry1cry2* mutant, for instance, are defective in the blue light but not the red light-induced hypocotyl inhibition response (Mockler et al., 1999). Unlike *cry1cry2*, the *phot1phot2* mutant is blocked in the phototropin-dependent chloroplast, stomatal and phototropic movements and lacks blue light-induction of calcium currents in mesophyll cells (Sakai et al., 2001; Kinoshita et al., 2001; Stoelzle et al., 2003). The *phyAphyB* double mutant is impaired in hypocotoyl length inhibition under both red and far-red light and shows an early-flowering phenotype (Cerdán and Chory, 2003). The phytochromes C, D, and E, which are still functional in *phyAphyB*, generally fulfil their physiological functions in combination with either PHYA or PHYB (Schepens et al., 2004).

Our data show that signalling events mediated by the blue light receptors cryptochrome and phototropin are dispensible for local resistance responses of inoculated Arabidopsis leaves, i.e. SA accumulation, defence gene expression, the HR, and basal or

specific resistance towards *P. syringae*. Moreover, many defence reactions triggered by *Psm avrRpm1*, including expression of *PAL1* and HR development, occur without functional phytochrome signalling (Figs. 4-7). The phytochrome-independency of pathogen-induced *PAL1*-expression in leaves was not necessarily expected, because light-dependent activation of the phenylpropanoid pathway in roots occurs in a phytochrome-dependent manner (Hemm et al., 2004). A slight attenuation of SA production and early *PR-1* gene expression is evident in the *phyAphyB* mutant, together with a modest decrease in specific and basal resistance. This indicates that the phytochrome pathway to a limited scale affects the SA resistance pathway at infection sites, which qualitatively parallels earlier findings in Arabidopsis (Genoud et al., 2002). Quantitatively, however, Genoud et al. (2002) report a larger dependency of local resistance on phytochrome signalling, including a requirement of the system for HR development. These discrepancies might arise from the different experimental systems used in both studies. Genoud et al. infected Arabidopsis accession Ler and mutants in the Ler background with the incompatible strain *P. syringae* pv. *tomato* DC3000 harbouring *avrRpt2*, which activates defence signalling pathways through the Rps2 resistance protein. By contrast, we studied Rpm1-mediated specific resistance as well as basal resistance in accession Col with *P. syringae* pv. *maculicola* ES4326 (± *avrRpm1*) strains. However, our data are both qualitatively and quantitatively comparable to the findings of Chandra-Shekara et al. (2006), who report that the light-dependent HR, *PR-1* expression and resistance of Arabidopsis accession Di-17 towards turnip crinkle virus are phytochrome-independent.

According to the present findings, cross-talk with photoreceptor signalling is not causative for the strong light-dependency of SA production, *PAL1* expression, up-regulation of *PR-1*, and HR development in *Psm avrRpm1*-inoculated leaves (Zeier et al., 2004), leaving a possible direct or indirect role of photosynthesis to enable these defences. SA biosynthesis proceeds through the shikimate pathway which requires erythrose-4-phosphate and phosphoenolpyruvate as metabolic precursors. Through the pentose phosphate pathway and glycolysis, respectively, availability of both metabolites is connected to the plants carbohydrate status. Light might thus positively influence SA levels through photosynthesis and increased production of biosynthetic carbon precursors. Metabolizable sugars have been shown to positively influence secondary metabolism and defence gene expression in Arabidopsis, because they promote lignification in dark grown roots and induce *PR* transcript levels in seedlings (Rogers et al., 2005; Thibaud et al., 2004). As SA biosynthesis via isochorismate synthase occurs in plastids (Strawn et al., 2007), photosynthetic activity might be required to supply reducing equivalents and energy for SA accumulation. At least for HR execution, intact chloroplasts and associated ROS production seem to play an important role

(Genoud et al., 2002; Liu et al., 2007). The impact of carbohydrate status and chloroplast function on pathogen-induced defence activation, however, requires further attention.

Although phytochrome signalling only moderately influences defence responses at inoculation sites, the present data clearly demonstrate that activation of whole plant resistance during SAR depends on phytochrome photoperception. This finding provides a mechanistic explanation for the previously observed light-dependency of SAR (Zeier et al., 2004). Phytochrome signalling seems to specifically control SA-associated systemic defences such as SA accumulation and *PR-1*-expression, but not SA-independent systemic defences such as *PR-2* expression. This is interesting, because it suggests that at least two independent systemic signalling pathways are activated after a local pathogen inoculation. Thereof, only the SA pathway provides protection against a *P. syringae* challenge infection. Considering the broad-spectrum character of SAR (Dean and Kuć, 1985), this does not necessarily exclude a contribution of SA-independent pathways to an enhanced resistance response against other microbial pathogens. Our data also show that intact phytochrome signalling is required for pathogen-induced expression of *FMO1* in non-inoculated leaves. FMO1 is required for SAR in Arabidopsis, its overexpression confers increased plant resistance, and mutant plants unable to express the gene in distant tissue after a local infection, including *phyAphyB*, are all SAR-deficient (Mishina and Zeier, 2006; Bartsch et al., 2006; Koch et al., 2006). During the SAR process, long-distance signal(s) generated in inoculated leaves are thought to travel through the plant and trigger resistance in distant tissue (Grant and Lamb, 2006; Park et al., 2007). In comparison to a local infection event, however, these long-distance signals are relatively low defence stimuli, and for a sufficiently strong resistance response to occur in systemic leaves, they must be amplified. We have recently proposed an amplification mechanism to occur in systemic leaves in which FMO1 and other SAR regulators are involved to boost incoming SAR signals (Mishina and Zeier, 2006). In an extended model that is consistent with our previous and current findings, phytochrome photoperception regulates signal amplification of such weak defence stimuli and is therefore especially required for low stimuli responses such as SAR, whereas it gets almost dispensable when stronger stimuli at infection sites trigger more massive local defence responses.

Although the extent of induced defence reactions in a single inoculated leaf is generally higher than in a single systemic leaf (Mishina et al., 2008), the sum of systemic defences might well exceed defence reactions at infection sites. In fact, the SAR-induced state can entail considerable costs due to the allocation of resources from primary metabolism (van Hulten et al., 2006; Walters and Heil, 2007), and these costs might be procured by light-driven photosynthetic metabolism. The phytochrome system might monitor

light availability and accommodate photosynthetic resources to the relatively costly increase in whole plant resistance.

5.5 Materials and Methods

5.5.1 Plant Materials and Growth Conditions

Arabidopsis thaliana L. Heynh. (Arabidopsis) ecotype Col-0 was used for the daytime experiments. To investigate the role of photoreceptors in plant defence, the following double mutants were used: *cry1cry2* (*cry1-304 cry2-1*; Mockler et al., 1999), *phot1phot2* (*phot1-5 phot2-1*; Sakai et al., 2001), and *phyAphyB* (*phyA-211 phyB-9*; Cerdán and Chory, 2003). Col-0 is the genetic background for both *cry1cry2* and *phyAphyB*, and Col-3 (gl-1) is background for *phot1phot2*.

The *phyAphyB* plants were put on Murashige and Skoog medium containing 3% sucrose for germination, and seedlings were transferred to soil mixture (see below) after ten days. All other lines were already sown and grown on an autoclaved mixture of soil (Klasmann, Beetpfanzensubstrat Typ R.H.P.16), vermiculite and sand (10:0.5:0.5). Plants were kept in a controlled environmental chamber (J-66LQ4, Percival) with a 9 h day period from 9.00 hours to 18.00 hours (photon flux density 70 µmol m^{-2} sec^{-1}, temperature 21°C) and a 15 h night period (temperature 18°C). For experiments, 6-week-old, naïve and unstressed plants showing a uniform appearance were used. If not otherwise indicated, pathogen treatments were performed at 10.00 hours.

5.5.2 Growth of Plant Pathogens and Inoculation

Pseudomonas syringae pv. *maculicola* ES4326 lacking (*Psm*) or harbouring *(Psm avrRpm1*) the avrRpm1 avirulence gene were grown at 28°C in King's B medium containing the appropriate antibiotics (Zeier et al., 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to a final optical density (OD) of 0.01 (SAR induction), 0.005 (determination of local gene expression and metabolite levels), or 0.002 (bacterial growth assays). The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1-ml syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed 3 d after infiltration (0,002 OD) by homogenising discs originated from infiltrated areas of three different leaves in 1ml 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 d. All pathogen experiments depicted in the figures were repeated at least twice with similar results.

5.5.3 Daytime experiments

Arabidopsis plants were infiltrated with bacteria at different daytimes (9.00 hours, 13.00 hours, 19.00 hours and 01.00 hours), and resistance responses were scored at constant times after inoculation. Batches of bacteria resulting from the same overnight culture were used for each inoculation series. To minimize relative ageing effects of bacteria, overnight cultures were prepared five days before the inoculation experiment was started. Purified bacterial batches were stored at 4°C until use. Inoculation series were repeated with permutated starting times.

5.5.4 Characterization of Systemic Acquired Resistance

Three lower leaves of a given plant were first infiltrated with a suspension of *Psm* (OD 0.01), or with 10 mM $MgCl_2$ as a control. Two days after this primary inoculation, non-treated upper leaves were harvested for SA determination and gene expression analysis, or plants were inoculated on three upper leaves with *Psm* (OD 0.002). Growth of *Psm* in upper leaves was assessed 3 d later.

5.5.5 Analysis of Gene Expression

Analysis of gene expression was performed as described by Mishina and Zeier (2006). Expression levels of *PR-1* (At2g14610), *PR-2* (At3g57260), *PAL1* (At2g37040) and *GST1* (At1g02930) were determined by Northern blot analysis, and *FMO1* (At1g19250) expression was analysed by RT-PCR. The following primers were used for PCR: 5'-CTTCTACTCTCCTCAGTGGCAAA-3' (FMO1-forward), 5'-CTAATGTCGTCCCATCTT-CAAAC-3' (FMO1-reverse). Hereby, the actin2 gene (At3g18780) was amplified as a control with the primers 5'-TCGCCATCCAAGCTGTTCTCT-3' (ACT2-forward), 5'-CCTGGACCTG-CCTCATA-CTC-3' (ACT2-reverse).

5.5.6 Determination of Defence Metabolites

Determination of free SA, glycosidic SA, jasmonic acid and camalexin levels in leaves was realised by a modified vapour-phase extraction method and subsequent gas chromatographic/mass spectrometric analysis according to Mishina and Zeier (2006). Total SA contents were calculated by summing up free and glycosidic SA levels.

5.5.7 Quantification of microscopic HR lesions and assessment of H_2O_2 production

The extent of microscopic HR lesion formation and H_2O_2 production were assessed by the Trypan blue and diaminobenzidine staining procedures, respectively, which are described in Zeier et al. (2004).

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6. Membrane stigmasterol accumulation enhances plant disease susceptibility to bacterial pathogens

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6.1 Summary

Upon inoculation with pathogenic microbes, plants induce an array of metabolic changes which potentially contribute to induced resistance, either by participating in defence signalling or by directly exerting antimicrobial activity. When analyzing leaf lipid composition during the *Arabidopsis thaliana* - *Pseudomonas syringae* interaction, we found that accumulation of the phytosterol stigmasterol is a significant plant metabolic process occurring upon bacterial infection. Stigmasterol is synthesized from β-sitosterol by the cytochrome P450 CYP710A1 via C22-desaturation. Arabidopsis *cyp710A1* mutant lines impaired in pathogen-inducible expression of the C22-desaturase and concomitant stigmasterol accumulation are more resistant to both avirulent and virulent *P. syringae* strains than wild-type plants, indicating that induced sterol desaturation in the wild-type favours pathogen multiplication and plant susceptibility. Stigmasterol formation is triggered through perception of pathogen-associated molecular patterns (PAMPs) such as flagellin and lipopolysaccharides, and by production of reactive oxygen species (ROS), but does not depend on the salicylic acid (SA)-, jasmonic acid (JA)-, or ethylene defence pathways. Isolated microsomal and plasma membrane preparations exhibit a similar increase in the stigma-/β-sitosterol ratio than whole leaf extracts after leaf inoculation with *P. syringae*, indicating that the produced stigmasterol is incorporated into plant membranes. The elevated contents of stigmasterol in membranes after pathogen attack do not influence SA-mediated defence signalling but attenuate the pathogen-induced expression of the defence regulator FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1). Thus, through PAMP exposure, ROS generation, and sterol desaturation, *P. syringae* interferes with an FMO1-associated resistance pathway in Arabidopsis to promote disease susceptibility.

6.2 Introduction

Several metabolic pathways are known to be activated in *Arabidopsis thaliana* (Arabidopsis) leaves upon inoculation with the hemibiotrophic bacterial pathogen *Pseudomonas syringae*. Some metabolites accumulate, albeit with different temporal characteristics or to varying levels, in leaves that have been inoculated with non-adapted, virulent, or avirulent bacterial strains, whereas others are only produced in a particular type of interaction (Mishina and Zeier, 2007a; Mishina *et al.*, 2008). Pathway activation might be either triggered by the perception of pathogen-associated molecular patterns (PAMPs), by the recognition of type III secretion system (TTSS)-secreted effector proteins, or by a combination of both.

The biosynthesis of the phenolic defence hormone salicylic acid (SA), and its conversion to derivatives such as SA β-glucoside (SAG), SA glucose ester, and methyl salicylate (MeSA) constitute a major and general metabolic event in *P. syringae*-inoculated leaves (Dean and Delaney, 2008; Attaran *et al.*, 2009). By contrast, jasmonic acid (JA) and related oxylipins such as the JA biosynthetic precursor OPDA and the non-enzymatically produced phytoprostanes are predominantly produced upon inoculation with avirulent, hypersensitive response (HR)-inducing strains, or with high inoculum densities of compatible strains (Grun *et al.*, 2007; Mishina and Zeier, 2007b). Since both treatments result in strong tissue necrosis, production of JA-related oxylipins appears to be associated with the disruption of leaf tissue (Mishina and Zeier, 2007b). Likewise, unsaturated C16 and C18 fatty acids such as linoleic and linolenic acid accumulate in *P. syringae*-treated Arabidopsis leaves (Yaeno *et al.*, 2004), with linolenic acid representing the initial precursor of JA biosynthesis. Moreover, virulent and avirulent but not non-host *P. syringae* strains evoke accumulation of the indole alkaloid camalexin, a tryptophan-derived phytoalexin of Arabidopsis that provides effective protection against necrotrophic but not (hemi-)biotrophic pathogens (Thomma *et al.*, 1999; Zhou *et al.*, 1999). Besides accumulation of phenolics (SA and its derivatives) and alkaloids (camalexin), the isoprenoid pathway, the third major metabolic pathway involved in plant secondary metabolite biosynthesis, is activated to yield production of the volatile terpenoids TMTT, β-ionone, and α-farnesene (Attaran *et al.*, 2008). Finally, the levels of the phytohormone abscisic acid (ABA), which is involved in several plant responses to abiotic stress, rise in leaves infected with compatible *P. syringae* (de Torres *et al.*, 2009). Strikingly, among the various pathogen-induced metabolites mentioned above, the only compound unequivocally required for resistance against *P. syringae* infestation is SA (Nawrath and Métraux, 1999; Wildermuth *et al.*, 2001). JA and ABA even favour pathogen susceptibility by interfering with SA biosynthesis and signalling (Kloek *et al.*, 2001; de Torres *et al.*, 2009).

Some hundred Arabidopsis genes are strongly up-regulated in *P. syringae*-inoculated leaves, many of them encoding proteins with putative functions in primary and secondary

metabolism (Zimmermann *et al.*, 2004). This suggests that several metabolic events induced in Arabidopsis leaves upon pathogen attack might still await discovery. An important metabolic route for plant development and survival is the sterol biosynthetic pathway. Sterols are indispensable compounds in plants and other eukaryotes because they are structural constituents of membranes where they regulate fluidity and permeability (Schaller, 2003). Whereas cell membranes of mammals and fungi are generally composed of one predominant sterol, cholesterol and ergosterol, respectively, plants have a more complex sterol mixture (Hartmann, 1998). For instance, the sterol profile of Arabidopsis accession C24 plants includes β-sitosterol as the major compound (64%), along with 24-methyl cholesterol (11%), stigmasterol (6%), isofucosterol (3%) and brassicasterol (2%) and several other minor sterols (Schaeffer *et al.*, 2001). It is as yet unknown whether a relationship between the sterol composition of plant membranes and plant defence or disease resistance exists.

We report here that C22-desaturation of the predominant Arabidopsis phytosterol β-sitosterol via the cytochrome P450 enzyme CYP710A1 and concomitant accumulation of stigmasterol constitutes a significant metabolic process in *P. syringae*-inoculated Arabidopsis leaves. Stigmasterol formation in leaves is triggered by perception of bacterial PAMPs and generation of ROS, but is independent of the SA-, JA- or ethylene-associated signalling pathways. Through mutant analysis, we show that stigmasterol accumulation attenuates a specific defence signalling pathway which results in enhanced susceptibility against *P. syringae*. Increased proportions of stigmasterol in microsomal membrane and plasma membrane isolates after bacterial attack indicate that pathogen-induced changes in the sterol composition of leaf membranes influence plant defence responses and affect the outcome of particular plant-pathogen interactions.

6.3 Results

6.3.1 β-sito- to stigmasterol conversion via CYP710A1 constitutes a significant metabolic process in *P. syringae*-inoculated Arabidopsis leaves

Publicly available microarray experiments indicate that several hundred Arabidopsis genes are substantially up-regulated in Arabidopsis leaves upon *P. syringae*-inoculation, among them the cytochrome P450 gene *CYP710A1* (Zimmermann *et al.*, 2004; Fig. S1a). Expression of *CYP710A1* is strongly induced with both the avirulent, HR-inducing strain *Psm avrRpm1* and the virulent *Psm* strain (Fig. 1a). CYP710A1 has been previously described as a sterol desaturase which introduces a double bond at the C22 position of the saturated sterol side chain of β-sitosterol to produce stigmasterol (Morikawa *et al.*, 2006; Arnqvist *et al.*, 2008; Fig. 1d). We have thus determined the sterol composition of mock- and pathogen-treated Col-0 leaves by chloroform/methanol extraction and subsequent GC/MS analysis (Fig. 1b, c). β-sitosterol constitutes the predominant sterol in both control and *P. syringae*-treated leaves. However, whereas in control leaves, the levels of stigmasterol are only faint (Fig. 1b), we observed a significant peak of stigmasterol in extracts from *P. syringae*-treated leaves (Fig. 1c), indicating its induced production after bacterial treatment.

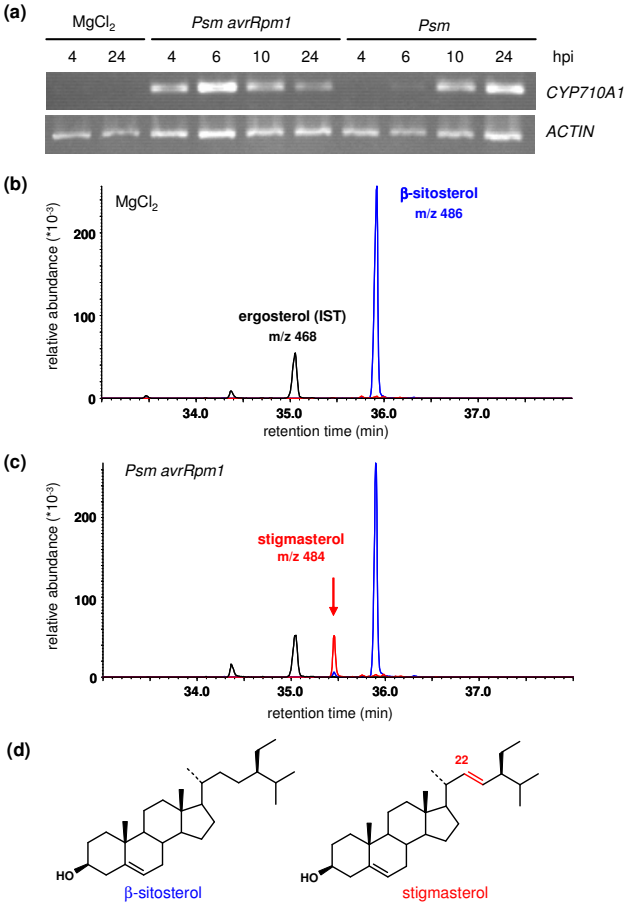

Figure 1. Expression of *CYP710A1* and GC/MS-based determination of sterols in leaves of Arabidopsis ecotype Col-0 after bacterial inoculation. (a) RT-PCR analysis of *CYP710A1* expression after inoculation with *P. syringae* pv. *maculicola* (*Psm*; virulent strain) or *Psm avrRpm1* (avirulent strain). Control leaves were infiltrated with 10 mM $MgCl_2$ and collected at 4 h and 24 h post treatment. The actin gene *ACT2* was amplified for internal standardization. (b, c) GC/MS analyses of lipid extracts from Arabidopsis leaves. Ion chromatograms at m/z 486 (blue), m/z 484 (red), and m/z 468 (black) are shown. The retention times of β-sitosterol, stigmasterol, and ergosterol (used as internal standard) are 35.9 min, 35.5 min, and 35.1 min, respectively. (b) Extract from $MgCl_2$-treated control leaves (24 hpi). (c) Extract from *Psm avrRpm1*-inoculated leaves (24 hpi).

(d) Chemical structures of β-sitosterol and stigmasterol.

A detailed timecourse analysis revealed that stigmasterol starts to accumulate in leaves between 10 and 24 hours after inoculation (hpi) of both avirulent *Psm avrRpm1* and virulent *Psm* (Fig. 2a). The leaf contents of stigmasterol raise to about 15 µg g^{-1} fresh weight (FW) until 48 hpi in both the incompatible and the compatible interaction, and from then on essentially remain constant. In $MgCl_2$-infiltrated control leaves, the stigmasterol content does not change significantly during the course of analysis and remains low at 0.1 to 0.2 µg g^{-1} FW. Moreover, the leaf levels of β-sitosterol are constantly high at 150 to 200 µg g^{-1} FW, exhibiting no significant alteration over time in $MgCl_2$-, *Psm avrRpm1*- or *Psm*-treated leaves (Fig. 2b). At 48 h after treatment, the molar ratio of stigmasterol to β-sitosterol is about 0.09 in *P. syringae*-inoculated leaves and 0.0007 in control leaves, reflecting a pathogen-triggered increase in leaf stigmasterol content of more than a 100-fold (Fig. 3c).

According to the T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress), several Arabidopsis lines with putative T-DNA insertions in the *CYP710A1* gene exist. By applying the PCR-based protocol described by Alonso *et al.* (2003), we identified two homozygous insertion lines from the Salk collection, *cyp710A1-1* (Salk_112491) and *cyp710A1-2* (Salk_014626), both with predicted T-DNA inserts in the promoter region of the *CYP710A1* gene. Neither insertion line shows any morphological nor growth phenotype that would distinguish it from wild-type plants. However,

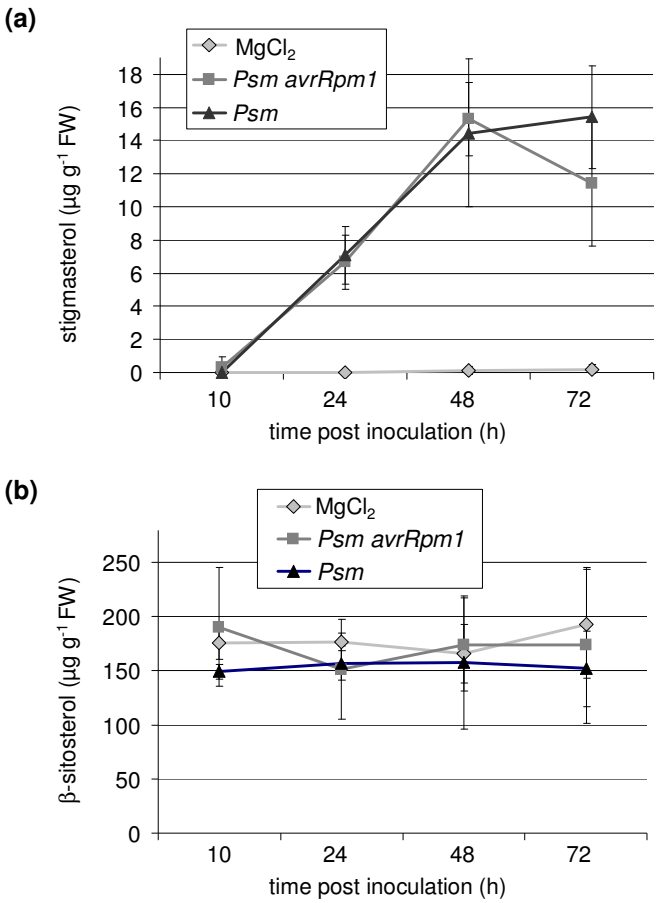

Figure 2. Time course analyses [hours (h) after infiltration] of stigmasterol and β-sitosterol levels in Col-0 leaves after treatment with 10 mM $MgCl_2$ (diamonds), *Psm* (triangles), or *Psm avrRpm1* (squares).
(a) Leaf stigmasterol contents and (b) leaf β-sitosterol contents.
Mean values of µg substance g^{-1} leaf fresh weight (± SD) from three independent samples are given.

compared to wild-type Col-0 plants, *cyp710A1-1* and *cyp710A1-2* are strongly impaired in *P. syringae*-induced expression of *CYP710A1* and the accumulation of stigmasterol (Fig. 3). This indicates that *P. syringae*-induced β-sito- to stigmasterol conversion predominantly, if not exclusively, results from the pathogen-induced expression of *CYP710A1* and the concomitant increase in CYP710A1-mediated sterol C22-desaturase activity.

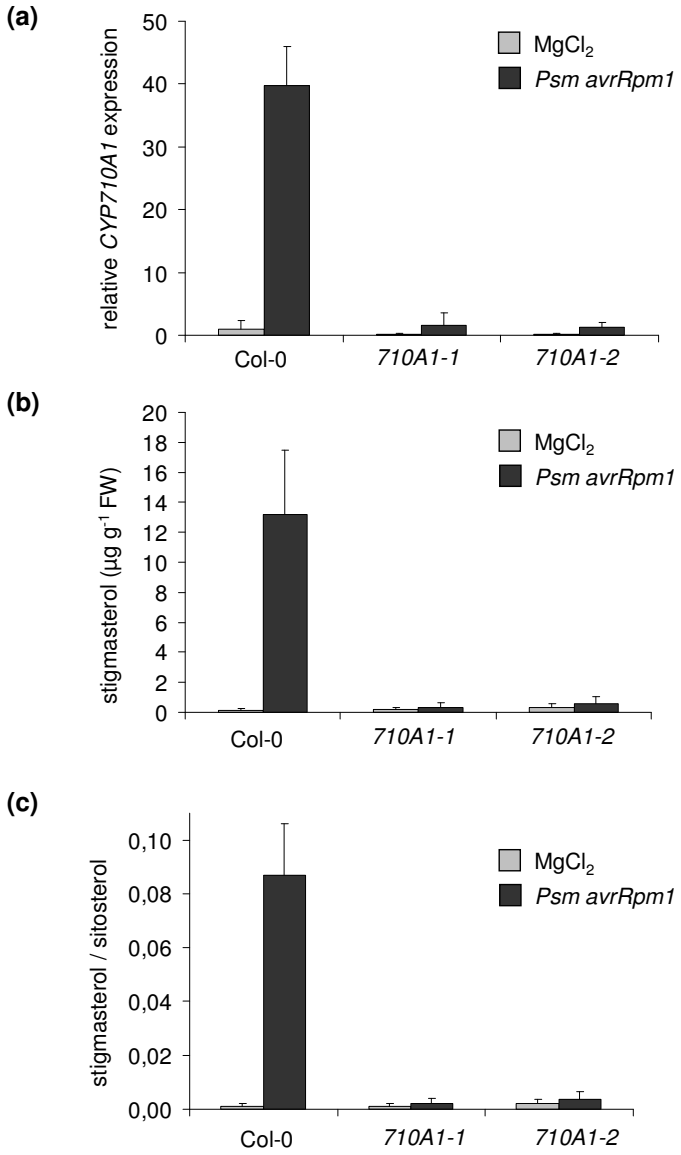

Figure 3. Expression of the *CYP710A1* gene, levels of stigmasterol, and stigmasterol/β-sitosterol ratios in leaves of Col-0, *cyp710A1-1*, and *cyp710A1-2* plants at 48 h after treatment with $MgCl_2$ (light bars) or *Psm avrRpm1* (dark bars).

(a) Relative expression levels of *CYP710A1*, as assessed by quantitative real-time PCR analysis. Expression values were normalized to those of the reference gene (At4g05320), and expressed relative to the wild-type $MgCl_2$ sample. For each expression value of one sample, three PCR-replicates were performed and averaged. The depicted bars represent mean values (± SD) of three biologically independent samples.

(b) Leaf stigmasterol levels.

(c) Molar stigmasterol/β-sitosterol ratios.

6.3.2 Mutational defects in *CYP710A1* lead to increased resistance towards *P. syringae*

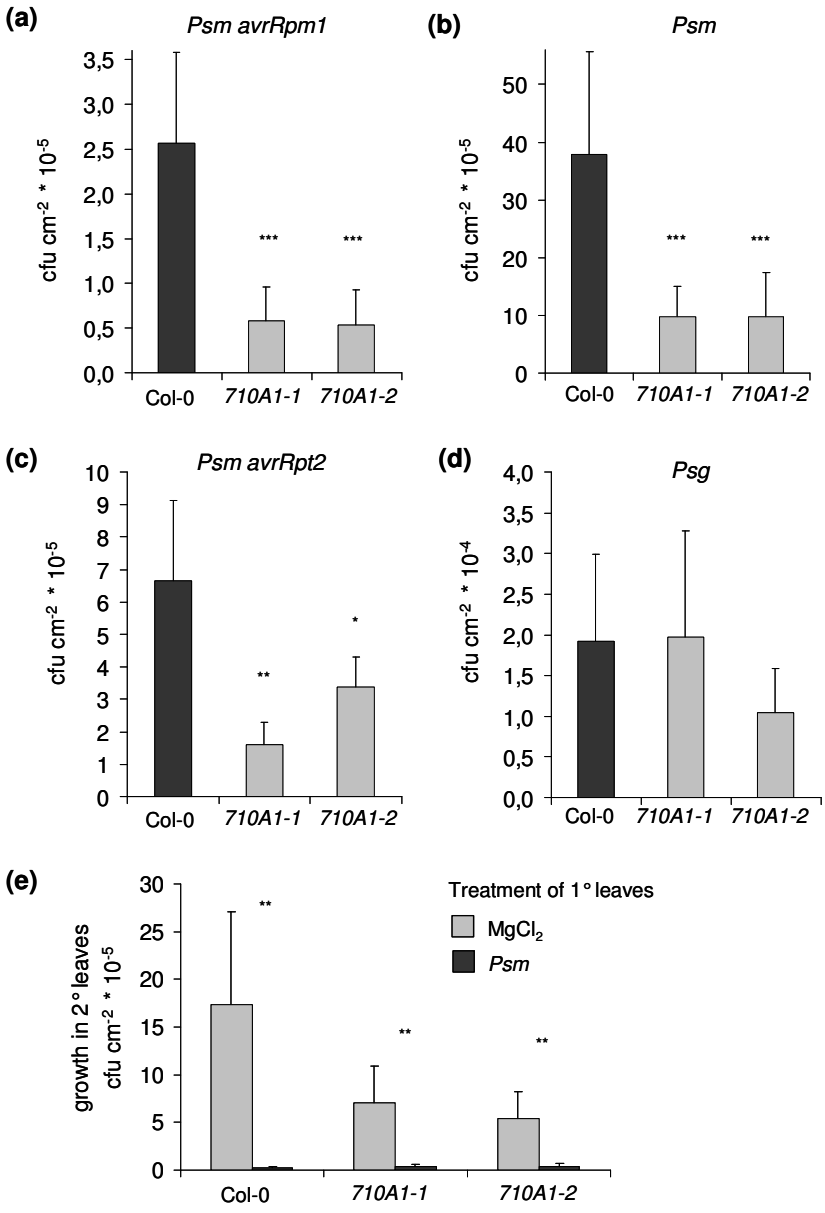

Figure 4. Resistance of Col-0, *cyp710A1-1*, and *cyp710A1-2* plants towards *P. syringae* leaf inoculation.

(a-d) Local resistance to avirulent, virulent, and non-adapted *P. syringae*.

Bacterial numbers of (a) *Psm avrRpm1* (applied in titers of OD 0.002), (b) *Psm* (OD 0.002), (c) *Psm avrRpt2* (OD 0.002), and *Psg* (OD 0.1) at three days after inoculation. Bars represent mean values (± SD) of colony forming units (cfu) per square centimetre from at least six parallel samples. Asterisks denote *cyp701A1* values with statistically significant differences to the wild-type value (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; Student's t-test). To ensure the uniformity of infiltrations, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for leaves of the different lines (data not shown).

(e) Systemic acquired resistance. Plants were pre-treated with either 10 mM $MgCl_2$ or *Psm* (OD 0.005) in three lower (1°) leaves. Two days later, three upper leaves (2°) were challenge-infected with *Psm* (OD 0.002). Bacterial growth in upper leaves was assessed three days after the 2° leaf-inoculation. Asterisks denote statistically significant differences of bacterial growth in 2° leaves between *Psm*- and $MgCl_2$-pre-treated plants of a particular line (**: $P < 0.01$).

To determine whether the considerable amount of accumulating stigmasterol would affect plant disease resistance, we comparatively determined multiplication of different *P. syringae* strains in leaves of Col-0, *cyp710A1-1*, and *cyp710A1-2* plants (Fig. 4). Multiplication of the avirulent *Psm avrRpm1* strain is about five times less pronounced in each of the *cyp710A1* mutant lines than in the Col-0 wild-type at 3 days post inoculation (dpi; Fig. 4a). Similarly, the compatible *Psm* strain and another HR-inducing strain, *Psm avrRpt2*, exhibits significantly lower multiplication in the *cyp710A1* lines than in Col-0 (Figs. 4b, c). By contrast, bacterial numbers of the non-adapted *Psg* strain are not statistically different in Col-0, *cyp710A1-1*, and *cyp710A1-2* leaves at 3 dpi (Fig. 4d). Together, this indicates that the stigmasterol produced in response to *P. syringae* in wild-type plants favors bacterial multiplication of avirulent or virulent bacteria and therefore enhances disease susceptibility towards adapted strains, whereas non-host resistance remains unaffected.

Upon local inoculation of *P. syringae*, systemic acquired resistance (SAR), a state of enhanced broad-spectrum disease resistance, develops throughout the whole plant (Cameron *et al.*, 1994; Mishina and Zeier, 2007b). To test whether sterol desaturation would play a role during the SAR process, we inoculated lower (1°) leaves of Col-0 or *cyp710A1* mutant plants with the SAR-inducing *Psm* strain, and challenge-inoculated upper (2°) leaves 2 days later. Upon the 1° pathogen-treatment, the *cyp710A1* lines are able to restrict bacterial growth during the 2° infection to the same extent as Col-0 plants, indicating that SAR is fully established in *cyp710A1* mutants and therefore does not dependent on stigmasterol production (Fig. 4e).

6.3.3 Stigmasterol formation is triggered by recognition of bacterial PAMPs

The conversion of β-sitosterol to stigmasterol in inoculated leaves is not only triggered by virulent and avirulent strains of *Psm* but also by the corresponding *Pst* strains (Fig. 5a). Stigmasterol accumulation solely occurs in pathogen-treated leaves but not in leaves distant from the initial inoculation site, suggesting that sterol C22-desaturation is a local but not a systemic plant response (Fig. 5a). Moreover, inoculation with the type III secretion-deficient *Pst hrpA*$^{-}$ strain or the non-adapted *Psg* strain induces a certain degree of local stigmasterol formation, which is statistically significant but lower than the accumulation in response to (a)virulent strains (Fig. 5a).

The common responsiveness to different *P. syringae* pathovars and the TTSS-defective *Pst* strain indicate that β-sito- to stigmasterol conversion might be initially triggered by the perception of conserved bacterial structures such as PAMPs rather than by recognition of specific pathogen determinants such as TTSS effectors. We therefore investigated whether exogenous application of two well-characterized bacterial PAMPs,

flagellin, the proteinaceous building unit of the bacterial flagellum, and LPS, a major component of the outer membrane of Gram-negative bacteria, would promote β-sito- to stigmasterol conversion. Infiltration of 200 nM flg22, a peptide corresponding to the elicitor active epitope of flagellin (Gomez-Gomez *et al*., 1999), into Col-0 leaves indeed provokes a considerable accumulation of stigmasterol at 48 h after treatment (Fig. 5b). Significant increases of stigmasterol contents also occur when leaves are treated with gel-purified LPS preparations from *Pseudomonas aeruginosa* or *Escherichia coli* in a concentration of 100 µg ml^{-1} (Fig. 5b). Thus, single treatments of leaves with flagellin or LPS are sufficient to induce stigmasterol formation, suggesting that bacterial PAMP recognition initiates β-sitosterol C22-desaturation after *P. syringae* inoculation in Arabidopsis leaves.

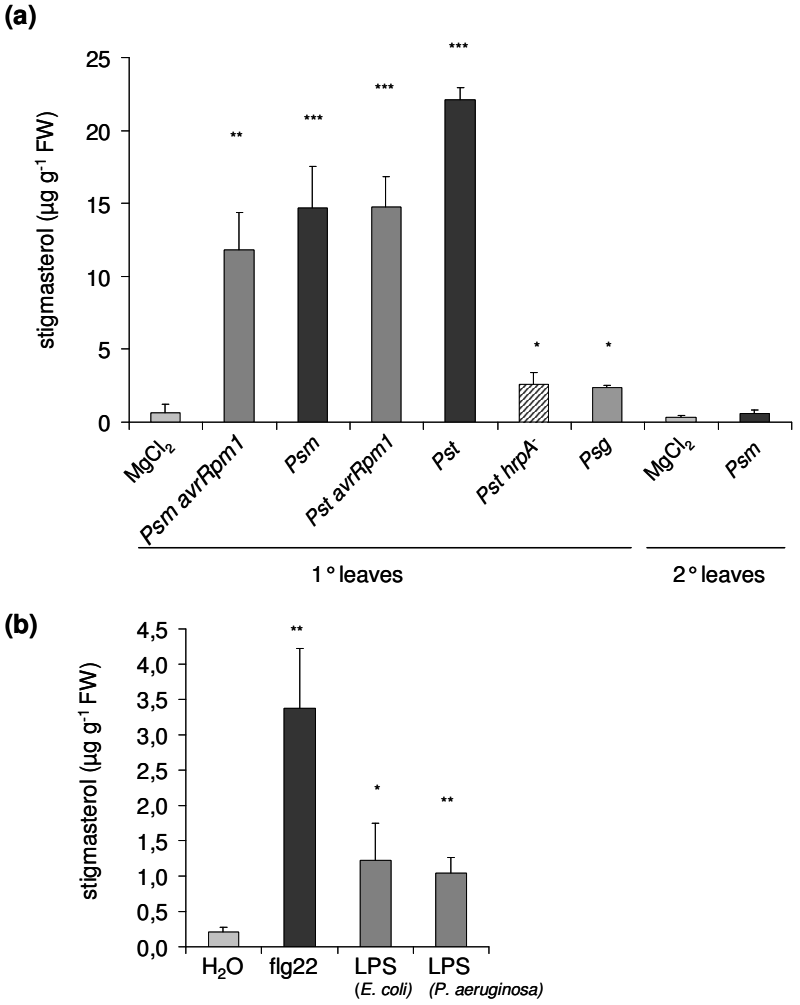

Figure 5. Leaf stigmasterol accumulation after inoculation with different *P. syringae* strains or bacterial PAMP treatments.

(a) Stigmasterol levels (means in µg g^{-1} FW ± SD of three independent samples) in treated (1°leaves) or non-treated, systemic leaves (2° leaves) of Col-0 plants at 48 h post 1° leaf infiltration. 10 mM $MgCl_2$ or suspensions of *P. syringae* pv. *maculicola* ES4326 (*Psm*, *Psm avrRpm1*), *P. syringae* pv. *tomato* DC3000 (*Pst*, *Pst avrRpm1*), *Pst hrpA*$^-$ (TTSS-defective), and *P. syringae* pv. *glycinea* race 4 (*Psg*; non-adapted) were applied to the 1° leaves.

(b) Stigmasterol levels (means ± SD) at 48 h post leaf treatment with 10 mM $MgCl_2$, 200 nM flg22, and 100 µg ml^{-1} lipopolysaccharide (LPS) purified from *E. coli* or *Pseudomonas aeruginosa*.

6.3.4 Stigmasterol production is activated through elevated ROS levels and occurs independently of SA-, JA-, and ethylene signalling

To investigate whether induced stigmasterol biosynthesis would be mediated by classical defence signalling pathways, we determined the leaf contents of stigmasterol in different well-characterized Arabidopsis defence mutants after *Psm avrRpm1* inoculation (Fig. 6a). A wild type-like stigmasterol production was observed in the SA pathway mutants *ics1 (sid2)* and *npr1*, which are defective in SA biosynthesis and downstream signalling, respectively (Nawrath and Métraux, 1999; Cao *et al.*, 1994). Additionally, pathogen-induced stigmasterol levels in leaves of the JA biosynthesis mutant *dde2* (von Malek *et al.*, 2002), the JA signalling mutant *jar1* (Staswick *et al.*, 1992), and the ethylene-insensitive mutant *etr1* (Bleecker *et al.*, 1988) are similar to those of Col-0 plants. Likewise, the defence mutants *fmo1*, *ndr1*, and

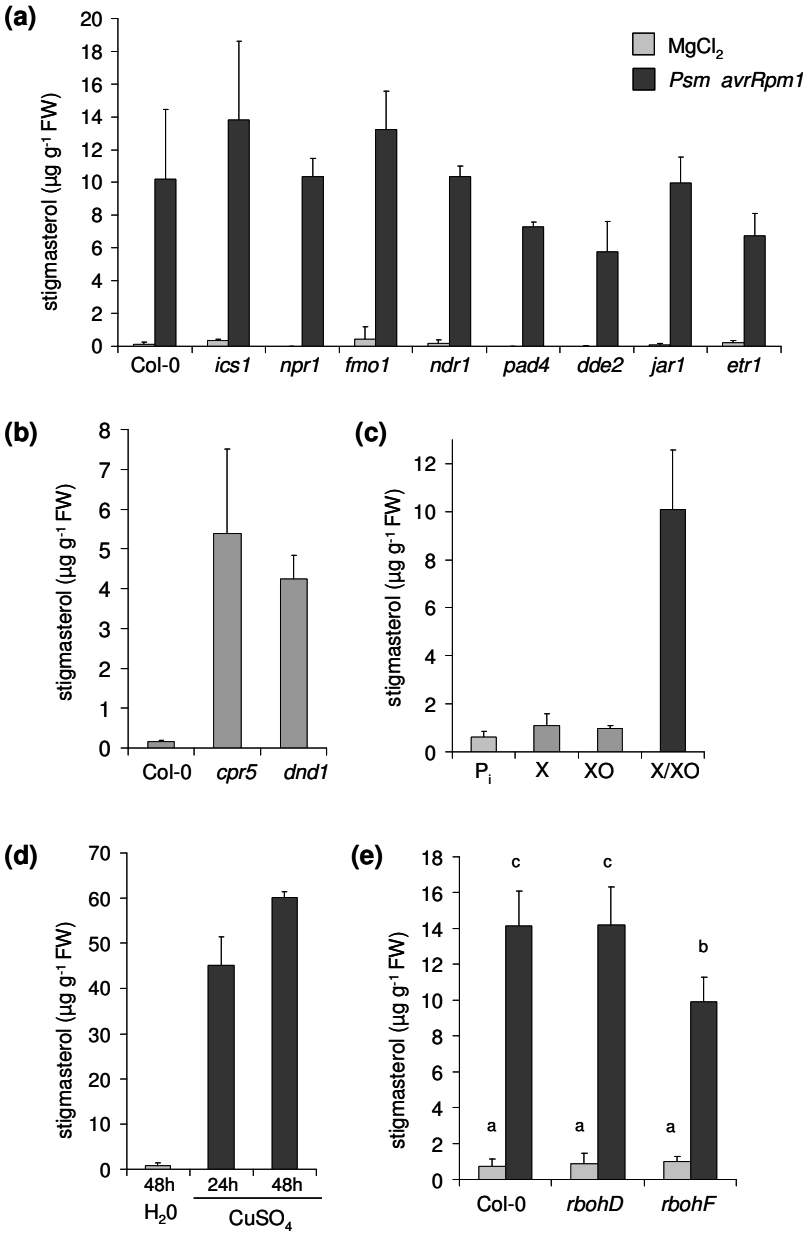

Figure 6. Induced stigmasterol synthesis in leaves of defence-related Arabidopsis mutants, and accumulation of stigmasterol in Col-0 leaves after treatment wih ROS generating substances.

(a) Leaf stigmasterol levels of *Psm avrRpm1*- or mock-inoculated defence mutants and the Col-0 wild-type at 48 hpi.

(b) Constitutive levels of stigmasterol in leaves of untreated Col-0, *cpr5*, and *dnd1* plants.

(c) Stigmasterol levels of Col-0 leaves treated with the superoxide-generating substrate / enzyme mix xanthine (X; 0.5 mM) / xanthine oxidase (XO; 0.5 U ml^{-1}), with control buffer (20 mM sodium phosphate), and with X or XO alone. Leaves were harvested from plants at 48 h post treatment.

(d) Stigmasterol levels of Col-0 leaves infiltrated with water or 10 mM $CuSO_4$. Leaves were harvested at the indicated times (h) post treatment.

(e) Leaf stigmasterol levels of *Psm avrRpm1*- or mock-inoculated *rboh* mutant and Col-0 wild-type plants at 48 hpi.

pad4, which are compromised in SAR and certain local defence responses (Mishina and Zeier, 2006; Bartsch *et al.*, 2006; Century *et al.*, 1995; Glazebrook *et al.*, 1997), are still able to induce stigmasterol accumulation in a wild-type-like manner. Thus, stigmasterol production occurs independently of several well-described defence routes, including the SA-, JA-, and ethylene-dependent pathways.

We noticed that the Arabidopsis *cpr5* and *dnd1* mutants, which possess increased pathogen resistance because of constitutively activated defence responses (Bowling *et al.*, 1997; Yu *et al.*, 1998), exhibit markedly elevated leaf stigmasterol levels in the absence of a pathogen contact (Fig. 6b). A common feature of *cpr5* and *dnd1* plants is a constitutive enhancement of leaf ROS levels (Mateo *et al.*, 2006). We therefore tested whether exogenous ROS application alone would be sufficient for the induction of stigmasterol biosynthesis. Infiltration of 0.5 mM xanthine / 0.5 U ml^{-1} xanthine oxidase, an enzyme-substrate mix which provides a continuous production of O_2^- in the low µM range (Delledonne *et al.*, 1998), provokes an accumulation of stigmasterol in Col-0 leaves that is comparable to the levels determined after *P. syringae* inoculation (Fig. 6c). Moreover, infiltration of leaves with 10 mM copper sulphate, a treatment that leads to massive ROS production and oxidative stress (Drazkiewicz *et al.* 2004), causes a vigorous elevation of leaf stigmasterol contents that exceeds the *P. syringae*-induced accumulation by a factor of three (Fig. 6d). Thus, exogenous supply of ROS-generating compounds is sufficient for the induction of stigmasterol biosynthesis in leaves.

A specific hallmark of the incompatible *P. syringae*-Arabidopsis interaction is the occurrence of an oxidative burst between 3 and 10 hpi, which is the consequence of R protein-mediated recognition of a pathogen-derived avirulence factor (Lamb and Dixon, 1997; Zeier, 2005). The Arabidopsis respiratory burst oxidase homologue *AtrbohD* has been shown to be required for the *Pst avrRpm1*-triggered oxidative burst, with the related *AtrbohF* gene exerting a minor impact (Torres *et al.*, 2002). A very similar statement can be made for the *Psm avrRpm1*-Col-0 interaction because *atrbohD* mutant plants are almost fully impaired in the early H_2O_2 burst at 4 hpi, whereas *atrbohF* shows only a slightly diminished H_2O_2 accumulation (Fig. S2a). Both *atrbohD* and *atrbohF*, however, are able to significantly increase their leaf stigmasterol levels after *Psm avrRpm1* inoculation, indicating that the early oxidative burst following avirulence protein recognition plays a minor role as a trigger for stigmasterol synthesis (Fig. 6e).

6.3.5 Stigmasterol accumulation in *Botrytis cinerea*-infected leaves does not influence resistance against the fungal necrotroph

P. syringae is usually considered as a biotrophic or hemibiotrophic phytopathogen (Glazebrook, 2005). To investigate whether β-sito- to stigmasterol conversion also plays a role in interactions of plants with pathogens undertaking a typically necrotrophic lifestyle, we performed leaf infection experiments with *Botrytis cinerea*, a fungus able to cause soft rot disease on more than 200 plant species including Arabidopsis. Inoculation of Arabidopsis leaves with *B. cinerea* spores results in an early production of ROS which is required for pathogenicity of the fungal necrotroph (Govrin and Levine, 2000).

Spray inoculation of Col-0 leaves with *B. cinerea* strain B05.10 results in a marked increase of stigmasterol levels at 48 hpi quantitatively similar to the elevation detected after *P. syringae*-infection. Comparatively, stigmasterol accumulation is strongly attenuated in leaves of *B. cinerea*-infected *cyp710A1-1* plants (Fig. 7a). To test whether the induced production of stigmasterol would influence the plants' susceptibility to *B. cinerea*, we droplet-inoculated the centre of Col-0 and *cyp710A1-1* leaves and followed the extent of tissue maceration, which manifested itself as radial outgrowing lesions (Stefanato *et al.*, 2009). No apparent differences in the extent of disease lesions were obvious, however, and the lesion diameters in Col-0 and *cyp710A1-1* leaves at 4 dpi show no quantitative differences (Fig. 7b). Thus, although *B. cinerea*-infection induces sterol C22-desaturation in Arabidopsis leaves, the accumulating stigmasterol does not influence resistance against the necrotrophic fungus.

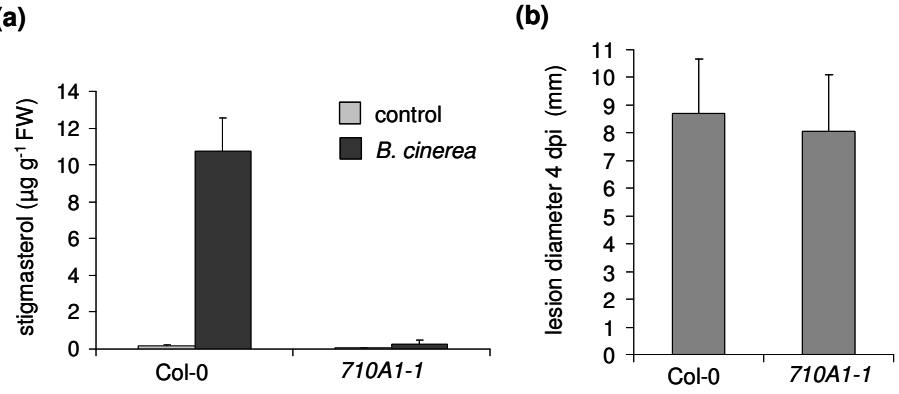

Figure 7. Stigmasterol accumulates upon *Botrytis cinerea* infection in Col-0 leaves without affecting resistance to the fungal necrotroph.

(a) Stigmasterol levels in control leaves of Col-0 and *cyp710A1-1* plants and in leaves spray-inoculated with *B. cinerea* spores (48 h post treatments).

(b) Resistance of Col-0 and *cyp710A1-1* plants towards *B. cinerea* droplet-infection. The diameter of radially outgrowing lesions was determined at 4 dpi. Means ± SD of at least six independent leaf samples are given in mm.

6.3.6 Stigmasterol production negatively affects expression of the positive defence regulator FMO1

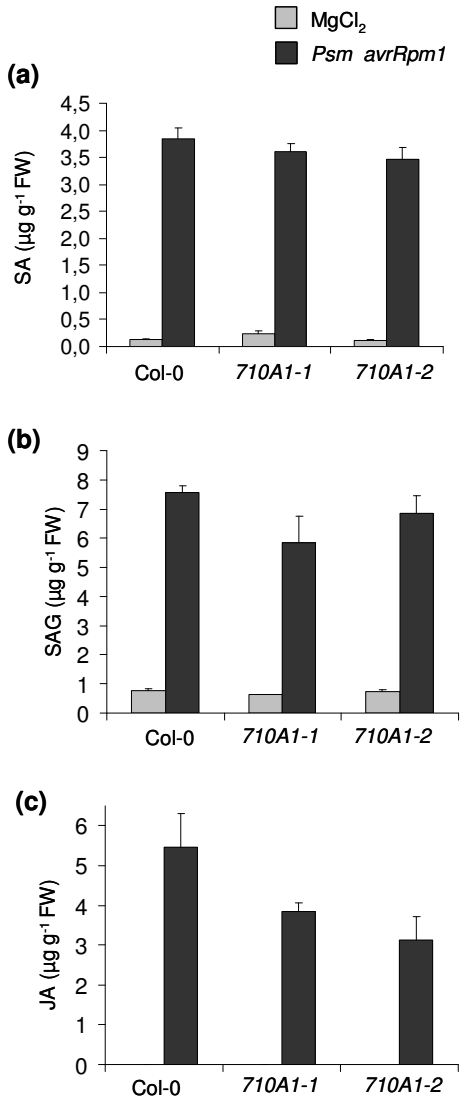

Figure 8. Defence responses in Col-0 and *cyp710A1* plants. Accumulation of defence-related metabolites in leaves after *Psm avrRpm1* (OD = 0.005) inoculation (dark bars) or $MgCl_2$-treatment (light bars).
(a) Salicylic acid (SA) levels at 10 hpi.
(b) Levels of glucoside-bound SA (SAG) at 10 hpi.
(c) Jasmonic acid (JA) levels at 10 hpi.

To better understand the basis of the elevated resistance of *cyp710A1* towards *P. syringae* infection, we comparatively examined the extent of inducible defence responses in Col-0 and *cyp710A1* plants. Since the SA-associated defence pathway significantly contributes to restrict *P. syringae* multiplication in leaves (Nawrath and Métraux, 1999), we first analyzed SA accumulation and expression of the SA-inducible *PR-1* gene. Compared to the wild-type, neither of the *cyp710A1* mutant lines exhibits constitutively elevated levels of free SA, glycosidic SA, or *PR-1* transcripts, excluding that the enhanced resistance phenotype of *cyp710A1* would rely on a constitutive activation of SA defence signalling (Figs. 8a, 8b, 9a). Furthermore, upon *Psm avrRpm1* inoculation, Col-0, *cyp710A1-1*, and *cyp710A1-2* plants induced the synthesis of free and glycosidic SA, and the expression of *PR-1* to similar levels, suggesting that the SA pathway is not hyper-activated in *cyp710A1* mutants after pathogen contact (Figs. 8a, 8b, 9a). The same statement is true for the oxidative burst, JA accumulation, and the hypersensitive cell death response following *Psm avrRpm1* inoculation, because Col-0 and *cyp710A1* mutant plants induced these responses to similar degrees (Figs. 8c, 9c, S3a, S3b).

The flavin-dependent monooxygenase FMO1 positively regulates Arabidopsis pathogen resistance by a yet unknown mechanism (Bartsch *et al.*, 2006; Mishina and Zeier, 2006). Expression of *FMO1* in pathogen-inoculated tissue occurs independently of SA signaling, and over-expression of *FMO1* in Arabidopsis is sufficient to enhance plant resistance to *P. syringae* and to *Hyaloperonospora arabidopsidis* (Koch *et al.*, 2006; Bartsch *et al.*, 2006). We found that induced expression of *FMO1* at 24 hpi is significantly higher in *Psm avrRpm1*-inoculated leaves of *cyp710A1* mutant plants than in Col-0 plants (Fig. 9b), indicating that stigmasterol accumulation in Col-0 attenuates an FMO1-dependent resistance pathway and by this means elevates disease susceptibility.

A well-described response of plant cells upon perception of bacterial (e.g. flagellin, elongation factor Tu) or fungal (e.g. ergosterol, chitin) elicitors is extracellular alkalinization, which has been measured in the growth medium of suspension-cultured cells or directly in the leaf apoplast (Granado *et al.*, 1995; Felix *et al.*, 1999; Kunze *et al.*, 2004; Felle *et al.*, 2005). The fungal sterol ergosterol triggers extracellular alkalinization in tomato cells in picomolar concentrations. The side chain double bond at C22 is a common structural element of ergosterol and stigmasterol. In contrast to other plant or animal sterols, stigmasterol also elicits medium alkalinization in tomato suspension cells, although the required concentrations are in the low micromolar range and thus much higher than for fungal ergosterol (Granado *et al.*, 1995). This prompted us to test whether plant-derived stigmasterol synthesized after pathogen attack would directly act as an elicitor and contribute to a medium alkalinization response in *P. syringae*-inoculated Arabidopsis leaves. We therefore determined the pH values of apoplastic washing fluids obtained from leaves after mock-, *Psm avrRpm1* or *Psm*-treatment. Inoculations with both *Psm avrRpm1* and *Psm* trigger a marked pH increase of 0.4-0.5 units in apoplastic fluids from both Col-0 and *cyp710A1-1* leaves, with no statistically significant differences between Col-0 and *cyp710A1-1* (Fig. 9d). This suggests that *P. syringae* inoculation evokes an apoplastic alkalinization of Arabidopsis leaves independently of stigmasterol accumulation and that stigmasterol is not a direct elicitor of the alkalinization response.

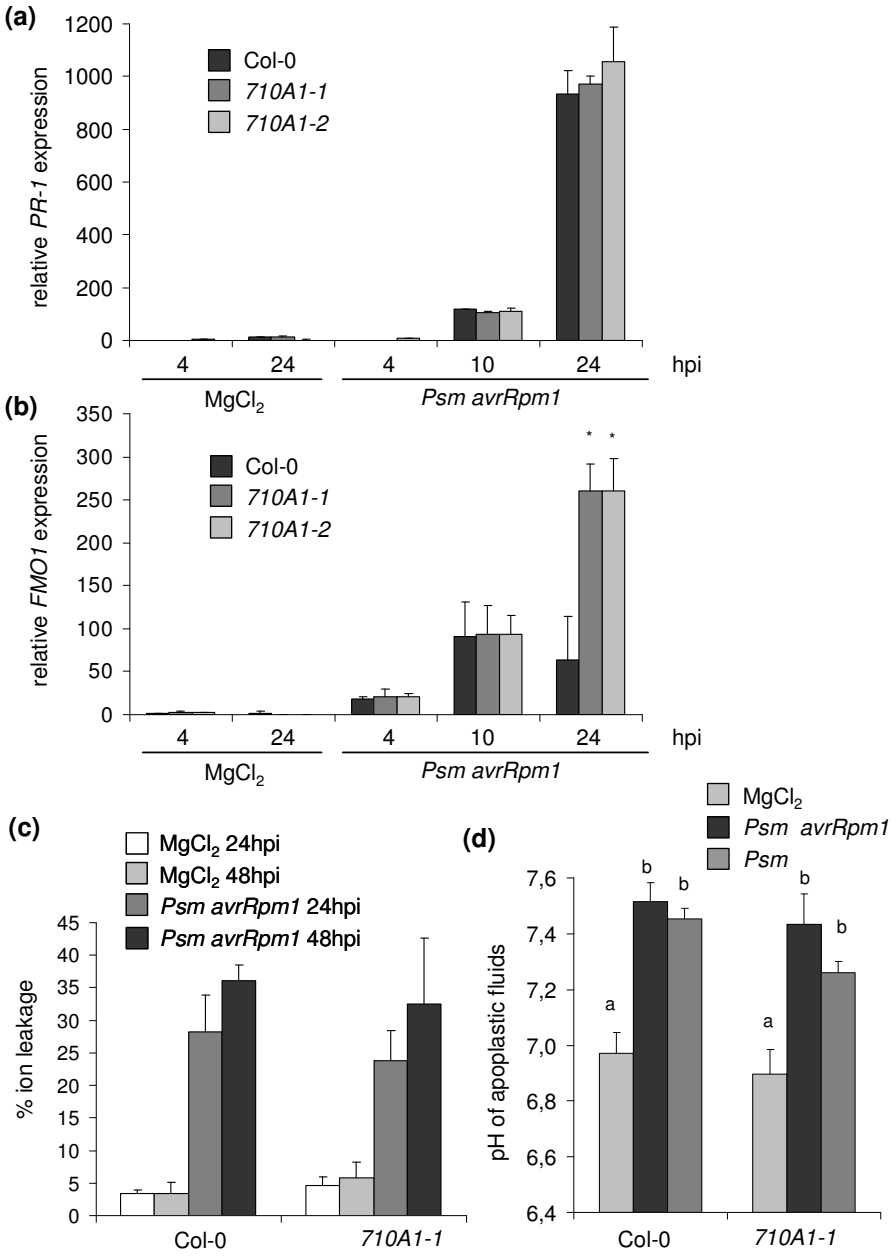

Figure 9. Defence responses in Col-0 and *cyp710A1* plants. Induced expression of defence-related genes, ion leakage from leaves, and alkalinization of apoplastic fluids after *P. syringae*-inoculation.

(a, b) Relative expression of defence-related genes, as assessed by quantitative real-time PCR analyses (see Fig. 3a for details).

(a) Relative expression of the SA-inducible gene *PR-1*.

(b) Relative expression of *FLAVIN-DEPENDENT-MONOOXYGENASE1* (*FMO1*).

(c) Ion leakage from leaves at the indicated times after $MgCl_2$- or *Psm avrRpm1*-treatment to assess the hypersensitive cell death response. The measured values are expressed in % of the values obtained after leaf boiling (100 % values). Means (± SD) of four independent samples are given.

(d) pH values of apoplastic washing fluids from leaves at 2 d post treatment with 10 mM $MgCl_2$, *Psm*, or *Psm avrRpm1*. Bars represent mean values (± SD) of three independent samples. Different characters symbolize statistically significant differences ($P < 0.05$).

6.3.6. Pathogen-induced stigmasterol is integrated into plant membranes

Since sterols are known as characteristic constituents of biological membranes, we investigated whether the pathogen-induced accumulation of stigmasterol in leaves would manifest itself in a higher content of the C22-unsaturated sterol in isolated plant membrane fractions after inoculation. We therefore isolated microsomal membrane fractions by leaf homogenizing and ultracentrifugation, and subsequently enriched these fractions for plasma membranes (PMs) by two-phase partitioning (Laloi *et al.*, 2007). Microsomal and PM isolates were then subject to chloroform/methanol extraction to determine their sterol composition by GC/MS. Similar to the situation in whole-leaf extracts (Fig. 3c), the stigmasterol/β-sitosterol ratios strongly increase in both the microsomal and the PM fractions of Col-0 leaves upon *Psm avrRpm1* inoculation from about 0.002 to 0.06, whereas the ratios in microsomal or PM isolates from *cyp710A1-1* leaves remain at basal values (Fig. 10a, b). This indicates that the stigmasterol synthesized after pathogen contact in wild-type leaves is predominantly incorporated into plant membranes.

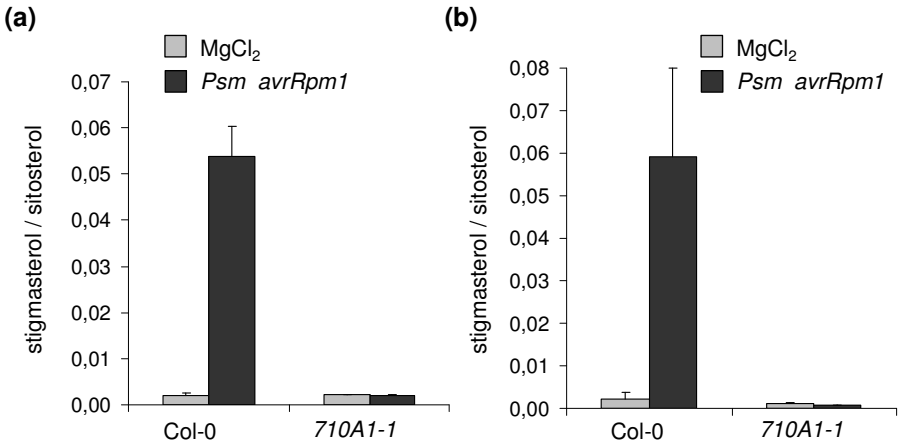

Figure 10. Stigmasterol/β-sitosterol ratios in microsomal membrane pellets and plasma membrane fractions isolated from Col-0 and *cyp710A1-1* leaves at 48 h post mock- or *Psm avrRpm1*-treatment. Means ± SD from three independent samples are given.

(a) Microsomal membrane isolates.

(b) Plasma membrane fractions isolated thereof.

6.4 Discussion

We describe here the conversion of β-sitosterol to stigmasterol as a plant metabolic process triggered after bacterial and fungal pathogen infection. During the interaction of Arabidopsis plants with virulent and avirulent *Pseudomonas syringae* strains, stigmasterol is produced in leaves between 10 h and 48 h after inoculation and reaches levels of about 15 µg g^{-1} FW (Fig. 2a). Comparatively, rises in total levels of the defence hormone salicylic acid (i.e. the sum of free SA, glycoside-bound SA, and MeSA) in *P. syringae*-inoculated Col-0 leaves amount to 10-15 µg g^{-1} FW at 48 hpi under our routine experimental conditions (Mishina *et*

al., 2008; Attaran *et al.*, 2009). Thus, the *P. syringae*-induced accumulation of leaf stigmasterol is quantitatively similar to the elevation of total SA, illustrating that, on a quantitative basis, the C22-desaturation of β-sitosterol represents a considerable metabolic process in *P. syringae*-challenged Arabidopsis leaves. Stigmasterol production is not specifically evoked upon leaf inoculation with the hemibiotrophic bacterium *P. syringae*, but also after infection with the necrotrophic fungus *Botrytis cinerea* (Fig. 7a). Since the *CYP710A1* gene is also up-regulated in Arabidopsis leaves infected with the biotrophic fungus *Golovinomyces cichoracearum* or the hemibiotrophic oomycete *Phytophtora infestans* [Fabro *et al.*, 2008; Genevestigator microarray analysis (Zimmermann *et al.*, 2004)], it is likely that stigmasterol is produced in a range of mechanistically different plant-pathogen interactions.

In recombinant protein assays and in *in planta* overexpression studies, the CYP710A family members CYP710A1, CYP710A2, and CYP710A4 are able to catalyze the C22-desaturation of β-sitosterol to stigmasterol (Morikawa *et al.*, 2006; Arnqvist *et al.*, 2008). Our *cyp710A1-1* and *cyp710A1-2* mutant analyses show that CYP710A1 is predominantly, if not exclusively, responsible for the pathogen-induced synthesis of stigmasterol in Arabidopsis leaves (Figs. 3, 7a, 10). This is explicable by the fact that *CYP710A1* is strongly up-regulated upon pathogen infection (Figs. 1a, S1a), whereas *CYP710A2* and *CYP710A4* are not pathogen-responsive and expressed in leaves at comparatively low basal levels (Fig. S1b, c). CYP710A-mediated C22-desaturation of β-sitosterol to stigmasterol is evolutionary conserved among land plants, since several functional isoforms of different higher plants and of the moss *Physcomitrella patens* have been characterized (Morikawa *et al.*, 2009). Interestingly, whereas stigmasterol is a minor sterol in leaves of unstressed higher plants, it is the major sterol in *P. patens*, suggesting taxonomical differences of the stigmasterol/β-sitosterol ratio which might have functional implications (Morikawa *et al.*, 2009). However, the biological relevance of β-sitosterol C22-desaturation in higher and lower plants is not clear until now.

Despite its partial conversion to stigmasterol, the levels of the CYP710A1 substrate β-sitosterol do not decrease but essentially remain constant after pathogen inoculation (Fig. 2b). It is thus likely that the proportion of β-sitosterol desaturated after pathogen contact is replenished through the sterol biosynthetic pathway. The first pathway-specific reaction of the sterol branch of the isoprenoid pathway in all eukaryotes is catalysed by squalene synthase, which converts two molecules of farnesyl diphosphate into the linear C30 terpenoid squalene. This is followed by epoxidation of squalene to 2,3-oxidosqualene through squalene epoxidase (Benveniste, 2004). Until recently, the next step was believed to principally differ in photosynthetic and non-photosynthetic eukaryotes. Whereas 2,3-oxidosqualene is known to be converted to lanosterol in fungi and animals, it has been

assumed to be specifically cyclized into cycloartenol in plants (Schaller, 2004). However, Ohyama *et al.* (2009) recently identified a LANOSTEROL SYNTHASE1 (LAS1) in Arabidopsis and described that higher plants possess a dual biosynthetic pathway leading to phytosterols, in which the major part of the metabolic flux occurs via cycloartenol, and a minor part takes place via LAS1 and lanosterol. Several enzymatic steps involving methyl transferases, reductases, isomerases, demethylases and desaturases are required to convert cycloartenol or lanosterol to β-sitosterol (Schaller, 2004). Publicly available microarray data indicate that, besides *CYP710A1*, only a few Arabidopsis genes supposedly involved in phytosterol biosynthesis (listed in Benveniste, 2004) are up-regulated in leaves upon *P. syringae* inoculation: the expression of the two squalene epoxidase isoforms *SQE5* (At5g24150) and *SQE6* (At5g24160), as well as *LAS1* (At3g45130) are moderately enhanced at 24 h post infection with avirulent or virulent *Pst* strains (Fig. S4). It is thus possible that a replenishment of the β-sitosterol pool after its partial, pathogen-induced conversion to stigmasterol takes place via enhanced expression of squalene epoxidase and lanosterol synthase genes, and thus proceeds via the recently discovered plant lanosterol pathway.

Our data reveal that induced stigmasterol formation is independent of the SA- and JA/ET defence pathways (Fig. 6a). Moreover, exogenous application of two typical bacterial PAMPs, flagellin and LPS, are a suffcient trigger for stigmasterol production (Fig. 5b). Stigmasterol also accumulates when Arabidopsis leaves are exogenously supplied with ROS-generating substances (Fig. 6c, d). During the incompatible Arabidopsis-*Psm avrRpm1* interaction, ROS are massively produced around 4 h post infection, and this early oxidative burst is triggered via recognition of the bacterial effector avrRpm1 by the plant resistance protein Rpm1 (Fig. S2a; Bisgrove *et al.*, 1994). By contrast, the early oxidative burst is absent in the compatible Arabidopsis-*Psm* interaction (Fig. S2b). Our finding that stigmasterol is produced with similar kinetics in response to the incompatible and the compatible *Psm (± avrRpm1)* strain between 10 and 48 hpi (Fig. 2a) argues against a marked function for the early oxidative burst as a trigger of stigmasterol biosynthesis. This statement is corroborated by the fact that *atrbohD* mutants, in which the early *Psm avrRpm1*-induced H_2O_2 accumulation is strongly attenuated (Fig. S2a), exhibit a wild-type-like stigmasterol production after avirulent *P. syringae* inoculation. Both avirulent and virulent *P. syringae* possess bacterial PAMPs, and PAMP perception is known to initiate the endogenous generation of ROS (Felix *et al.*, 1999; Meyer *et al.*, 2001). Adapted *P. syringae* bacteria strongly multiply in the leaf apoplast between 10 and 48 hpi (Katagiri *et al.*, 2002; Mishina and Zeier, 2007b), and the concomitant exposure to bacterial PAMPs might give rise to a continuous production of ROS at later infection times. Consistent with this assumption, non-enzymatically produced oxylipins, whose formation mirrors increases in ROS, are

formed in later stages of both the incompatible and the compatible Arabidopsis-*P. syringae* interaction (Grun *et al.*, 2007). We thus propose a persistent, PAMP-induced ROS formation at later stages (between 10 and 48hpi) after *P. syringae* inoculation as a likely stimulus for β-sitosterol C22-desaturation. A similar ROS trigger probably also exists in *B. cinerea*–infected leaves, since the necrotrophic fungus evokes ROS formation during infection (Govrin and Levine, 2000).

We have shown that the induced accumulation of leaf stigmasterol favours apoplastic *P. syringae* multiplication and thus leads to enhanced disease susceptibility (Fig. 4a, b, c). The elevated levels of stigmasterol do not influence the SA and JA defence pathways but attenuate expression of the flavin-dependent monooxygenase *FMO1* which acts as a positive regulator of Arabidopsis disease resistance (Figs. 8, 9; Bartsch *et al.*, 2006; Koch *et al.*, 2006; Mishina and Zeier, 2006; Schlaich, 2007). Therefore, the pathogen-induced elevation of stigmasterol in leaves obviously suppresses a specific, FMO1-associated resistance pathway, and this might be sufficient for resistance attenuation. Stigmasterol does not act as a direct elicitor of the extracellular alkalinization response evoked by *P. syringae* (Fig. 9d). Furthermore, the enhanced stigmasterol/β-sitosterol ratio in leaves manifests itself also in microsomal membrane isolates and in the plasma membrane fraction obtained thereof (Fig. 10). This suggests that the stigmasterol produced after pathogen inoculation is integrated into plant membranes and thus alters the membrane sterol composition. Since a varying ratio of individual sterols can alter the physical characteristics of lipid bilayers (Hartmann, 1998), we propose that the negative effect of induced stigmasterol production on resistance relies on an influence on the biophysical properties of plant membranes rather than on a direct signalling effect of free sterol molecules. Our findings that stigmasterol accumulation modulates resistance to *P. syringae* but not to *B. cinerea* might be related to the different lifestyles of the two pathogens, which are hemibiotrophic and necrotrophic, respectively.

Arabidopsis plants develop SAR in upper leaves about two days after the inoculation of lower leaves with avirulent or virulent *P. syringae* (Cameron *et al.*, 1994; Mishina and Zeier, 2007b; Mishina *et al.*, 2008). The putative lipid transfer protein (LTP) DIR1 is an essential SAR component that is necessary for the generation or translocation of a mobile long-distance signal (Maldonado *et al.*, 2002). DIR1 was suggested to act as a chaperone for a potential lipid signal (Grant and Lamb, 2006). Sterols might be good candidates for LTP-transportable lipids because they can bind to elicitins, small cystein-rich fungal proteins with structural similarity to plant LTPs (Mikes *et al.*, 1998). In this context, it is interesting to note that stigmasterol formation and SAR establishment are induced by similar molecular determinants: PAMPs and ROS (Fig. 5b, Fig. 6b, c; Mishina and Zeier, 2007b; Alvarez *et al.*, 1998). Thus, we initially considered stigmasterol, which accumulates at the site of pathogen infection, as a good candidate for a mobile SAR signal. However, the SAR-positive

phenotype of *cyp710A1* mutant plants clearly argues against a role for stigmasterol during SAR. Moreover, our finding that stigmasterol levels are not elevated in systemic, non-infected leaves corroborates that the sterol is only involved in the modulation of local resistance but not in SAR (Fig. 5a).

How might an increased stigmasterol/β-sitosterol ratio in the membrane affect plant disease resistance? Compared to β-sitosterol, stigmasterol bears an additional double bond at the sterol side chain. This renders the alkyl chain less flexible and therefore influences the solubility, packing properties, and the ability to accommodate in lipid bilayers. Experimental evidence exists that incorporation of stigmasterol in di- and monosaturated bilayers exerts a lower ordering effect on the bilayer hydrocarbon chains than integration of β-sitosterol (Hodzic *et al.*, 2008). Likewise, stigmasterol reduces the permeability of soybean phosphatidylcholine bilayers much less efficiently than β-sitosterol (Schuler *et al.*, 1991). Distinct sterol molecules might also differently affect the activities of membrane-situated enzymes (Hartmann, 1998). For instance, stigmasterol was found to stimulate the activity of maize root H^+-ATPase, whereas its C22-saturated counterpart β-sitosterol did not (Grandmougin-Ferjani *et al.*, 1997). Several reports propose the existence of sterol- and sphingolipid-enriched membrane microdomains in plants which can be isolated as detergent-resistant membranes (Mongrand *et al.*, 2004; Borner *et al.*, 2005; Bhat and Panstruga, 2005; Laloi *et al.*, 2007). These so-called lipid rafts might play a role as platforms for the recruitment of molecular components involved in plant defence signalling (Bhat *et al.*, 2005). A prerequisite for the existence of membrane rafts is the ability of sterols to induce liquid-ordered membrane phases (Zappel and Panstruga, 2008). Whether individual phytosterols have different microdomain-inducing abilities is not yet established. However, it is conceivable that a pathogen-induced change in the stigmasterol/β-sitosterol ratio can influence the number and/or the physicochemical properties of ordered membrane (micro)domains and thereby modulate plant defence signalling.

6.5 Experimental procedures

6.5.1 Plant material and growth conditions

Arabidopsis thaliana L. Heynh. (Arabidopsis) plants were grown on an autoclaved mixture of soil (Einheitserde Typ P, Gebr. Patzer GmbH, Sinntal-Jossa, Germany) vermiculite and sand (10:0.5:0.5) and kept in a controlled environmental chamber (J-66LQ4, Percival) with a 9 h day period (temperature 21 °C), a 15 h night period (temperature 18 °C), and a relative humidity of 70%. All experiments were done with 6-week-old, naïve and unstressed plants exhibiting a uniform appearance.

The *cyp710A1-1* and *cyp710A1-2* mutants which correspond to the SALK T-DNA insertion lines SALK_112491 and SALK_014626, respectively, both have Col-0 background. To identify homozygous T-DNA insertion lines by PCR, the method described by Alonso *et al.* (2003) was applied using the following gene specific primers: *CYP710A1-1*-forward, 5'-CAAATTGCAATGGTATACCGG-3'; *CYP710A1-1*-reverse, 5'-TTGTGTTTTATACGACATTA-CTCGTG-3'; *CYP710A1-2*-forward, 5'-AGAGAGTTGCTCGACGAGAAG-3'; *CYP710A1-2*-reverse, 5'-GCGCTATACGC-AACTTGAAAC-3'. T-DNA specific primers were equivalent to those proposed by Alonso *et al.* (2003).

Further experiments were performed with the following mutants: *sid2-1* (Nawrath and Métraux, 1999), *npr1-2* (NASC ID: N3801), *fmo1* (Mishina and Zeier 2006), *ndr1* (Century *et al.*, 1995), *pad4-1* (Glazebrook *et al.*, 1997), *dde2-2* (von Malek *et al.*, 2002), *jar1-1* (Staswick *et al.*, 1992), *etr1* (Bleecker *et al.*, 1988), *cpr5* (Bowling *et al.*, 1997), *dnd1* (Yu *et al.*, 1998), *rbohD* and *rbohF* (Torres *et al.*, 2002). Wild-type plants in the Col-0 background were used as control plants unless otherwise stated.

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6.5.2 Growth and inoculation of *Pseudomonas syringae*

The following *Pseudomonas syringae* strains were used for inoculation experiments: *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) and *Pseudomonas syringae* pv. *tomato* (*Pst*) lacking (*Psm, Pst*) or harbouring the *avrRpm1* avirulence gene *(Psm avrRpm1, Pst avrRpm1*), a *Psm* strain harbouring the *avrRpt2* avirulence gene (*Psm avrRpt2)*, a *Pst* strain defective in the *hrpA*$^-$-gene (*Pst hrpA*$^-$), and *Pseudomonas syringae* pv. *glycinea* race 4. All *P. syringae* strains were grown at 28 °C in King's B medium containing appropriate antibiotics. Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to a final optical density (OD) of 0.005 for determination of local gene expression, local metabolite levels, SAR induction and pH determination of apoplastic washing fluids, and 0.002 for bacterial growth assays of virulent and avirulent strains (*Psm, Pst, Psm avrRpm1, Psm avrRpt2, Pst avrRpm1*). The non-adapted *Psg* and the TTSS-deficient strain *Pst hrpA*$^-$ were always applied in bacterial titers of OD 0.1. For ion leakage studies, suspensions of *Psm avrRpm1* in titers of OD 0.1 were used.

The bacterial suspensions were infiltrated via the abaxial leaf side using a 1-ml syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed 3 d after infiltration by homogenising discs originated from infiltrated areas of three different leaves in 1 ml 10 mM $MgCl_2$, plating of appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28 °C for 2 d.

6.5.3 Growth and inoculations of *Botrytis cinerea*

The *Botrytis cinerea* strains B05.10 was cultured on potato dextrose agar (Stefanato *et al*., 2009). Plates were flooded with 0.05 % Tween 80 and the spores mechanically scraped off. Spores were purified from mycelium, transferred into sterile water, and concentration of spores was determined. For inoculations, spores were transferred in quarter strength potato broth in a concentration of 2.5 x 10^5 ml^{-1}. For the determination of phytosterols, plants were sprayed with the spore suspension. For the assessment of symptom development, 2 µl droplets of the same suspension were placed on the upper leaf sides. The diameter of the lesions was determined after 4 days. Quarter strength potato broth was used as control treatment in both assays.

6.5.4 flg22 and LPS treatments

The flg22 peptide and purified LPS from *E. coli* and *P. aeruginosa* were diluted in 10 mM $MgCl_2$ to final concentrations of 200 nM (flg22) and 100 µg ml^{-1} (LPS), and infiltrated into leaves. The flg22 peptide, representing the elicitor active domain of bacterial flagellin (Felix *et al*., 1999), was synthesized by Mimotopes (www.mimotopes.com). The chromatographically purified LPS preparations were acquired from Sigma-Aldrich (www.sigma-aldrich.com; L3024, L8643). Control infiltrations were performed with 10 mM $MgCl_2$.

6.5.5 Copper sulphate and Xanthine/Xanthine oxidase treatments

Copper sulphate (Merck, Darmstadt, Germany) was infiltrated into the sample leaves in a concentration of 10 mM to induce high oxidative stress in the leaves. To investigate the influence of ROS, the O_2^--producing combination of xanthine (X) and xanthine oxidase (XO) was applied in a concentration of 0.5 mM X and 0.5 units ml^{-1} XO in 20 mM sodium phosphate buffer (pH 6.5). XO from buttermilk and X were obtained form Sigma-Aldrich (www.sigma-aldrich.com). Control treatments were performed with deionised H_2O (for copper sulphate), and sodium phosphate, as well as single X or XO treatments (for X/XO).

6.5.6 Determination of phytosterol contents

For the determination of stigmasterol and β-sitosterol, plant lipids were extracted from 120 mg (FW) of frozen leaf samples. Leaf tissue was homogenized with methanol:chloroform (2:1, v/v), internal standard (10 µg ergosterol) was added, and lipids were extracted for 0.5 h at 70 °C. After addition of 500 µl of H_2O, the mixture was thoroughly shaken and centrifuged for phase separation. The lower, organic phase was removed and the phase separation step

was repeated. The collected organic phases were combined, dried over Na_2SO_4, and the volume was reduced in a stream of nitrogen. Samples were derivatised for gas chromatography by adding of 40 µl of pyridine and 40 µl of BSTFA (N,N-bis-trimethylsilyltrifluoroacetamide) and subjected to a vapour-phase extraction procedure according to Mishina and Zeier (2006), using volatile collector traps packed with Super-Q absorbent (VCT1/4X3-SPQ; Analytical Research Systems) and a final evaporation temperature of 200 °C for 5 minutes. Samples were eluted from the collector trap by addition of 1 ml methylene chloride. The sample volume was reduced with nitrogen to 40 µl and subject to gas chromatography-mass spectrometry analysis. 2 µl of the sample were separated on a gas chromatograph (GC 6890 N; Agilent Technologies; www.agilent.com) with a silica capillary column (ZB-1ms, 30 m length, 0.25 mm internal diameter, 0.25 µm film thickness; www.phenomenex.com) and a 5975 mass spectrometry detector (Agilent Technologies; www.agilent.com). For the quantification of sterols, peaks descending from selected ion chromatograms (m/z 486 for β-sitosterol, m/z 484 for stigmasterol, m/z 468 for ergosterol) were integrated and the corresponding peak areas were related to the peak area of the internal standard ergosterol. Experimentally determined correction factors for each sterol/standard combination were considered.

6.5.7 Analysis of gene expression

For analysis of gene expression, RNA samples and the corresponding cDNA were prepared from frozen leaves as outlined in detail by Mishina and Zeier (2006).

Pathogen-induced expression of *CYP710A1* (At2g34500; Fig. 1A) in wild-type leaves was determined by reverse-transcription PCR also according to Mishina and Zeier (2006). The following primers were used for PCR: 5'-ATTAGATACAGGCCTCCTGCAA-3' (*CYP710A1-forward*), 5'-CAAAAGCTAGGAA-GTTGCGTTT-3' (*CYP710A1*-reverse). The *ACTIN2* gene (At3g18780) was amplified as a control with the primers 5'-TCGCCATCCAAGCTGTTCTCT-3' (*ACT2*-forward), 5'-CCTGGACCTGCCTCATACTC-3' (*ACT2*-reverse). Gene expression in other experiments was investigated using quantitative real-time PCR as described in detail by Attaran *et al.* (2009), using the SensiMIxPlus SYBR Green kit (Quantance) and the Rotor-Gene 2000 (Corbett Research). The expression levels of *CYP710A1* (At2g34500), *PR-1* (At2g14610), and *FMO1* (At1g19250) were analysed with the following gene specific primers: 5'-AAGAAGCTCTTCGGTGACCA-3' (*710A1*-forward), 5'-GCTGGAGGGCAGAGTAAGTG-3' (*710A1*-reverse), 5'-GTGCTCTTGTTCTTCCCTCG-3' (*PR-1*-forward), 5'-GCCTGGT-TGTGAACCCTTAG-3' (*PR-1*-reverse), 5'-TCTTCTGCGTGCCGTAGTTTC-3' (*FMO1*-forward), 5'-CGCCATTTGACAAGAAGCATAG-3' (*FMO1*-reverse). The *UBQ10* gene (At4g05320), which is non-responsive to *P. syringae*-inoculation, was used as the reference gene (Czechowski *et al.*, 2005). Data were analysed using Rotor-Gene 6000 software and

values were normalized to those for the reference gene and relative to the $MgCl_2$-treated wild-type control sample.

6.5.8 Characterization of systemic acquired resistance

Three lower leaves of a given plant were first infiltrated with a suspension of *Psm* (OD 0.005), or with 10 mM $MgCl_2$ as a control. Two days after this primary inoculation, non-treated upper leaves were harvested for SA determination, or plants were inoculated on three upper leaves with *Psm* (OD 0.002). Growth of *Psm* in upper leaves was assessed 3 d later.

6.5.9 Determination of defence metabolites

Determination of free SA, glycosidic SA, and jasmonic acid levels in leaves was performed using vapour-phase extraction and subsequent gas chromatographic/ mass spectrometric analysis according to Mishina and Zeier (2006).

6.5.10 Assessment of the HR by ion leakage

Infected and control leaves were cut from plants and washed with deionized water. Individual leaves were put in small glass vessels filled with deionized water and slightly shaken for 2 hours. Conductivity of the water solution was measured using the conductivity meter B173 from Horiba (HORIBA Instruments Limited, UK). Afterwards, samples were boiled for 0.5 h and the conductivity after total leaf collapse was measured. The percentage of the first measurement over the second measurement was used to assess the pathogen-induced level of membrane damage compared to the level of total damage after boiling.

6.5.11 Collection and pH determination of apoplastic washing fluids

48 h after infection, treated leaves were cut from plants and washed several times with deionized water. After covering them with fresh water, leaves were subject to vacuum infiltration (vacuum pump MZ 2C, Vaccubrand, Wertheim, Germany) for 20 minutes. Water on the leaf surfaces was removed with paper tissue and apoplastic washing fluids were collected by centrifugation of the leaves at 500*g* for 5 min. For each sample, 60 leaves from 20 different plants were used and their washing fluids combined. pH values were determined using an InoLap pH Level1 pH meter (www.wt.w.com).

6.5.12 Isolation of membranes

Microsomal membrane and plasma membrane isolates were obtained according to Laloi et al. (2007) with few modifications. Leaf material was homogenized in the presence of a buffer containing 330 mM sucrose, 1 M Tris-HCl, 0.5 M EDTA, 1 mM DTT, and complete protease inhibitor cocktail tablets (Roche, www.roche.com). The homogenate was filtrated and centrifuged at 15,000*g*. The supernatant was again centrifuged at 100,000*g* for 1 h yielding microsomal pellets which were either resuspended in 10 mM KH_2PO_4 (pH 8.2) for phytosterol determination or in a buffer containing 10 % sucrose, 6 mM KCl, 5 mM K_2HPO_4 (pH 7.8) and protease inhibitor tablets for plasma membrane isolation. Plasma membranes were isolated by two-phase partitioning of the microsomal pellet samples between 6.4 % PEG (Applichem) and 6.4 % dextran T500 (Applichem) in 3 mM KCl, 10 % sucrose, 50 mM K_2HPO_4 (pH 7.8). Centrifugation at 1,500*g* for 5 min resulted in phase partitioning with an upper PEG-enriched phase containing the plasma membranes. For purification, phase-partitioning was repeated for both phases. The PEG-enriched phase was separated and centrifuged for 1 h at 100,000*g*. The resulting plasma membrane pellets were resuspended in a buffer containing 1 mM DTT and 50 mM Tris-HCl (pH 7.4) and used for phytosterol determination.

6.5.13 Quantification of microscopic HR lesions and assessment of H_2O_2 production

The extent of microscopic HR lesion formation and H_2O_2 production were assessed by the Trypan blue and diaminobenzidine (DAB) staining procedures, respectively, which are described in Zeier *et al.* (2004).

6.5.14 Reproducibility of experiments and statistical analyses

All experiments depicted in the figures were repeated at least twice with similar results and statistical analyses were performed using Student's *t* test.

6.6 References

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6.6 Supplementary data

Fig. S1

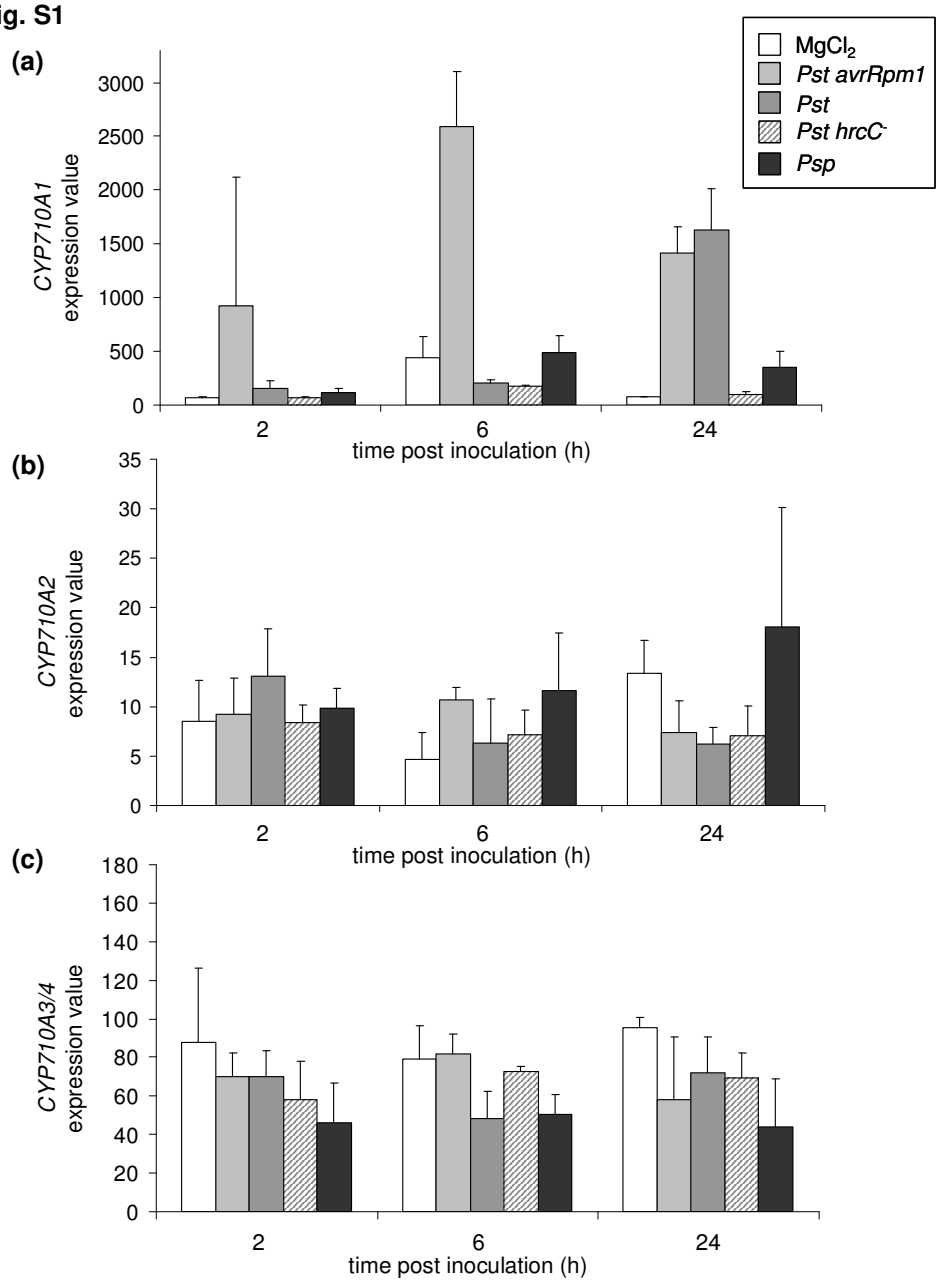

Figure S1. Expression levels of *CYP710A* isogenes in Arabidopsis leaves upon challenge with different strains of *Pseudomonas syringae* pv. *tomato* (*Pst*) or *Pseudomonas syringae* pv. *phaseolicola* (*Psp*) according to publicly available microarray analyses (TAIR-ME00331: Response to virulent, avirulent, type III secretion system deficient and nonhost bacteria). Means (± SD) of Affymetrix expression values originating from three independent replicates are shown. The data were normalized according to the Affymetrix MAS 5.0 Scaling Protocol.

(a) *CYP710A1* (gene ID At2g34500, probe ID 266995_at)

(b) *CYP710A2* (gene ID At2g34490, probe ID 266966_at)

(c) mixed *CYP710A3/CYP710A4* probe (gene IDs At2g28850/60, probe ID 266218_s_at)

Fig. S2

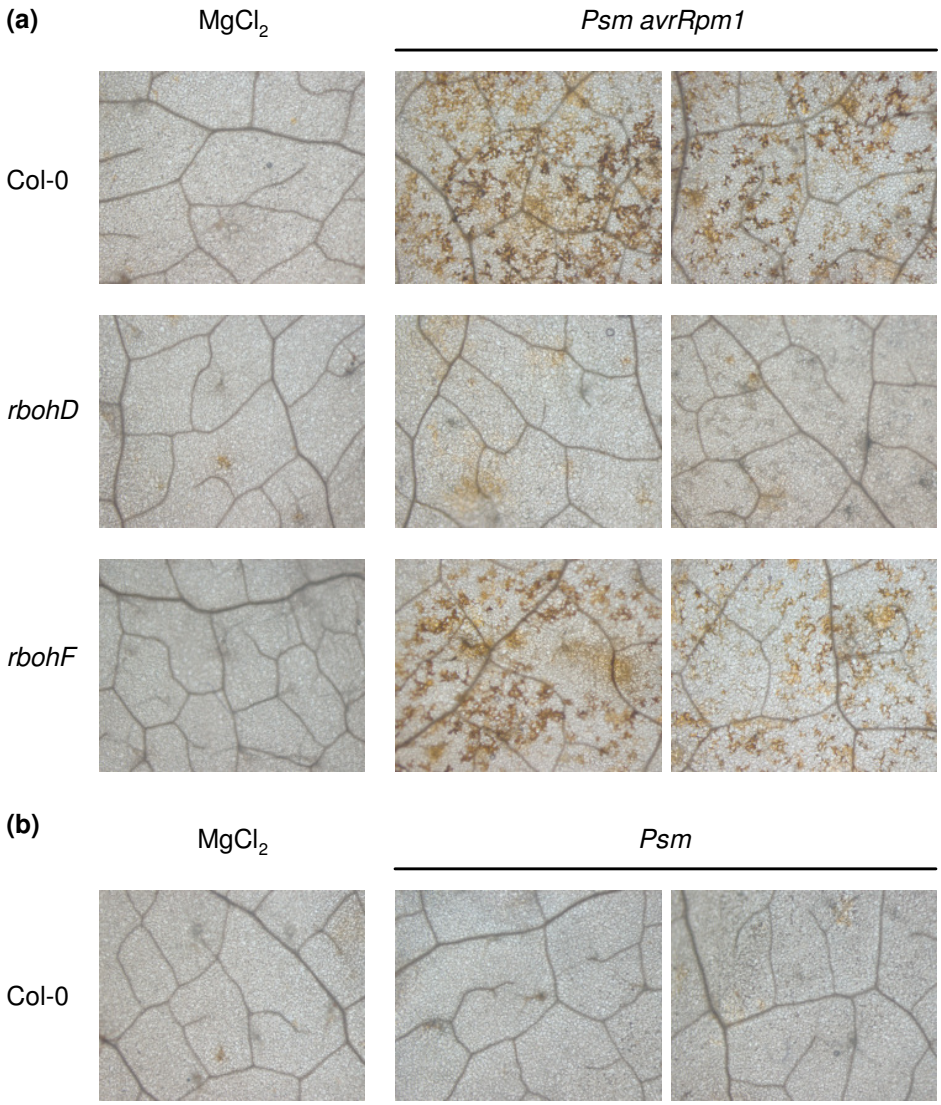

Figure S2. Diaminobenzidine (DAB) staining to assess the oxidative burst in Arabidopsis leaves upon *Psm* (±*avrRpm1*) inoculation at 4 hpi. Control leaves were infiltrated with 10 mM $MgCl_2$. A dark-brown pigmentation indicates H_2O_2 production. Staining patterns of representative leaves are shown (100-fold magnification).

(a) Col-0, *rbohD*, and *rbohF* plants inoculated with avirulent *Psm avrRpm1*.

(b) Col-0 plants inoculated with virulent *Psm*.

Fig. S3

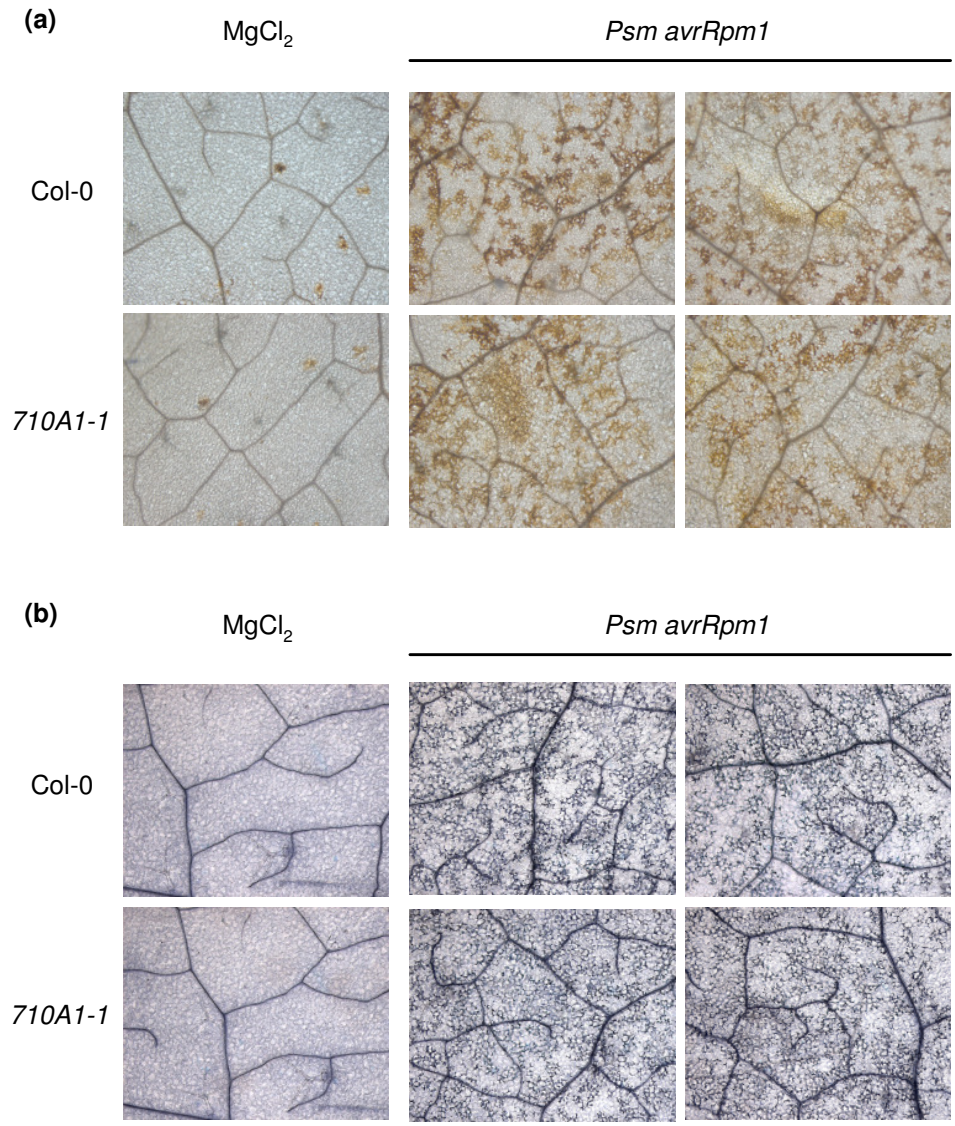

Figure S3. Assessment of the oxidative burst and microscopic HR lesions in Col-0 and *cyp710A1-1* leaves after *Psm avrRpm1* inoculation. Control leaves were infiltrated with 10 mM $MgCl_2$.

(a) DAB staining to assess the oxidative burst in leaves at 4 hpi. A dark-brown pigmentation indicates H_2O_2 production. Staining patterns of representative leaves are shown (100-fold magnification).

(b) Trypan blue staining to assess the HR in leaves at 24 hpi. A dark-blue pigmentation indicates microscopic cell death. Staining patterns of representative leaves are shown (100-fold magnification).

Fig. S4

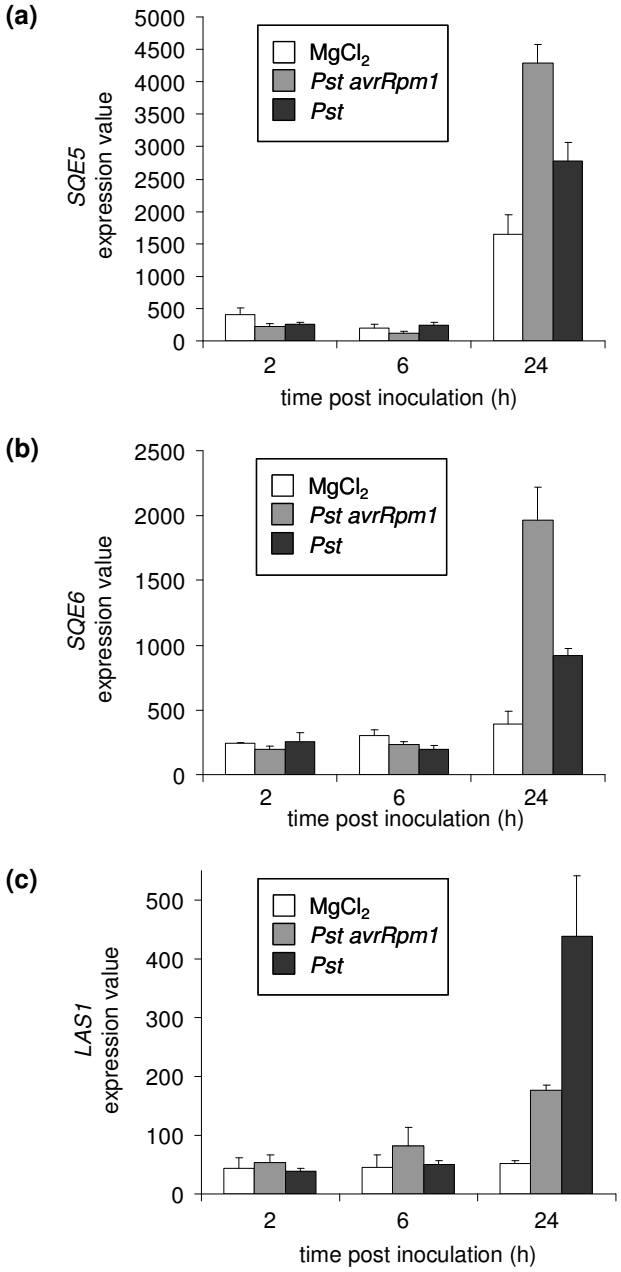

Figure S4. Expression levels of sterol biosynthesis pathway genes up-regulated in Arabidopsis leaves upon challenge with different strains of *Pseudomonas syringae* pv. *tomato* (*Pst*) according to publicly available microarray analyses (TAIR-ME00331: Response to virulent, avirulent, type III secretion system deficient and nonhost bacteria). Means (± SD) of Affymetrix expression values originating from three independent replicates are shown. The data were normalized according to the Affymetrix MAS 5.0 Scaling Protocol.

(a) *SQUALENE EPOXIDASE5* (*SQE5*; gene ID At5g24150, probe ID 249774_at)

(b) *SQUALENE EPOXIDASE6* (*SQE6*; gene ID At5g24160, probe ID 249775_at)

(c) *LANOSTEROL SYNTHASE1* (*LAS1*; gene ID At3g45130, probe ID 252611_at)

7. Discussion and perspectives

7.1 Light availability and daytime affect defence responses in plants

The first part of this thesis highlights the light dependency of plant-pathogen interactions. Light is an external factor that crucially influences metabolism, physiology, and development of plants. The availability of light is strongly connected with the survival of plants, since it is indispensable for photosynthesis and, thereby, represents a beneficial component for the plant's fitness. In the last years, many influences of light on plant resistance responses to microbial infections were observed. It was shown that the HR-associated programmed cell death, which occurs upon infection with avirulent bacteria, but also lesion formation in response to viral pathogens, are obviously light-dependent in different model plants (Guo et al., 1993; Genoud et al. 2002; Mateo et al., 2004; Zeier et al., 2004; Chandra-Shekara 2007). One study on Arapidopsis demonstrated that protoplasts treated with the fungal toxin fumosin B1 developed a higher degree of programmed cell death in the light than in darkness (Asai et al., 2000). A double mutant of *acd11* (*accelerated cell death*) and NahG, for instance, only exhibited hypersensitive cell death after treatment with BTH after light exposition of the plants (Brodersen et al., 2002). Moreover, the *lsd1* (*lesion simulating disease 1*) mutant shows a constitutive cell death phenotype under long day conditions with more than 16h of photoperiod, but not in short days (Mateo et al., 2004).

Whereas some plant responses to pathogens are not impaired in the dark, SA-associated plant defence reactions are strongly dependent on the presence of light after pathogen inoculation. Zeier et al. (2004) studied resistance responses of Arabidopsis under different light conditions: high light, medium light, and darkness (500, 70, and 0 μmol^{-2} s^{-1} PFD, respectively). In the dark, plants exhibited markedly lower resistance to the avirulent *Psm avrRpm1* strain than in the light. Moreover, plants accumulated higher levels of SA, and showed a stronger expression of the *PR-1* defence gene in response to *Psm avrRpm1*-inoculation than in darkness. Following up on these results, I have investigated the light dependency of plant defences within a day/night cycle similar to natural occurring light conditions, including a light period from 09:00 to 18:00 and a darkened night period during the remaining time. Inoculations of plants were done at four different day times: 01:00 (night), 09:00 (morning), 13:00 (midday), and 19:00 (evening). According to the length of light availability after the first hours post infection, I observed differences in the strength of SA-dependent defence responses and SA accumulation itself. Morning inoculation, for instance, resulted in high levels of SA, earlier expression of *PR-1*, stronger HR development, and enhanced resistance against *Psm avrRpm1* than inoculations in the evening or in the night.

These data indicate that the amount of SA correlates with the number of light hours available in the first ten hours after inoculation.

A relationship between pathogen resistance and the circadian clock was previously reported. Thus, a conceivable contribution of the circadian rhythm to the observed daytime-dependent tendencies should be discussed. Sauerbrunn and Schlaich (2004) demonstrated the existence of pathogenesis-related genes expressed in a rhythm according to the circadianic clock. *PCC1* (*pathogen and circadian controlled 1*), for instance, turned out to be a gene regulated by the plants' inner clock, and its overexpression resulted in resistance against otherwise virulent oomycetes. The glycine-rich RNA-binding protein 7 (GRP7) is as well controlled by the circadian clock, and a mutation lacking the gene's function results in enhanced susceptibility against *Pst DC3000* (Fu et al., 2007). The TTSS effector HopU1 causes ADP-ribosylation of GRP7 and, hereby, interferes with the transcription of genes involved in plant defence (Fu et al., 2007). GRP7 is predominantly generated in the guard cells, where it contributes to the regulation of stomatal opening and closure (Kim et al., 2008). Besides, recognition of PAMPs induces stomatal closure as well and functions in resistance against bacterial infection (Melotto et al., 2008). By using pressure infiltration of the bacterial suspensions in all experiments but especially in the daytime studies, I intended amongst others to avoide this influence of differentially opened or closed stomata, which could be caused by recognition of PAMPs or the circadian clock. To specifically examine a contribution of the circadian clock to the observed daytime-dependend variations in pathogen-induced SA accumulation, I also performed control measurements in the morning and in the evening with plants continuously kept in the dark or in the light. For instance, morning inoculations of continuously dark-kept plants give rise to only a weak accumulation of SA, which is similar to the SA produced after evening or night inoculations of plants situated in the light/dark cycle. Conversely, evening inoculation of plants kept under continuous light did not result in a lower SA accumulation than morning inoculations of plants situated in the light/dark cycle. These observations essentially rule out a contribution of the circadian clock on the observed daytime dependency of pathogen-induced SA production, and corroborate the importance of the light factor in this context. The number of available light hours during the early infection period correlates with the intensity of SA-related resistance responses, but without an apparent influence of the circadian clock. The presence of light in the early hours after pathogen inoculation critically contributes to the activation of early plant defence. Morning inoculation showed even higher SA accumulation and earlier *PR-1* gene expression than midday infection. The requirement of a certain extended light period was also reported from other pathosystems. Chandra-Shekara (2006) reported that, within the incompatible interaction of Arabidopsis accession Di-17 and *Turnip crinkle virus,* an HR or a considerable *PR-1* gene expression failed to appear when the initial light period

after inoculation was shorter than 6 hours. In summary, the data of the current work show that the light dependency of plant defence responses manifests itself not only when rather artificial situations are applied (e.g. complete darkness; Zeier et al, 2004), but also when naturally occurring light/dark cycles are included in the experimental setting.

Besides, the results of this work also indicate that pathogens could take advantage of daytime-dependent alterations in resistance by selecting night hours for infection and attack. The formation of primary haustoria of *Erysiphae graminis* f. sp. *hordei* on barley, for example, is accelerated in the darkness (Edwards, 1993). We can only speculate if this effect is caused by a reduced resistance capacity of the plant in the nighttime. Genererally, it is also conceivable that light affects the virulence of pathogens. Oberpichler et al. (2008) demonstrated reduced numbers of flagella of *Agrobacterium tumefaciens* cultered in the light. This resulted in an impaired bacterial virulence including reduced bacterial root attachment in tomato and smaller tumor formation in cucumber. Within the studies of the current work, I tried to avoid different extents of light-dependent virulence by culturing and keeping the bacterial suspensions in the dark until inoculation times.

Finally, these results enforce a strong admonition to all researchers of plant biology and phytopathology to start comparative experiments at the same daytime. In doing so, the receipt of reproducible results is promoted and the described influences of the daytime are avoided.

7.2 SAR but not local defence responses requires phytochrome signalling

According to the results and data discussed above, we can conclude that at the site of pathogen inoculation, many induced defence responses, in particular SA pathway-related responses, are light-dependent. Zeier et al. (2004) previously demonstrated that the capacity of Arabidopsis to establish SAR is also dependent on the presence of light. One aim of this project was thus to investigate in more detail how light positively regulates local and systemic resistance responses. As plants can perceive and translate light signals via photoreceptors, I studied the defence behaviour of a collection of photoreceptor double mutants: *phytochromeAphytochomeB (phyAphyB), cryptochrome1cryptochrome2 (cry1cry2), and phototropin1phototropin2 (phot1phot2).*

The results of these studies indicate that impaired photoperception did only slightly affect defence responses at inoculation sites. In response to the avirulent strain *Psm avrRpm1*, SA levels were slightly enhanced or reduced in *cry1cry2* and *phyAphyB*, respectively, but did not result in an altered resistance against this pathogen. *phyAphyB* showed an impaired basal resistance resulting in higher bacterial growth of the virulent *Psm*

strain in leaves. All tested photoreceptor mutant lines and also wild-typ plants showed similar patterns of pathogen-induced gene expression and a similar extent of HR. Contrasting to our data, Genoud et al. (2002) reported that phytochome signalling has a stronger influence on local resistance responses and is indispensable for HR. A possible reason for these divergent results might be related to the use of distinct experimental systems in the two studies. Genoud and colleagues used Arabidopsis wild-type and mutant plants within the Landsberg erecta background, and inoculated them with the bacterial strain *Psm avrRpt2*, whose defence activation is based on recognition by the Arabidopsis resistance protein Rps2. This bacterial strain initiates a different signalling pathway than *Psm avrRpm1.* Wu and Yang (2010) recently showed that Rps2-mediated resistance is further dependent on functional CRY1 signalling, but these experiments were done under continous light conditions. Under these circumstances, plants might be stressed by light and the impact of photoreceptors might probably be enhanced or altered compared to the light situation including naturally occuring day and night times used in the present work. On the other hand, results from Chandra-Shekara et al. (2006) are in agreement with the present data, because they showed that the light-dependent development of an HR in the Arabidopsis accession Di-17 after infection with *Turnip crinkle virus* is independent of functional phytochromes. Regarding the results of this work, however, we need to highlight that the strong, *PsmavrRpm1*-triggerd local defence responses occurring in the presence of light are essentially not related to functional photoreceptor signalling. Therefore, I can summerise that the signalling pathways underlying *Psm avrRpm1*-mediated defence responses at inoculations sites do not decisively overlap with phytochrome-, cryptochrome- or phototropin-associated signalling pathways.

Since an impairment of individual light signalling pathways does not critically influence local defences against *Psm avrRpm1*, the molecular basis of their light dependency might be directly or indirectly associated with photosynthesis. SA biosynthesis, for instance, requires the shikimate pathway which uses the photosynthesis-derived carbohydrates erythrose-4-phosphate and phosphenolpyruvate as precursors. Moreover, photosynthesis does not only deliver carbon building blocks for the biosynthesis of defence metabolites, but is also the source of plant energy. Thereby, photosynthesis enables many metabolic processes by ensuring high energy status and supplying reduction equivalents. The HR, for instance, requires functional chloroplasts, in which the photosynthetic reactions take place (Genoud et al., 2002). The pathogen-induced biosynthesis of SA from isochorismate synthase occurs in the plastids (Wildermuth et al, 2001), and this could reflect another connection between plant defence responses and photosynthesis. Besides, the chloroplasts are described to initiate pathogen-induced and light-dependent ROS formation (Liu et al., 2007). Chemical reduction of the plastoquinone pool, an essential element of the photosynthetic electron transport chain

located in the chloroplasts, results in an increased resistance against virulent *Pst DC3000* (Mühlenbrock et al., 2008). Generally, the light dependency of some pathogen defence responses seem to be closely connected to photosynthesis-related processes, but the underlying mechanism are still poorly understood. Investigating plant-pathogen interactions in the dark in the presence or absence of exogenously applied sugars could provide an answer to the question whether the production of important defence metabolites is limited by the lack of photosynthetic precursors in the dark. For instance, previous studies show that the expression of the pathogensis-related gene *PR-2* can be induced by application of metabolizable sucrose (Thibeaud et al., 2004). Studies using inhibitors of photosynthesis or experiments with plants situated in a carbon dioxide-free atmosphere might also help to improve our understanding of the light-dependency of plant defence responses and disease resistance.

In contrast to the minor impact of photoreceptors on defence responses at inoculation sites, I found that the establishment of SAR requires a functional phytochrome signalling pathway. SAR, as determined by comparative bacterial growth analyses in distant leaves of pathogen- and mock-pre-treated plants, did not develop in *phyAphyB* double mutants. Moreover, the mutants failed to elevate SA levels and expression of *PR-1* at the systemic level after a localized pathogen inoculation. Interestingly, systemic *PR-2* gene expression was not impaired highlighting the SA independency of *PR-2* expression previously reported by Nawrath and Métraux (1999). This might indicate that SA-dependent and SA-independent systemic responses branch early after perception of the SAR activating signal. Alternatively, SA-dependent and independent responses might be triggered by different SAR activating signals, which would require the existence of more than one mobile SAR long-distance signal. These studies also show that the expression of *PR-2* has almost no contribution to enhanced systemic resistance, and this suggests that systemic resistance is mainly due to SA-dependent responses. Additionally, *phyAphyB* fails to express the flavin-dependent monooxygenase *FMO1* in distant leaves of pathogen-treated plants. FMO1 was previously shown to be a necessary component for SAR establishment (Mishina and Zeier, 2006). Hence, a putative SAR-facilitating factor derived from the phytochrome pathway might function upstream of FMO1.

Besides, the present data confirm that an activated SAR state is closely associated with the systemic expression of *FMO1,* and that, alongside *PR*-1, FMO1 is an excellent SAR marker gene. Mishina and Zeier (2006) have suggested the existence of a positive feedback-loop occurring in distant, non-inoculated leaves of SAR-induced plants, which is involved in amplifying incoming SAR long-distance signals to allow the establishment of enhanced systemic resistance. This possible amplification loop might involve FMO1, ROS, NDR and NPR1, and the postulated phytochrome-dependent factor might regulate this amplification

mechanism (Fig. 1). However, the findings of this work explain that the light dependency of SAR previously demonstrated by Zeier et al. (2004) is caused and conditioned by functional phytochrome photoperception, providing a mechanistic explanation for this phenomenon. This further provokes the question about the benefit of photochrome-controlled SAR activation. Establishing a state of enhanced resistance in a whole plant demands high costs of energy and requires many precursors from primary metabolism. As both components are derived from photosynthesis and thereby, from the availibitity of light, the phytochromes might function as a trigger to enable SAR activation only when sufficient light, and thus energy and precursor metabolites, is available.

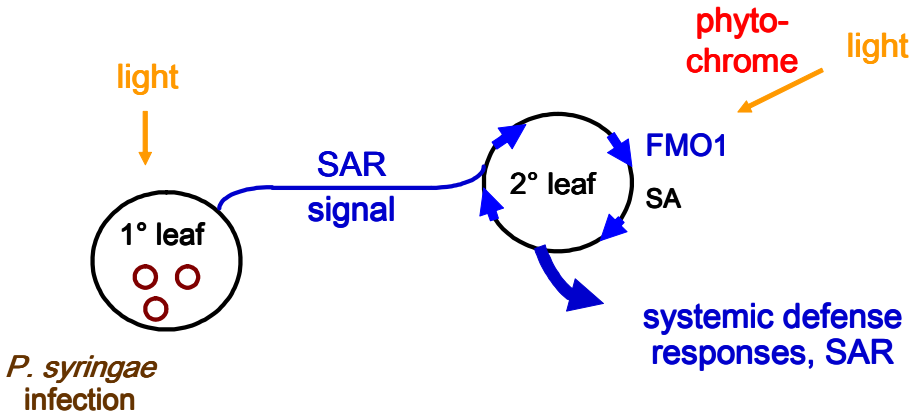

Figure 1. Light influences defence responses at the inoculation site and in distant tissue (Figure provided by J. Zeier)

Taken together, the results of this work indicate that a crosstalk between the phytochrome signalling pathway and the events leading to SAR activation exists. However, there is no influence of the blue light signalling pathways initiated by phototropins and cryptochromes on SAR. Further studies might identify the exact connection point of the phytochrome and the SAR signalling pathways. Many genes and proteins of light signalling downstream of the phytochromes are identified and corresponding mutants could be used therefore. Signalling events downstream of both phytochromes include PIF3 (PHYTOCHROME INTERACTING FACTOR 3), NPDK2 (NUCLEOSIDE DIPHOSPHATE KINASE 2) and PSI2 (PHYTO-CHROME SIGNALLING 2; Gyula et al., 2003). Furthermore intermediates of the branched signalling pathways of each phytochrome are known (e.g. PHYA: FAR1, FHY3, PAT1, LAF1, LAF6; PHYB: GI, ELF3, ZTL; Gyula et al., 2003). SAR assays with the single mutants, *phyA* and *phyB*, might furthermore reveal whether PHYA alone, PHYB alone, or the simultaneous function of both phytochromes is necessary for SAR activation. Recently, a new promoter element, *FORC*^{A}, was described to mediate responses and crosstalk between defence and light signalling (Evrard et al., 2009). Future studies could examine the involvement of this promoter element in light dependencies of local and systemic plant responses to pathogens.

In summary, it can be concluded that both the activation of resistance at the site of pathogen inoculation and of systemic acquired resistance are light-dependent processes. For responses at the inoculation site, cross-talk of defence signalling with the phytochrome, cryptchrome, and phototropin pathways is weak, and light might be mainly necessary to drive photosynthetic processes leading to the production of metabolites, energy, and reduction equivalents for defence activation. In contrast, SAR is tightly controlled by the phytochrome signalling pathway, which seems to accommodate light availability and the capacity to increase plant resistance at the systemic level. Naturally, this does not rule out an additional control element for SAR that is based on photosynthesis.

7.3 Pathogen treatment induces stigmasterol production

Upon infection with pathogens, plants induce a multitude of metabolic changes with potential impact on plant resistance. The current work characterised the phytosterol stigmasterol as a major metabolite that accumulates in Arabidopsis leaves in response to *P. syringae* infection. Stigmasterol biosynthesis comprises the desaturation of β-sitosterol which is the direct precourser. The presented data demonstrate that untreated Arabidopsis leaves only contain traces of stigmasterol. Upon infection with different *P. syringae* strains, stigmasterol is produced between 10 h and 48 h after infection, and finally reaches levels of about 15 µg g^{-1} FW. Stigmasterol formation is not only detected upon leaf inoculation with the hemibiotrophic bacterial pathogen *P. syringae*. Accumulation of the unsaturated sterol was also observed after infection with the necrotrophic fungus *Botrytis cinerea* and the biotrophic fungus *Golovinomyces cichoracearum* (Supplementary Fig. 1). This indicates that stigmasterol accumulation occurs within a broad range of plant-pathogen interactions. Furthermore, this sterol C22-desaturation event is triggered by PAMPs and inducible through exogenous ROS, whereas a contribution of the classical plant defence pathways involving SA-, JA-, or ethylene signalling appears to be low or even not existing. The *CYP710A* gene family members *CYP710A1*, *CYP710A2,* and *CYP710A4* are described to be involved in sterol C22-desaturation in Arabidopsis (Morikawa et al., 2006; Arnquist et al., 2007). The current data reveal that *CYP710A1* is predominantly mediating the pathogen-induced stigmasterol formation, because two independent T-DNA insertion lines lacking *CYP710A1* expression do not accumulate stigmasterol upon pathogenic infection. Although the biological relevance of stigmasterol accumulation is still not clear, CYP710A1-mediated C22-desaturation to stigmasterol seems to be an evolutionary conserved mechanism in many plants. Even the moss *Physcomitrella patens,* which contains stigmasterol as the predominant sterol, is described to contain functional gene isoforms (Morikawa et al., 2009). The presented results indicate that the stigmasterol/β-sitosterol ratio is strictly regulated but can be modulated upon

external stimuli. Moreover, plant species-dependent differences in the stigmasterol/β-sitosterol ratio exist. Such differences in the relative sterol composition might have implications for diverse physiological processes in plants. Furthermore, it might be of interest to study variations in the stigmasterol/β-sitosterol ratio in other plants with a view to resistance against pathogens. Especially, economical important crop plants could be used as objects for further studies.

7.4 Stigmasterol promotes plant susceptibility to bacterial pathogens by a still unkown mechanism

A second essential point concerning the functional relevance of phytosterols in plant-pathogen interactions was based on results obtained from bacterial growth assays with *CYP710A1*-deficient T-DNA lines. I found that a lack of pathogen-induced stigmasterol accumulation in c*yp710A1* mutant lines results in increased resistance to avirulent and virulent *P. syringae* strains. In other words, C22-desaturation of β-sitosterol to stigmasterol in the wild-type benefits growth of the hemibiotrophic bacteria and promotes plant susceptibility. By contrast, stigmasterol accumulation was not found to affect the susceptibility to the necrotrophic fungus *Botrytis cinerea*. This difference might be related to the different lifestyles of both pathogens.

How does stigmasterol enhance the susceptibility of plants to *P. syringae*? The obtained data demonstrate that the pathogen-induced changes in the stigmasterol/β-sitosterol ratio do not affect the SA-mediated defence pathway, which is a major route for resistance against biotrophic and hemibiotrophic pathogens. However, a lack of pathogen-induced stigmasterol accumulation in the *cyp710A* mutants leads to enhanced expression of the defence regulator FMO1. Overexpression of FMO1 was previously described to enhance basal resistance (Koch et al., 2006; Bartsch et al., 2006) and thereby, provides a possible explanation for the enhanced resistance phenotype in the absence of elevated stigmasterol contents. Figure 2 provides a summary of our results concerning pathogen-triggered stigmasterol accumulation. Stigmasterol levels are also described to increase in ripening tomato fruits (Whitaker and Gapper, 2008). If this is caused by enhanced levels of ROS or degradation of membranes, and if this correlates with an enhanced susceptibility of ripe fruits is not known.

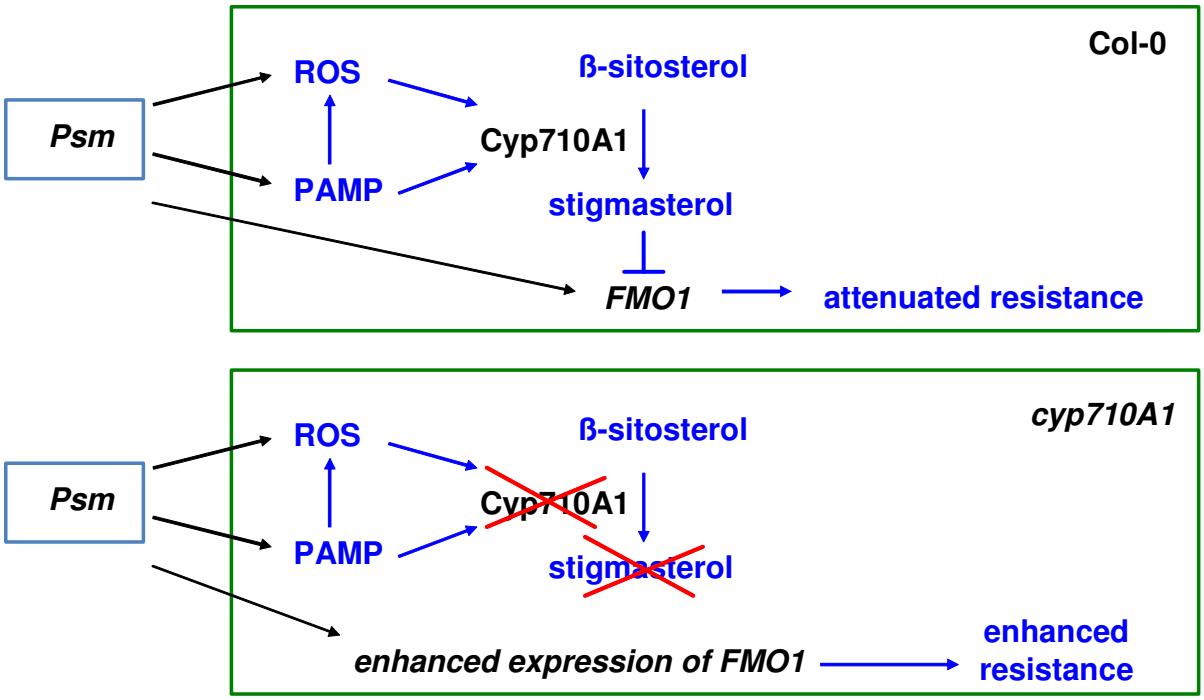

Figure 2. Simplified model for the function of pathogen-induced stigmasterol accumulation.

Phytosterols are typical components of plant membranes and influence their properties. According to the present data, the pathogen-induced change in the stigmasterol/β-sitosterol ratio manifests itself in isolated microsomal fractions and plasma membranes. In both fractions, I measured similar phytosterol ratios as in leaf extracts. It is thus likely that the alteration of this ratio affects the properties of membranes. The additional double bond of stigmasterol in the sterol side chain might decrease the ordering poperties of membranes and thus lead to an increased permeability of plant membranes. Experimental evidence that an increase in the stigmasterol contents of lipid bilayers does affect the physicochemical properties of membranes exists (Schuler et al., 1991; Hartmann, 1998; Hodzic et al., 2008). Changes in the membrane sterol composition might also affect the activities of membrane-situated enzymes. For instance, stigmasterol but not β-sitosterol activates and stimulates an H^+-ATPase in maize roots (Grandmougin-Ferjani et al., 1997). Stigmasterol might also influence the composition and existence of sterol- and sphingolipid-enriched membrane domains which could serve as signalling platforms in plant defence (Bhat and Panstruga, 2005; Zappel and Panstruga, 2008). Alterations of these so-called lipid rafts, which can be isolated as detergent-resistant membranes (DRMs; Simons and Ikonen, 1997; Mongrand et al., 2004; Laloi et al., 2007), may also be a result of elevated stigmasterol levels and might provide an approach for further investigations. A recent study shows that cold acclimation, for example, induces changes of the lipid composition of DRMs in Arabidopsis and that this alteration of lipid composition is mainly caused by an increase in the amount of free sterols in DRMs (Minami et al., 2009). A similar mechanism is also

conceivable for pathogen-induced alterations of DRMs, maybe due to accumulation of stigmasterol. Currently, the research on plant DRMs is advancing rapidly and, this could contribute to a deeper understanding of the mechanisms underlying stigmasterol-enhanced susceptibility. Further experiments might address whether the formation or composition of DRMs is altered after pathogen-induced sterol desaturation, and a comparative analysis of wildtype and *cyp710A1* mutant plants might be performed in this context. A proteomic analysis of DRMs could show if, for instance, a special protein is released from DRMs after stigmasterol accumulation, and might thus present a possible signal for plant susceptibility or resistance inside the cell. A similar event might be conceivable for other DRM-bound compounds such as sphingolipids or sterols.

Furthermore, a role for sterols in endocytosis is discussed in the literature. Sterol composition affects auxin-regulated and clathrin-dependent endocytosis. Auxin signalling mutants are impaired in their sterol contents, and mutants of sterol biosynthesis show altered auxin-regulated endocytosis (Boutté and Grebe, 2009). Pathogen-induced endo- and exocytotic processes are also an essential response in plant resistance signalling. For instance, the perception of flagellin in Arabidopsis results in endocytosis of the flagellin receptor FLS2 (Robatzek, 2007). It might be worth investigating in future studies whether changes in the membrane sterol composition would alter certain endocytotic reactions in plants. In particular, these studies might answer the question whether stigmasterol accumulating in membranes would modify endocytotic processes involved in plant resistance and susceptibility.

The practical work of this thesis did not deal with a putative direct role of stigmasterol as a substance favouring bacterial multiplication. However, it is known that prokaryotes are not able to synthesise sterols on their own. For instance, the oomycete group of *Phytophtora* is lacking sterol biosynthesis and acquires sterols exogenously from their environement or hosts (Marshall et al., 2001). How far phytopathogenic bacteria such as *P. syringae* depend on the uptake of different phytosterols as part of their nutrition is not known. Applications of different phytosterols into overnight cultures of *P. syringae* might reveal whether there is a growth promoting or inhibiting influence of specific sterols on bacteria. Similar studies indicate that stigmasterol enhances in cultures the growth of *Phytophtora sojae* by causing the down-regulation of elicitin genes that are putative avirulence signals of oomycetes (Yousef, 2009).

Further insights afford the investigation of *CYP710A1* overexpressing lines with constitutively enhanced levels of stigmasterol (Morikawa et al., 2006; Arnquist et al., 2007). According to the results of this thesis, such lines should exhibit enhanced disease susceptibility to *P. syringae*.

In summary, we can conclude that β-sitosterol desaturation and stigmasterol accumulation constitutes a significant metabolic process occurring upon infection with different pathogens. This process is mediated by recognition of PAMPs and leads to a lowered basal resistance against hemibiotrophic bacterial pathogens but not against necrotrophic fungi. The nature of this susceptibility promoting state is not yet clear but might be realted to a negative regulation of the expression of positive defense regulators such as FMO1.

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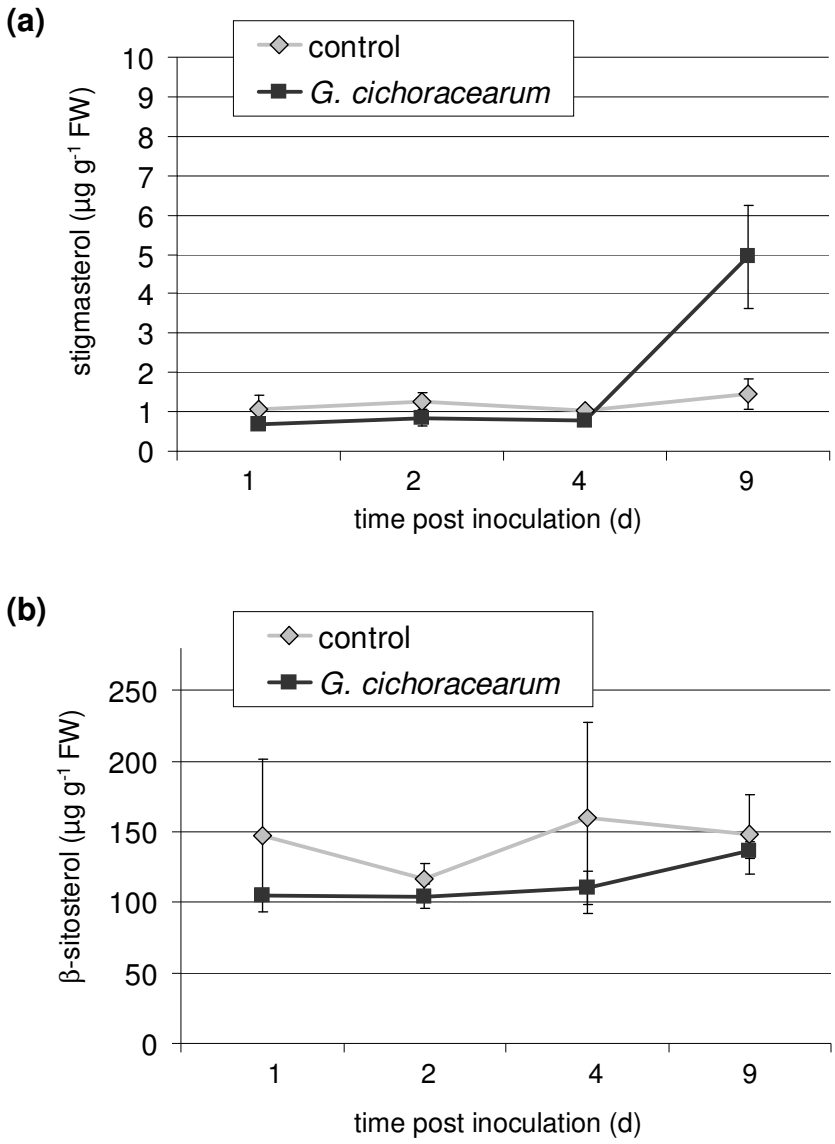
8. Supplemental Figures



Supplemental figure 1. Time course analysis [days (d) after infiltration] of stigmasterol and β-sitosterol levels of Col-0 leaves infected with the biotrophic fungus *Golovinomyces cichoracearum* (black squares). Levels of control plants are marked with grey diamonds. A similar tendency was observed in two independent experiments.
(a) Leaf stigmasterol contents and (b) leaf β-sitosterol contents.
Mean values of µg substance g^{-1} leaf fresh weight (± SD) from three independent samples are given.

9. Abbreviations

ABA	abscisic acid
avr	avirulence
BAK1	BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1
Bgh	*Blumeria graminis* f. sp. *hordei*
BR	Brassinosteroid
BTH	Benzo-1,2,3-thiadiazole-7-carbothioic acid S-methyl ester
CC	coiled-coiled
cfu	colony-forming units
Cor	coronatin
coi1	*coronatin insensitive 1*
CRY	CRYPTOCHROM
DAMP	Damage-associated molecular pattern
DMAPP	dimethylallyl diphosphate
dpi	days post infection
DW	dry weight
eds	*enhanced disease susceptibility*
ETI	effector-triggered immunity
FAD	flavin adenine nucleotide
flg22	22-amino acid, elicitor active flagellin peptide
FLS2	FLAGELLIN SENSING 2
FMO	FLAVIN-DEPENDENT MONOOXYGENASE
f. sp.	forma specialis
FW	fresh weight
HR	hypersensitive response
HMGCoA	3-HYDROXY-3-METHYLGLUTARYL-CoenzymA
HMGR	3-HYDROXY-3-METHYLGLUTARYL-CoA-REDUCTASE
hpi	hours post infection
hrp	*hypersensitive reaction and pathogenicity*
ics	*isochorismate synthase*
INA	2,6-dichloro-isonicotinic acid
IPP	isopentenylpyrophosphate
ISR	induced systemic resistance
JA	jasmonic acid
JAR1	JASMONATE RESISTANT 1
LOV	light, oxygen, voltage

LPS	lipopolysaccharides
LRR	leucine-rich repeat
LTP	LIPID TRANSFER PROTEIN
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
MeJA	methyl jasmonate
MeSA	methyl salicylate
NBS	nucleotide binding side
PAD4	PHYTOALEXIN DEFICIENT 4
PAL	PHENYLALANINE AMMONIUM LYASE
PAMP/MAMP	Pathogen/Microbe-associated molecular pattern
pen	*penetration*
PFD	photon flux density
PGN	peptidoglycan
PHOT	PHOTOTROPIN
PHR	photolyase homology region
PHY	PHYTOCHROME
PRR	pattern recognition receptor
Psg	*Pseudomonas syringae* pv. *glycinea*
Psm	*Pseudomonas syringae* pv. *maculicola*
Psp	*Pseudomonas syringae* pv. *phaseolicola*
Pst	*Pseudomonas syringae* pv. *tomato*
PTI	PAMP-triggered immunity
pv.	pathovar
R	resistance
Rboh	respiratory burst oxidases homologues
RK	receptor kinase
RLK	receptor-like kinase
RLP	receptor-like protein
ROS	reactive oxygen species
SA	salicylic acid
SAG	salicylic acid β-glycoside
SAG101	SENESCENCE-ASSOCIATED GENE 101
SAR	systemic acquired resistance
sid	*salicylic acid induction-deficient*
SMT	STEROL METHYLTRANSFERASE
TIR	Toll receptor-like and interleukin-like domain
TTSS	type three secretion system

10. Supplement

10.1 List of publications

Griebel T, Zeier J. Membrane stigmasterol accumulation enhances plant disease susceptibility to bacterial pathogens (*submitted*)*

Attaran E, Zeier TE, 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.

Griebel T, Zeier J (2008). Light regulation and daytime dependency of inducible plant defences in Arabidopsis: phytochrome signalling controls systemic acquired resistance rather than local defence. Plant Physiol. 147: 790-801.*

Mishina TE, Griebel T, Geuecke M, Attaran E, Zeier J (2008). New insights into the molecular events underlying systemic acquired resistance. In *Biology of Molecular Plant-Microbe Interactions*, Vol 6 (CD-ROM), 2007 IS-MPMI Symposium Proceedings, eds. Lorito L, Woo S & Scala F.

(* publications included into this thesis)

10.2 Poster presentations

Griebel T, Zeier J. Plant defence responses in Arabidopsis: light dependency and photoreceptor signalling. 20th meeting “Molekularbiologie der Pflanzen” (molecular biology of plants), Dabringhausen, Germany, 27.02.-02.03.2007

Griebel T, Zeier J. Plant defence responses in Arabidopsis: light dependency, photoreceptor signalling and carbohydrate metabolism. 8th Congress "Molecular Plant-Microbe Interactions", Sorento, Italy, 21.07.-28.07.2007

Griebel T, Zeier J. PAMP-induced sterol C-22 desaturation in plant-pathogen interaction, “6th Tri-national Arabidopsis Meeting”, Cologne, Germany 16.-19.09.2009

10.3 Curriculum vitae

Personal Data:

Thomas Griebel

Date of Birth:	12th October 1980
Place of Birth:	Bad Neustadt a. d. Saale, Germnay
Family status:	unmarried
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Educational Background:

01/2007-12/2009	Scholarship holder of the Research Training Group 1342 of the German Research Foundation: *"Molecular and functional analysis of lipid-based signal transduction systems",*
11/2006-02/2010	scientific employee at the Julius-von-Sachs-Institute, University of Würzburg, Germany, in the lab of PD Dr. Jürgen Zeier, 'Local and systemic resistance in *Arabidopsis thaliana* in response to *Pseudomonas syringae: impact of light and phytosterols*'
09/2006	„Diplom"- examination in biology (over-all grade: very good) Subjects: Botany (Ecophysiology and Plant Ecology) Biochemistry Zoology (Cell and Developmental Biology)
12/2005-09/2006	„Diploma"-Thesis: „Light dependency of salicylic acid-mediated plant defence mechanisms in Arabidopsis", supervised by PD Dr. Zeier, Julius-von-Sachs-Institute of Biological Sciences, University of Würzburg, Germany
10/2001-09/2006	Studies of biological sciences, University of Würzburg, Germany
06/2000	Abitur (German secondary school leaving examination)
09/1991- 06/2000	Secondary School, Rhön-Gymnasium, Bad Neustadt a. d. Saale, Germany
09/1987- 07/1991	Primary School, Schönau a. d. Brend, Germany

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10.5 Erklärung

Erklärung gemäß § 4 Abs. 3 Ziff. 3, 5 und 8
der Promotionsordnung der Fakultät für Biologie
der Julius-Maximilians-Universität Würzburg

Hiermit erkläre ich die vorliegende Dissertation selbstständig angefertigt zu haben und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Diese Dissertation hat weder in gleicher noch ähnlicher Form einem anderen Prüfungsverfahren vorgelegen. Ich erkläre weiterhin, dass ich früher weder akademische Grade erworben habe, noch versucht habe zu erwerben.

Würzburg, Februar 2010

……

Statement of the contribution of the thesis' author in the respective publications

Publication 1:

Griebel T, Zeier J (2008). Light regulation and daytime dependency of inducible plant defences in Arabidopsis: phytochrome signalling controls systemic acquired resistance rather than local defence. Plant Physiol. **147:** 790-801.*

Conception: 30%
Experimental contribution: 100%
Formulation of results: 30%

……. ……
Thomas Griebel PD Dr. Jürgen Zeier

Publication 2:

Griebel T, Zeier J. Membrane stigmasterol accumulation enhances plant disease susceptibility to bacterial pathogens (*submitted to The Plant Journal*).

Conception: 40%
Experimental contribution: 100%
Formulation of results: 40%

……. ……
Thomas Griebel PD Dr. Jürgen Zeier