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Research article

Suppression of UV-B stress induced flavonoids by biotic stress: Is there reciprocal crosstalk?☆

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ABSTRACT

Plants respond to abiotic UV-B stress with enhanced expression of genes for flavonoid production, especially the key-enzyme chalcone synthase (CHS). Some flavonoids are antioxidative, antimicrobial and/or UV-B protective secondary metabolites. However, when plants are challenged with concomitant biotic stress (simulated e.g. by the bacterial peptide flg22, which induces MAMP triggered immunity, MTI), the production of flavonoids is strongly suppressed in both *Arabidopsis thaliana* cell cultures and plants. On the other hand, flg22 induces the production of defense related compounds, such as the phytoalexin scopoletin, as well as lignin, a structural barrier thought to restrict pathogen spread within the host tissue. Since all these metabolites require the precursor phenylalanine for their production, suppression of the flavonoid production appears to allow the plant to focus its secondary metabolism on the production of pathogen defense related compounds during MTI. Interestingly, several flavonoids have been reported to display anti-microbial activities. For example, the plant flavonoid phloretin targets the *Pseudomonas syringae* virulence factors flagella and type 3 secretion system. That is, suppression of flavonoid synthesis during MTI might have also negative side-effects on the pathogen defense. To clarify this issue, we deployed an Arabidopsis flavonoid mutant and obtained genetic evidence that flavonoids indeed contribute to ward off the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Finally, we show that UV-B attenuates expression of the flg22 receptor FLS2, indicating that there is negative and reciprocal interaction between this abiotic stress and the plant-pathogen defense responses.

1. Introduction

Flavonoids (lat. *flavus* = yellow) are plant secondary metabolites with a diverse function in modulation of plant-environment interactions, e.g. protection against abiotic or biotic stresses (Falcone Ferreyra et al., 2012). Furthermore, they provide much to the flavor and color of fruits or vegetables and their free-radical scavenging activities suggest they have also a positive impact on human diet (Ross and Kasum, 2002). They are produced from phenylalanine via 4-coumaroyl-CoA. The chalcone synthase (CHS) is the key-enzyme for metabolite fluxes into the flavonoid pathway (Fig. 1). This enzyme converts 4-coumaroyl-CoA or 4-dihydrocoumaroyl-CoA to naringenin chalcone or phloretin, respectively (Gosch et al., 2010). Most flavonoid pathway genes (FPGs) are highly UV-B inducible (Schenke et al., 2011) and plants lacking flavonoids are hypersensitive to UV-B stress (Li et al., 1993; Landry et al., 1995). In *Arabidopsis thaliana* cell cultures flavonoids were efficiently induced by a fluence rate of 0.53 μmol m-2 s-1 (Schenke et al., 2011), but even a lower fluence rate of 0.1 μmol m-2 s-1 is sufficient to induce the CHS (Brown and Jenkins, 2008; Jenkins, 2014). The Arabidopsis *chs/f3h* loss of function mutant (N8585) is unable to produce flavonoids, readily visible because of the light-coloured seeds (Fig. 1) (Appelhagen et al., 2014). This mutant exhibits the highest susceptibility towards *Heterodera schachtii*, pointing to a role of flavonoids in defense against this cyst nematode (Jones et al., 2007) and silencing of CHS genes by RNAi in *Medicago truncatula* demonstrated the need of flavonoids in nodule formation by rhizobia (Wasson et al., 2006). Structurally, chalcone synthases are closely related to stilbene synthases (STS), which are for example involved in phytoalexin production, e.g. resveratrol from grape (Schröder and Schröder, 1990; Jeandet et al., 2010). Therefore, flavonoids play a role in plant defenses towards both abiotic and biotic stress. In this paper we focus on the role of flavonoids in the *Arabidopsis thaliana* response to biotic stress exerted by the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* (*Pst*), because there are contradicting observations concerning

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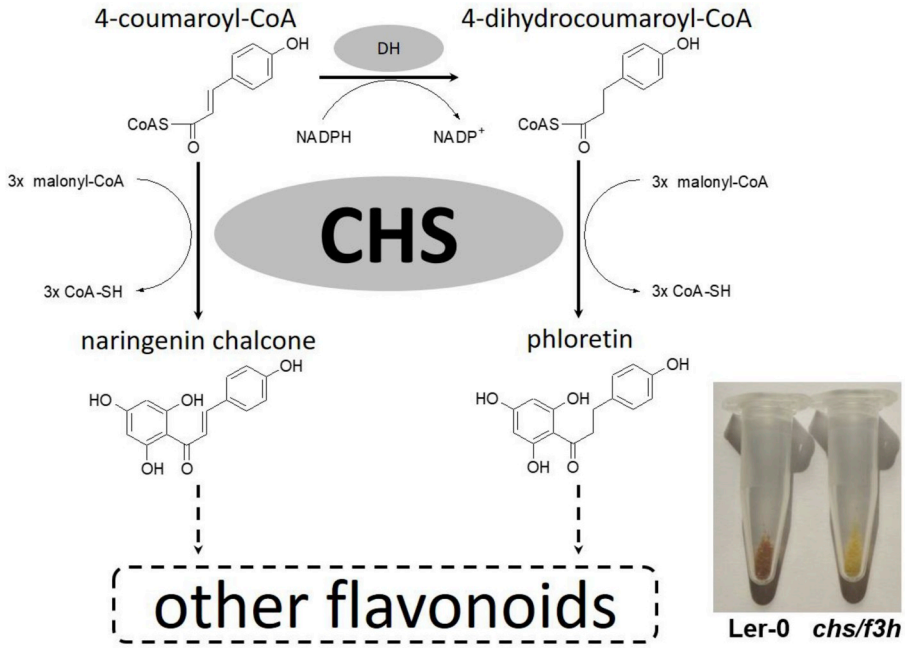

Fig. 1. Central role of the chalcone synthase (CHS) as entry-point for the flavonoid pathway in plants. A knock-out of the single copy gene in Arabidopsis results in plants void of flavonoids. Phloretin is reported to inhibit the T3SS and bacterial flagella of plant-pathogenic *Pst* DC3000 (Vargas et al., 2013).

flavonoid production after infection, which are reviewed in this introduction.

1.1. Flavonoid production is suppressed by biotic stress

Plants suffering biotic stress, either directly *via* pathogen infection or simulated by application of conserved microbe-associated molecular patterns (MAMPs) elicit a defense response, which is termed MAMP Triggered Immunity (MTI). Pathogens in turn have evolved effectors, which they translocate into the host cells in order to interfere with plant defense responses, called effector triggered susceptibility, ETS. Bacterial pathogens, such as *Pseudomonas syringae*, deliver their effectors with the Type 3 Secretion System (T3SS) into the host cells. Recognition of these effectors by the plant immune system results then in Effector Triggered Immunity, ETI (Jones and Dangl, 2006). Thus, it is advisable to treat plants with MAMPs in order to observe their pure, original and non-manipulated defense reaction. It is known for more than three decades that plants suppress the accumulation of flavonoids when facing biotic stress (Schenke et al., 2011). Especially down-regulation of the CHS expression contributes to this phenomenon and this mechanism involves prevention of histone acetylation, a hallmark for gene activation (Schenke et al., 2014). Fig. 2 shows the typical suppression of UV-B induced CHS expression by the MAMP flg22, which is a 22 amino acid conserved peptide from bacterial flagella. This kind of suppression is observed in several plants (Table 1).

Thus, there is a growing list of examples, which suggests this suppression of flavonoid production is a typical MTI defense reaction well conserved throughout the plant kingdom. Importantly, Lozoya et al. (1991) reported that elicitor treatment of parsley cell cultures completely suppressed flavonoid production, while the effect of UV-B on elicitor induced coumarins was only slightly slowing down their accumulation. Furthermore, a time course experiment to measure CHS transcript level showed that elicitor addition was able to reduce CHS expression at any time during UV-B treatment, indicating that as soon both stresses were applied simultaneously the biotic stress is getting the upper hand (Lozoya et al., 1991). This regulation is thought to enable the plant to redirect metabolic resources from the UV-B defense response to pathogen defense, for example to increase the production of phytoalexins (e.g. coumarins such as scopoletin) or lignin (Lo and

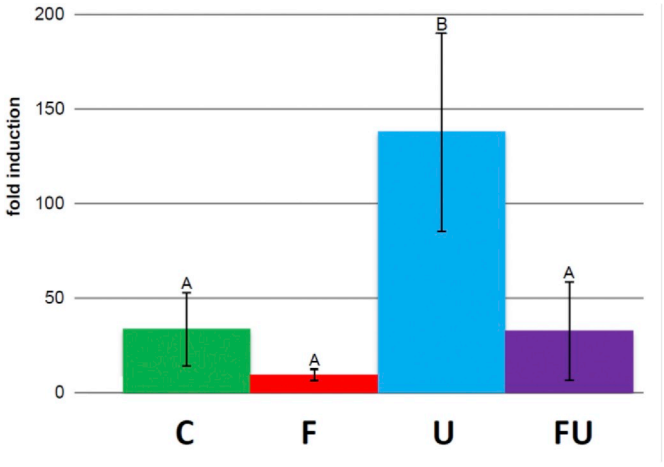

Fig. 2. Crosstalk between UV-B and biotic stress mimicked by elicitation with flg22. The CHS expression is suppressed by flg22. These data from Arabidopsis have been published in Zhou et al. (2017). Shown is the response to four different treatments: Water/VIS (C), flg22/VIS (F), UV-B/VIS (U) and both stresses simultaneously (FU). CHS gene expression level was investigated at 4 h and normalized to PP2A and Actin. Error bars represent standard error of triplicate experiments, and statistical significance was checked by a two-way ANOVA.

Nicholson, 1998; Schenke et al., 2011). It is supported by the observations that pathogens deploy effectors to boost anthocyanin production in order to reduce the lignin content (Tanaka et al., 2014) and that UV-B led to increased resistance towards *B. cinerea* independent of flavonoid production, but depending on enhanced sinapate accumulation required for the synthesis of syringyl-type ('defense') lignin (Demkura and Ballare, 2012). However, soluble sinapoyl-glucose might also directly inhibit the pathogen growth without the need of lignin formation as has been shown for the Arabidopsis - *Verticillium longisporum* interaction (König et al., 2014). Taken together it appears that during biotic stress (MTI) plants shut down the UV-B induced flavonoid pathway in order to produce other defense metabolites (This shall be "hypothesis one").

Tabel.1
Flavonoid production or CHS expression suppressed in plants under biotic stress.

Plant system	Suppressing biotic stress	Reference
parsley cells	*Phytophthora sojae* elicitor	Hahlbrock et al. (1981)
carrot cells	*Pythium aphanidermatum* elicitor	Gleitz et al. (1991)
sorghum plants	*Cochliobolus heterostrophus* infection	Lo and Nicholson (1998)
onion plants	*Botrytis allii* infection	McLusky et al. (1999)
Glehnia littoralis cells	Yeast extract elicitor	Ishikawa et al. (2005)
Arabidopsis seedlings	flg22 and elf18 elicitor	Saijo et al. (2009)
Arabidopsis cells	flg22 elicitor	Schenke et al. (2011)
Brassica napus seedlings	flg22 elicitor	this work (Fig. S1)

1.2. Flavonoid production is contributing to pathogen defense

On the other hand, it has been shown that several flavonoids can also contribute to pathogen defense responses, besides their role as UV-B protective sunscreens, having antiviral, antibacterial or antifungal activity (Cushnie and Lamb, 2005). For instance, phloretin ("Fig. 1") targets both motility and the T3SS of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Vargas et al., 2013). Accordingly, it has been observed that several flavonoids are induced in response to pathogen attack. Rutin, for example, accumulates in *Pst* infected tomato plants (López-Gresa et al., 2011) or in response to biocontrol treatment of potato against infection with *Verticillium dahliae* (El Hadrami et al., 2011). Though rutin is produced as UV-B protectant, it has also anti-bacterial properties (van der Watt and Pretorius, 2001). It serves as a signal released by host plant roots to attract e.g. the beneficial *Bacillus subtilis* (Singh et al., 2016) or mycorrizhal fungi (Lagrange et al., 2001) facilitating root colonization. Furthermore, rutin can enhance resistance to insects in tobacco (Misra et al., 2010) as well as to *Xanthomonas oryzae pv. oryzae*, *Ralstonia solanacearum* and *Pst* DC3000 in rice, tobacco and Arabidopsis, respectively (Yang et al., 2016). Therefore, rutin appears to mediate priming of plant resistance by inducing the early salicylic acid (SA) signalling pathway, quite similar to its aglycon quercetin (Yang et al., 2016; Jia et al., 2010). Quercetin exhibits anti-quorum sensing effects in *Pseudomonas aeruginosa* and reduces biofilm formation (Pejin et al., 2015). Other antimicrobial flavonoids are the rice phytoalexin sakuranetin, which is produced in response to the fungus *Pyricularia oryzae* (Kodama et al., 1992) or catechin, that inhibits infection hyphae formation by *Alternaria alternata* appressoria on strawberry leaves (Yamamoto et al., 2000) as well as rust infection of poplar (Ullah et al., 2017). It has been shown that catechin also interferes with *Pseudomonas aeruginosa* quorum sensing (Vandeputte et al., 2010). Taxifolin from kempas (*Koompassia malaccensis*) has antimicrobial activity against *Streptococcus sobrinus* (Kuspradini et al., 2009), while luteolinidin accumulated in sugar cane in response to *Colletotrichum falcatum* (Malathi et al., 2008). Luteolin accumulated in infected sorghum and affected spore germination of *Colletotrichum sublineolum* (Du et al., 2010). However, the strongest evidence for flavonoids as phytoalexins comes from isoflavone-derived pterocarpanes, which are described for the *Fabaceae* family (legumes), such as medicarpin, pisatin, glyceollin or phaseolin (Liu et al., 2006). Legumes contain generally a greater number of chalcone and stilbene synthase family proteins, which points to functional divergence. Samac and Graham (2007) concluded from other studies that suppression of the anthocyanin branch in soybean after infection with avirulent *P. syringae* pv *glycinea* and concomitant up-regulation of the flavone and isoflavone branch enhanced

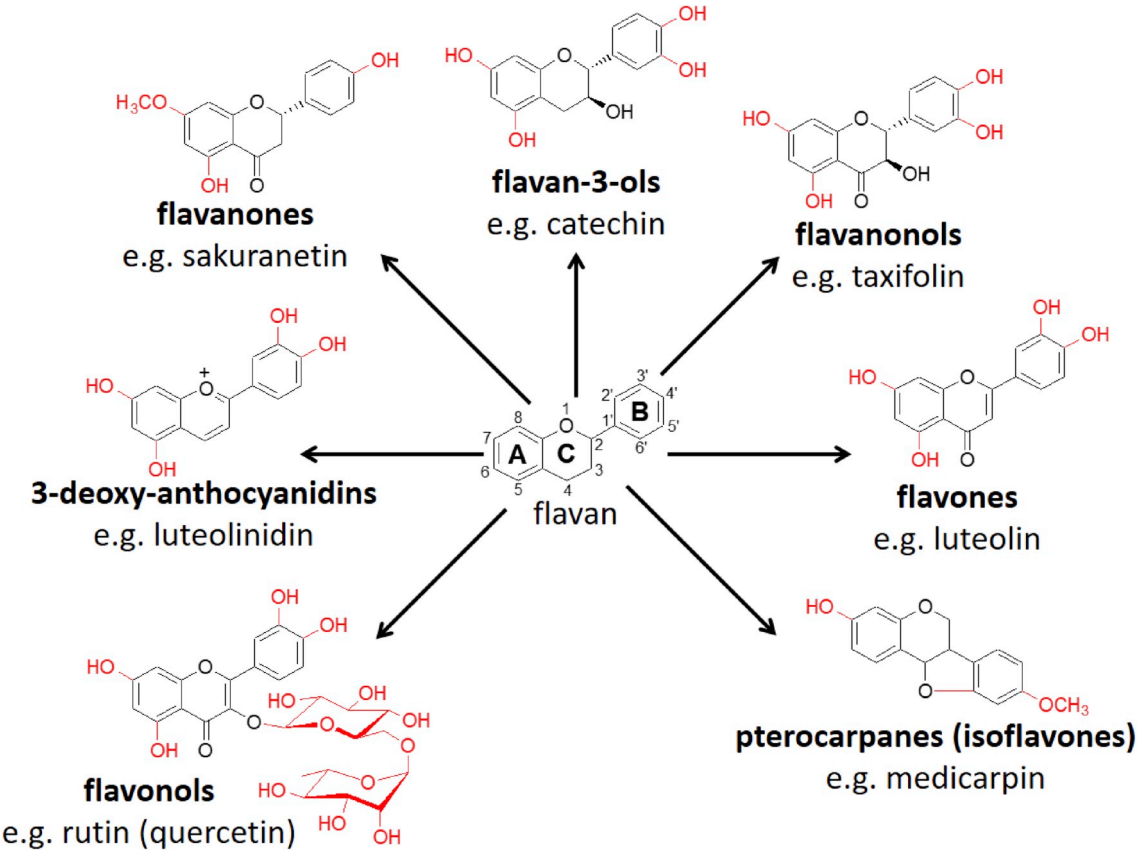

Fig. 3. Overview of selected flavonoid family members reported to have antimicrobial properties. The black skeleton structure is typical for each family, while the red substitutions are specific for the given example. For details see text. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

production of antimicrobial isoflavones, which is supported by Yu et al. (2003) describing that isoflavonoid synthesis was increased by a combination of ectopic expression of maize transcription activators and inhibition of the competing anthocyanin pathway. This suggests that flavonoid suppression is also present in legumes. It is likely that some chalcone/stilbene synthase copies are required to cope with biotic stress (phytoalexin and/or phytoanticipin production), some for UV-B protection and some to establish symbiotic interactions, e.g. with rhizobia or mycorrhiza (Steinkellner et al., 2007; Hassan and Mathesius, 2012). Such manifold involvement of flavonoids in plant-environment interactions is probably the evolutionary driving force to increase CHS/STS copy numbers in order to respond to both biotic and abiotic stress. These are just a few examples summarized in Fig. 3. Interestingly, some pathogens are able to degrade flavonoids, such as *Verticillium dahliae* metabolizing rutin (El Hadrami et al., 2015) and the rice blast fungus *Pyricularia oryzae* metabolizing the phytoalexin sakuranetin (Katsumata et al., 2017) or deploy flavonoid efflux pumps as shown for *Pst* (Vargas et al., 2011). In conclusion, plants produce flavonoids in response to infection because of their antimicrobial properties ("hypothesis two").

1.3. Aim of this study

Clearly both hypotheses are logically straight-forward, but also somehow mutually exclusive. Regarding pathogen defense, is the production of flavonoids now disadvantageous ("hypothesis one") or advantageous ("hypothesis two") for the plant? We try here to resolve this question by deploying the Arabidopsis *chs/f3h* double mutant completely lacking flavonoids and compared growth of the bacterial pathogen *Pst* between mutant and wild-type plants. Finally, we searched for indications whether the suppression of UV-B induced flavonoids by biotic stress is part of a unidirectional or reciprocal crosstalk, involving also suppression of biotic stress responses by UV-B.

2. Results

In a first experiment, we challenged the Arabidopsis *chs/f3h* double mutant void of flavonoids with different bacterial strains (Fig. 4). The results indicate that flavonoids contribute to restrict the bacterial growth in *Arabidopsis*. The *Pst* T3SS mutants grow less well than the virulent strain DC3000, which confirms the role of effector proteins to establish a compatible interaction and that the applied system is generally working.

To analyse the effect of flavonoids on *Pst*, we analysed bacterial gene expression in response to phloretin as well as naringenin by RNAseq (Table S1). Notably, the bacteria respond to these treatments with induction of multidrug efflux pumps, while the T3SS was not expressed.

In order to further support the role of flavonoids in plant defense against pathogens we investigated the effect of UV-B on *Pst* growth in *Arabidopsis* and infected UV-B pre-treated Arabidopsis plants with *Pst* (Fig. 5). However, we observed that the UV-B pre-treatment of Arabidopsis had no significant effect on the subsequent infection with two *Pst* strains, including the wild-type DC3000. Only for the *hrcQ* mutant the UV-B pre-treatment suppressed bacterial growth.

To investigate whether flavonoid pathway genes (FPGs) are generally suppressed in response to biotic stress and to search for antagonistically regulated defense genes we deployed a meta-analysis of publicly available gene expression data (Figs. S2 and S3) using GENEVESTIGATOR (Hruz et al., 2008). In Arabidopsis the FPGs were generally suppressed by biotic stress (Fig. S2A), but two of these stress treatments showed a strong up-regulation of the key marker gene CHS. The up-regulation in response to virulent *Pst* DC3000 was investigated in more detail, because of its relevance to this work (Fig. 6).

Additionally, two genes were identified in Arabidopsis as antagonistically regulated to the FPGs by treatments (Fig. S2) and in mutant backgrounds (Fig. S3). From the tested pathogen defense associated genes, the flg22-binding receptor like kinase FLS2 (Gómez-Gómez and Boller, 2000) and the glucosyltransferase UGT72E1 involved in monolignol 4-O-glucoside production (Lanot et al., 2006) were down-regulated when FPGs were up-regulated and *vice versa*. This temped us to investigate the expression of both genes in our Arabidopsis system, observing a negative effect of UV-B on *FLS2* but not on *UGT72E1* expression (Fig. 7), being indicative of a reciprocal crosstalk between UV-B and flg22 signalling.

Furthermore, we investigated differential CHS expression in response to biotic stress within other plant systems and selected several

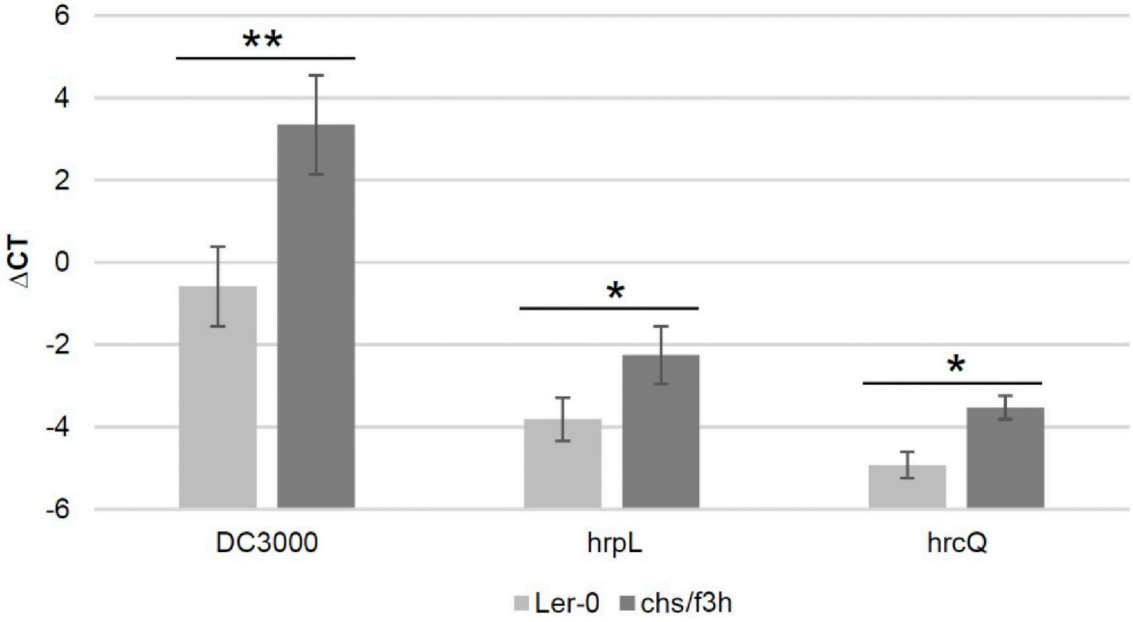

Fig. 4. Flavonoids dampen bacterial growth in Arabidopsis. Comparison of *Pst* DC3000 (WT), *hrpL* (transcription factor for virulence genes; Castillo-Lizardo et al., 2015) and *hrcQ* (T3SS-component; Badel et al., 2006) growth rate in Arabidopsis Ler-0 (WT) and *chs/f3h* mutant. Growth rates of wild-type (DC3000) and mutant (hrpL and hrcQ) Pst strains were analysed by qPCR of biomass marker genes. Statistical significance was checked by *t*-test (*: $p \leq 0.05$ **: $p \leq 0.01$) based upon 3 biological replicates.

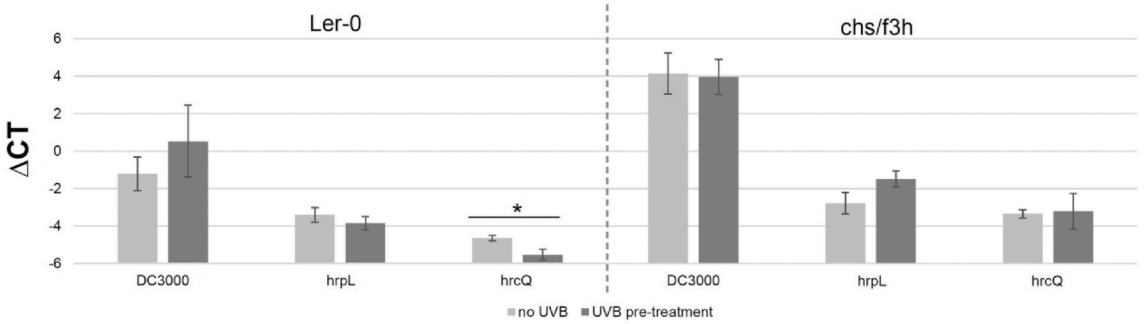

Fig. 5. Effects of UV-B pre-treatment on bacterial growth in Arabidopsis Ler-0 (WT) and *chs/f3h* mutant plants. Growth rates of wild-type (DC3000) and mutant (*hrpL* and *hrcQ*) *Pst* strains were analysed by qPCR of biomass marker genes. Statistical significance was checked by *t*-test (*: $p \leq 0.05$) based upon 3 biological replicates.

chalcone and stilbene synthase family proteins for a GENEVESTIGATOR meta-analysis (Fig. S4). In Arabidopsis only the CHS showed striking differential expression in response to biotic stress and elicitor application, while the other three chalcone and stilbene synthase genes were not strongly regulated in response to pathogens (Fig. S4A). Apparently, in almost all plant species investigated, some CHS/STS copies are suppressed in response to biotic stress.

3. Discussion

3.1. Flavonoids contribute in Arabidopsis to fend off pathogenic bacteria

Some aspects about suppression of flavonoid pathway genes (FPGs) during flg22-triggered MTI had not been dealt with so far. We have investigated if flavonoids really play also a role in the pathogen defense system *Arabidopsis thaliana – Pseudomonas syringae* pv. tomato DC3000 as suggested by Vargas et al. (2013) by deploying the flavonoid deficient Arabidopsis mutant *chs/f3h*. Reduced bacterial growth on the mutant would support hypothesis 1 (that less flavonoids means more resources for defense metabolites such as phytoalexins and lignin), while a better growth rate would support hypothesis 2 (that also flavonoids are produced against pathogens, thus less flavonoids means reduced anti-microbial activity). It appears that flavonoids indeed contribute to plant protection against the bacterial pathogen *Pst* DC3000, because bacterial growth was enhanced in the mutant (Fig. 4). We got also indications that UV-B pre-treatment could suppress bacterial growth, though only for the *Pst hrcQ* mutant (Fig. 5). Because this effect was lost in the *ch3/f3h* mutant, it is an indication that UV-B induced flavonoids were indeed involved in suppression of bacterial growth, supporting hypothesis 2. However, the majority of reports describing effects of UV-B on plant-pathogen interactions showed that disease symptoms rather increased (Table 2). Though we now show that flavonoids indeed contribute to pathogen defense in Arabidopsis, there is still ample evidence for suppression of flavonoid production during plant – pathogen interactions (Table 1), including Arabidopsis (Zhou et al., 2017).

Maybe it would be helpful to look on this crosstalk from a pathogen point of view. Several pathogens deploy effectors to manipulate their host and some effectors of the biotrophic pathogen *Ustilago maydis* can induce flavonoid/anthocyanin production thereby withdrawing resources from lignin formation (Tanaka et al., 2014). Thus, also *Pst* might theoretically deploy some of its T3SS effectors to influence flavonoid production. For example, it has been shown that the effector HopZ1 promotes *P. syringae* multiplication in soybean by targeting the enzyme 2-hydroxyisoflavanone dehydratase (GmHID1), which is required to produce the isoflavonoid and phytoalexin precursor daidzein (Zhou et al., 2011). Mechanistically, up-regulation of FPGs by *Pst* DC3000 infection could be achieved by effectors, reported to induce for example the Arabidopsis NCED3 gene resulting in elevated ABA levels (de Torres-Zabala et al., 2007 + 2009). ABA in turn appears to have a positive impact on FPG expression (Fig. S2 and discussion below). Our meta-analysis focusing on the Arabidopsis – *Pst* interaction indicates suppression of FPGs in response to infection with bacterial T3SS-deficient (*hrcC*) mutants, resembling MTI (Fig. 6). Infection with *Pst* DC3000, which deploys effectors, up-regulates FPG expression but this effect is limited to the key-enzyme CHS. Thus, such a putative T3SS effector has to be able to discriminate between the otherwise highly co-regulated FPGs. In this case CHS up-regulation might be a strategy of *Pst* DC3000 to interfere with lignin and phytoalexin production, similarly to that of *Ustilago maydis*. In another meta-analysis (Fig. S4) we found

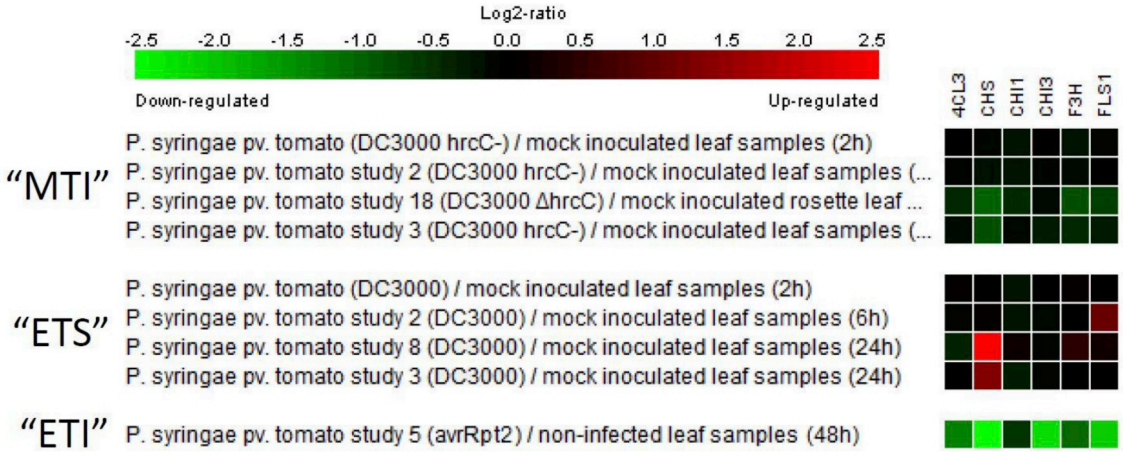

Fig. 6. Meta-analysis by GENEVESTIGATOR of the Arabidopsis – *Pst* interaction with respect to suppression of FPGs. The *Pst hrcC* mutant with defective T3SS is non-pathogenic and could resemble MTI, while *Pst* DC3000 is a virulent strain causing disease via ETS. The avirulent *Pst avrRpt2* strain provokes the Effector Triggered Immunity (ETI). Similar treatments were selected and indicate that CHS up-regulation is also time dependent, taking effect after 24 h from inoculation.

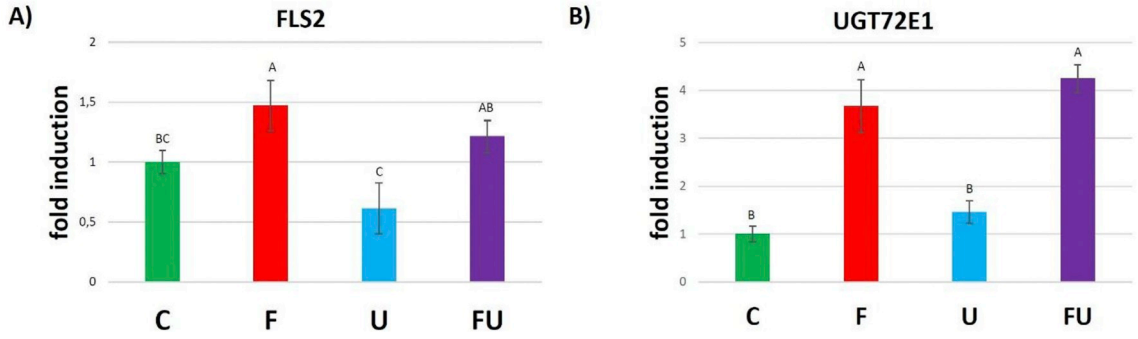

Fig. 7. Expression analysis of *FLS2* (A) and *UGT72E1* (B) in response to flg22 and UV-B alone or in combination. Gene expression levels were normalized to Actin. Gene expression was investigated after 4 h in response to four different treatments: Water/VIS (C), flg22/VIS (F), UV-B/VIS (U) and both stresses simultaneously (FU). Error bars represent standard error of triplicate experiments, and statistical significance was checked by a two-way ANOVA. However, the two-tailed p-value of an unpaired *t*-test equals 0.0462 for *FLS2* expression when comparing only the C and U treatment.

Table 2
Effects of UV-B light on plant-pathogen interactions.

Plant-pathogen interaction	Effect of UV-B light	Reference
susceptible cucumber *vs. Colletotrichum lagenarium* or *Cladosporium cucumerinum*	UV-B pre-treatment increased disease severity	Orth et al. (1990)
sugar beet *vs. Cercospora beticola*	disease severity increased in combined exposure to UV-B	Panagopoulos et al. (1992)
susceptible wheat *vs. Puccinia recondita*	UV-B exposure increased severity of rust disease	Biggs et al. (1984) in Manning and Tiedemann (1995)
Arabidopsis *vs. Botrytis cinerea*	Increased plant resistance, independent of flavonoids, but involving sinapate production	Demkura and Ballaré (2012)

CHS up-regulation also in soybean (infected with *Phakopsora pachyrhizi*) or wheat (infected with *Puccinia graminis*) pointing to crosstalk manipulation. Gene duplication events (as observed e.g. for CHS family members in legumes) could allow differential expression of the multiple alleles under diverse conditions, especially noticeable in the two legumes soybean and *Medicago truncatula*. That is, copies, which are predominantly induced by biotic stress, might enable the production of flavonoid phytoalexins in the presence of microbes (= activated MTI). Only for tomato and *Physcomitrella patens* there was no obvious CHS suppression by biotic stress treatments, but this might be due to the very limited number of such experiments in the database. Fig. 6 suggests furthermore, that the host plant in turn reinstalls suppression of flavonoid production by down-regulation of FPGs in response to ETI, which is resembled by infection with *Pst* expressing the *avrRpt2* gene (Tsuda et al., 2009). Thus, it is also possible that single up-regulation of the CHS in response to virulent *Pst* DC3000 is a plant reaction to favour production of compounds such as phloretin (Fig. 1), which targets the bacterial T3SS and flagella (Vargas et al., 2013), while no other flavonoids are produced. Here we show by RNAseq that bacteria respond to phloretin as well as naringenin with induction of multidrug efflux pumps in conditions where the T3SS is not expressed (Table S1). This indicates that *Pst* defense against plant flavonoids is mainly attributed to T3SS-independent expression of efflux pumps and not to effector deployment. Among these efflux pumps we detected MexAB-OprM, which is required for counteracting the detrimental effects of these flavonoids on *Pst* DC3000 and full virulence (Vargas et al., 2011). Considering that life began in aquatic environments with no atmosphere screening space radiation, phenylpropanoid metabolites such as flavonoids first served solely as UV-B protectants. Motile pathogens, such as bacteria posed later a new threat, thus - before developing something completely new - modified flavonoids were used to ward them off. Later, when plants began to conquer the land, they evolved another branch of the phenylpropanoid pathway that produced lignin. Upon pathogen recognition plants had now to decide whether to restrict the invasion with flavonoids or lignin. Probably lignin was more effective, hence the suppression of flavonoid production. Thus, it appears that CHS activation to produce antibacterial flavonoids is an ancient defense response, substituted with an evolutionary newer plant strategy to suppress the FPGs during MTI and ETI in order to induce more effective defenses, such as lignin and phytoalexins.

3.2. Crosstalk between UV-B and biotic stress appears to be reciprocal

As stated in Schenke et al. (2011), there was no apparent and statistically significant influence of UV-B on the expression of the flg22-inducible marker gene *FRK1*, hence it appeared that suppression is rather unidirectional and not reciprocal, which would be an important prerequisite to consider this regulation “real crosstalk” (Jansen et al. (THIS ISSUE)). A meta-analysis of Arabidopsis expression data (Fig. S2) generally confirmed that up-regulation of pathogen defense genes correlated with suppression of FPGs, whereas the opposite was not observable when focusing on the FRK1 gene. In order to detect conversely regulated pathogen defense genes, 200 Arabidopsis perturbation experiments were selected upon co-regulation of six FPGs with GENEVESTIGATOR. Fig. S2 shows that the FPGs are also inducible by sucrose and it is known that this effect can be attenuated by concomitant flg22 or elf18 treatment as well (Saijo et al., 2009). Thus, experiments with Arabidopsis grown on sucrose-containing medium have been excluded from our meta-analysis. In 60 experiments most of the 6 FPGs showed up-regulation, while in 140 cases these genes were collectively down-regulated (Figs. S2A and S3A). The FPGs were generally suppressed by biotic stress, but five treatments showed somehow up-regulation of the key marker gene CHS. However, 4 of these treatments came from very early time-points with no clear induction of pathogen defense responses and CHS up-regulation by *G. orontii* at 6 h (study 2) stands even in contrast to a comparable study showing no significant regulation pointing to treatment effects in study #2. Such effects could be for example light-induction when control plants were directly harvested after the night period and treated plants are exposed additionally to light conditions during infection. When comparing FPG expression from these 60 experiments with 3 sets containing each six genes involved in MTI (Figs. S2B and S3B; Pattern Recognition- and Co-

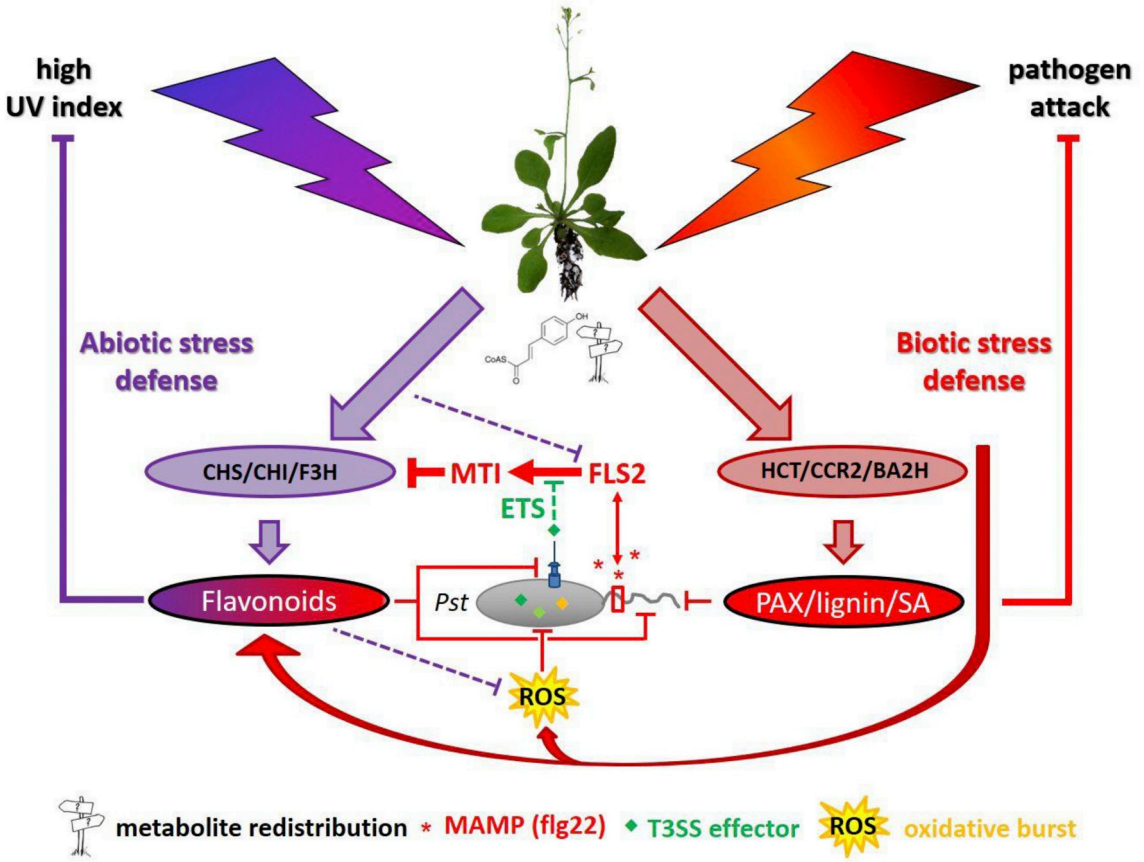

Fig. 8. Summary of different aspects in the interaction between abiotic UV-B and biotic pathogen stress responses. UV-B induces the production of flavonoids via activation of CHS and other FPGs, while pathogens trigger the production of phytoalexins (PAX), lignin or SA *via* HCT, CRR2 or BA2H, respectively. Reactive Oxygen Species (ROS) can be either produced in response to low or high doses of UV-B or during the oxidative burst in response to infection. Both hypotheses are incorporated in the center and potential links for suppression of biotic stress responses are indicated by dashed lines, suggesting reciprocal interaction between both abiotic and biotic stress signal transduction pathways.

Receptors, PRRs), involved in defense signalling, including FRK1 (Figs. S2C and S3C), or being involved in secondary metabolism for phytoalexin or lignin production (Figs. S2D and S3D), the FRK1 was suppressed only 7 times when FPGs were up-regulated. However, two other genes were suppressed 40 times (*FLS2*, encoding the flagellin receptor) or 33 times (*UGT72E1*, thought to be involved in lignin metabolism). Thus, we conducted an expression analysis of these two genes (Fig. 7) observing that these pathogen defense related genes were up-regulated in response to flg22 as expected. However, only the *FLS2* gene was negatively affected by UV-B and the co-treatment showed that biotic stress signalling initiated by flg22 application could override the negative effect of UV-B. In parsley cell cultures it was shown at the metabolite level that UV-B had also an attenuating effect on elicitor-induced coumarin production (Lozoya et al., 1991). Therefore, the negative effect of UV-B on basal FLS2 expression as suggested by the meta-analysis in Fig. S2B and confirmed in Fig. 7, respectively, adds a novel aspect to this now reciprocal crosstalk. It is further supported by the meta-analysis in Fig. S3B, showing that in the *uvr8* KO background FLS2 expression was increased. This points to a potential involvement of the UV-B receptor UVR8 in this crosstalk when it comes to UV-B effects on the biotic stress response. Because all FPGs are strongly down-regulated in the *uvr8* mutant (Fig. S3A), a further suppression by biotic stress cannot be investigated. An antagonistically regulation between FLS2 and the FPGs might still be biologically relevant, since attenuation of the flg22-induced MTI *via* dampening FLS2 expression under high UV-B levels could contribute to re-install an efficient UV-protection by enabling the production of flavonoids again.

Furthermore, plants can detect additional bacterial MAMPs, such as elf18 or lipopolysaccharides. However, other MAMP receptors, like EFR for elf18 recognition, are not so obviously down-regulated when FPGs are up-regulated (Fig. S2), whereas elf18-dependent MTI is also able to suppress the FPGs (Serrano et al., 2012). Therefore, testing long-time UV-B exposed plants for decreased FLS2 protein levels will be required in order to evaluate the effect of UV-B on overall flg22-induced defenses. Nevertheless, FLS2 activation can in turn inhibit light-induced stomatal opening (Zhang et al., 2008), supporting there is reciprocal crosstalk. Maybe it is critical to use the term crosstalk when arguing on different levels (e.g. stress perception, signal transduction, phytohormones, transcription factors/gene regulation or secondary metabolites). In this system we observe effects on the very end of UV-B signalling (expression of structural FPGs for metabolite production) and on the very beginning of flg22 signalling (on the stress perception level). Also a comparison on the level of secondary metabolism is complicated, since UV-B induced flavonoid secondary metabolites can contribute to both, UV-B protection (Li et al., 1993) as well as protection towards biotic stress exerted e.g. by infection with *Pst* (Figs. 3 and 4). Thus, this dual function of flavonoids makes this system additionally a nice example for cross-resistance (Jansen et al. (THIS ISSUE)).

Additional to induction of flavonoids, UV-B has been reported to trigger expression of PR1 and PDF1.2 in Arabidopsis *via* ROS production, albeit clear activation of PDF1.2 required unnaturally extensive (30 h) UV-B irradiation (Mackerness et al., 1999). This observation is further challenged by a report from Serrano et al. (2012), who demonstrated that naringenin pre-treatment attenuated the flg22-induced activation of PR-1 and PR-2 genes and that PR expression was increased at least in the CHS mutant line SALK_020583. Thus, the plant would

also benefit in this respect from CHS suppression during MTI. Intriguingly, Falcone Ferreyra et al. (2015) demonstrated that the Arabidopsis *dmr6* mutant is not a salicylic acid (SA) 3-hydroxylase, but it is involved in the production of flavones, such as apigenin. They suggest that pathogens could induce flavones to decrease SA levels, because the *dmr6* mutant has been shown to accumulate more SA. Whether flavone synthesis inhibits SA production or competes for a shared precursor (coumaric acid) remains to be elucidated. Furthermore, oxidative damage by UV-C has the potential to increase plant resistance, for example in the Arabidopsis - *Hyaloperonospora parasitica* interaction (Kunz et al., 2008). Flavonoids can scavenge such induced reactive oxygen species (Hideg et al., 2013), thus they might be also able to attenuate the oxidative burst, an important pathogen defense reaction that triggers the hypersensitive response (HR) against biotrophic pathogens. Consequently, those side effects may contribute to an altered plant resistance towards pathogens uncoupled from flavonoid production. If flavonoids in Arabidopsis are suppressed by biotic stress, but also contribute to plant defense it might be an option for the plant to store sufficient amounts as preformed phytoanticipins in the vacuole (Dixon and Pasinetti, 2010), which could allow a synthesis of both hypotheses. In order to visualize what is known and what we could expect from the crosstalk between the abiotic UV-B stress response and biotic bacterial infection Fig. 8 was drawn to give a quick overview of the above discussed findings.

3.3. Concluding remarks

All in all, it appears that the outcome of stress applications influencing the FPG expression/accumulation of flavonoids varies between different plant-pathogen systems and also depends on the time point of investigation. Naturally, most data concerning this regulation came from the model plant *Arabidopsis thaliana*, where it has been shown that the antagonistic regulation of FPGs involves positive regulators (e.g. the TFs MYB12 or MYB111) activated by UV-B and negative regulators, such as the TF MYB4, which can be induced by both UV-B and flg22 signalling (Schenke et al., 2011). Thus, MYB4 constitutes a shared component between two signal transduction pathways affecting each other. But can UV-B signalling really affect flg22 signalling? We have shown here indications for a reciprocal antagonism, because UV-B can suppress the FLS2 expression. However, these negative interactions do not take place on the same level, for example as the interplay between SA and abscisic acid (ABA), two important phytohormones regulating biotic and abiotic stress responses, respectively (Cao et al., 2011). ABA might also play a regulatory role in the context of the crosstalk discussed here, because the meta-analysis in Fig. S2 shows that FPG expression is rather up-regulated in response to some ABA treatments and negatively affected by SA application. Moreover, the GENEVESTIGATOR analysis based on Arabidopsis mutants (Fig. S3) adds some genetic indications, because the FPGs appear to be down-regulated in *aba1-1* and *abi1-1* (vs. Ler-0 background) or *abi4* and *aba1-6* (vs. Col-0 background). An involvement of ABA in UV-B responses is also supported by findings in maize, where it was shown that UV-B treatment increased ABA levels and ABA-mutants were more susceptible to UV-B radiation than the wild-type (Tossi et al., 2009, 2012). Additionally, in Arabidopsis UV-B could induce an increase in ABA levels (Rakitin et al., 2008) and in grape ABA contributes to UV-B protection (Berli et al., 2010). Furthermore, it appears there is a mutually antagonistic relationship between ABA and SA (Yasuda et al., 2008), which could also contribute to the regulation of FPGs as described above. Thus, the involvement of ABA could be beneficial for flavonoid production, while compromising plant immunity (Cao et al., 2011). Intriguingly, UV-B irradiation also induces ABA release from human cells (Bruzzone et al., 2012), suggesting that ABA is involved in a very ancient defense response to UV-B stress.

We conclude there is reciprocal crosstalk between abiotic and biotic stress signalling on the production of flavonoids, because of the several arguments presented here. Furthermore, flavonoids play a role in plant protection against both UV-B and pathogen infection, indicating there is potential for cross-resistance. Although the plant's pathogen defense response appears to prevail by suppressing the UV-B induced *FPG* up-regulation, a negative effect under rising UV-B levels depends not only on the inhibited *de novo* synthesis of flavonoids, but most likely also on storage capacities and flavonoid turn-over. Thus, the temporary suppression of flavonoid production by biotic stress might be bridged for a certain time. However, in light of the observed stratospheric ozone decline (Ball et al., 2018), this crosstalk could impact the plant's UV-B resistance/fitness under a continuous exposure to high UV-B levels.

4. Material & methods

4.1. Plant treatment with UV-B and flg22

Four *Brassica napus* seedlings were grown in pots with a soil/sand mixture under short day conditions (8 h light) and after two weeks transferred for two days to darkness in order to completely suppress *CHS* mRNA levels (Zhou et al., 2017). After two days in darkness two pots were sprayed with water, while the remaining two pots were sprayed with 1 mM flg22. To let flg22 taking effect the sprayed plants were incubated for two hours in darkness and were then exposed to UV-B or VIS-light as control for four h. A three mm thick glass plate was used to constrict the UV-B radiation emitted by two broadband UV-B lamps with an emission spectrum from 290 to 315 nm (Philips TL 20 W/12 RS) and two PROTEC. CLASS lamps (PLSL 18 W/21) for concomitant white light supply (12.69 m^2/s) to natural level which is sufficient to induce photomorphogenic UV-B response (0.53 µmol m2/s), while the control plants exposed only to VIS light were shielded with two additional layers of polyester plastic foil (Folanorm SF/AS 0.13 mm, Folex GmbH, Cologne, Germany). Thus, each biological replicate consists of the 4 treatments Water/VIS-light control (C), flg22 treatment (F), UV-B treatment (U) and the co-treatment flg22/UV-B (F/U). The treatment of *Arabidopsis thaliana* seedlings was similar as described in Zhou et al. (2017).

4.2. RNA isolation

Leaf material from *Brassica napus* was harvested into 2 ml tubes containing ca. 20 zirconium beads (Zircosil 1.2–1.7 mm, Mühlmeier mahltechnik, Germany), immediately flash-frozen in liquid nitrogen and stored at −80 °C for later RNA isolation. Total RNA was extracted with 1 ml TRIzol® reagent (Thermo Fisher Scientific) according to the manufacturer's instructions using a Precellys® Evolution ball-mill (bertin technologies) grinding for 3 × 20 s at 6800 rpm. Samples were incubated for 5 min at room temperature. Thereafter, 200 µl chloroform was added, vortexed vigorously and centrifuged at 10,000 rpm for 15 min at 4 °C. The clear supernatant was transferred into a sterile 1.5 ml tube and incubated at 4 °C for 30 min before being centrifuged at 12,000 rpm for 15 min at 4 °C. Then, the supernatant was discarded and RNA precipitate was washed twice with 1 ml 80% DEPC-ethanol and 1 ml 100% ethanol followed by centrifugation at 12,000 rpm for 5 min at 4 °C and discard the supernatant. The RNA pellet was dried in the air stream and stored at 4 °C after being dissolved in 50 µl DEPC-water.

4.3. cDNA synthesis and qPCR

1 µg total RNA was treated with RNase-free DNaseI (Fermentas) and then transcribed in a volume of 20 µl with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) into first strand cDNA according to suppliers' instructions. 2 µl of a 1:10 diluted cDNA preparation was mixed with 18 µl master mix as described in the manual of the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). PCR was performed on a CFX96 Touch Real-Time PCR Detection

System (Bio-Rad) using the following conditions: 3 min 95 °C; 40 × 15 s 95 °C, 30 s 59 °C, 30 s 72 °C, melting curve from 65 °C to 95 °C. Gene expression was determined using the delta C_T Method to calculate the fold-induction (compared to dark control samples) or the relative transcription levels (RTL) according to Pfaffl (2001). The data were normalized to PP2A and Actin as reference genes. Each data point is based on three independent biological replicates measured as two technical replicates each. The sequences of the gene-specific primer used in this study are given in Supplementary Table 2. Statistical analysis was carried out using a 2-Way ANOVA according to Minitab software (MINITAB, 2000).

4.4. Comparative transcriptome analysis

For RNA isolation, cells of *Pst* DC3000 were grown in LB with rifampicin (10 μg/ml) and incubated at 25 °C for 4–5 h to an OD_{660} = 0.5 in the absence or in the presence of sublethal concentrations of phloretin and naringenin [0.2 mM in dimethyl sulphoxide (DMSO)]. Cells were harvested, washed with sarkosyl 0.1% and cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C until RNA isolation. RNA was isolated for RNAseq, using TRI ReagentLS (Molecular Research Center, Cincinnati, OH, USA) as described before (Vargas et al., 2011). Residual DNA was removed with the RNase-free DNase I set (Roche). For comparative transcriptome analysis, library generation and sequencing was performed by Macrogen Inc. (Seoul, Republic of Korea) using Illumina HiSeq2000 to produce reads of 100 bp. Reads were clipped and trimmed to remove low quality nucleotides, Illumina adapters and rRNAs by using SeqTrimNext (Falgueras et al., 2010). Filtered reads were aligned to the *Pst* DC3000 reference genome with Bowtie (Langmead et al., 2009). SAMtools (Li et al., 2009) utilities were used to convert sam files to bam format, and to sort, count reads in order to analyse differential expression with RobiNA (Lohse et al., 2012). RNAseq data were deposited in the public database ArrayExpress at EBI under the assigned accession number E-MTAB-6426.

4.5. Bacterial preparation

Three strains of *Pseudomonas syringae pv. tomato* (*Pst*) were used in this study. Besides the wild-type DC3000, two mutant strains hrpL (transcriptional factor for virulence genes; Castillo-Lizardo et al., 2015) and *hrcQ* (T3SS-component; Badel et al., 2006) were cultured at 28 °C on solid NYGA medium (5 g/L bactopeptone, 3 g/l yeast extract, 20 ml/L glycerol, 15 g/L agar) supplemented with different antibiotics; for DC3000 50 mg/l Kanamycin and 50 mg/l Rifampicin, for HrpL 100 mg/l Kanamycin and for HrcQ 50 mg/l Spectinomycin. For infection assays a single colony was used to inoculate a fresh NYGA plate with the appropriate antibiotic. After 2 days 5 ml of 10 mM $MgCl_2$ were used to float off the bacteria and the suspension was then transferred into a 15 mL tube and centrifuged at 5000 rpm for 5 min. The supernatant was discarded and the bacterial pellet re-suspended with 2 mL $MgCl_2$. The OD600 was determined and set to 0.2 via dilution with 10 mM $MgCl_2$ yielding approximately 1 x 10^8 colony forming units/mL (cfu/mL). Before spray inoculation Silwet was added to a final concentration of 0,04%.

4.6. Arabidopsis thaliana treatment

Some plant-pathogenic fungi require UV light for sporulation or apothecia formation, leading to stronger disease symptoms on various vegetables under sunlight in greenhouse experiments without UV-absorbing (cut-off at 390 nm) vinyl films (Sasaki et al., 1985). On the other hand, UV-B reduced blister blight disease by *Exobasidium vexans* in tea plants, because the fungus is UV-B sensitive (Gunasekera et al., 1997). Thus, it is necessary to consider potential side-effects of UV-B on the pathogen itself. Orth et al. (1990) came to the conclusion that UV-B affected more the host plant than the fungal pathogen, because disease severity was similar in plants that received only UV-B pre-treatment and those that received additional UV-B treatment after the inoculation. This suggests that a pre-treatment with UV-B might be sufficient to observe effects. Since it is known that UV-B also damages *Pseudomonas syringae* (Miller et al., 2001), we decided to infect UV-B pre-treated Arabidopsis plants with *Pst*, assuming that they would have a higher flavonoid content than the untreated control. *Arabidopsis thaliana* Ler-0 (wild-type) and the flavonoid deficient mutant chs/f3h (N8585) were grown in pots with a soil/sand mixture under short day conditions (8 h light) for eight weeks. For the UV-B pre-treatment the plants were exposed for three days to six hours attenuated UV-B as described above, while the control plants were shielded additionally by two layers' polyester plastic foil. Before spray inoculation the plants were watered well and kept under a hood to increase the humidity for increased stomata opening. The plants were then sprayed with the bacterial suspensions and 10 mM $MgCl_2$ Solution including 0.04% Silwet as control until all leaves were dripping wet. The inoculated plants were placed back under a hood in the growth chamber (22 °C, short-day conditions) for 24 h. Then the hood was removed and the plants were left in the growth chamber until harvest at three days past inoculation (dpi). Samples were collected from two plants (three leaf discs each) for each of the three independent biological replicates, thus the final values represent six plants or 18 leaf discs in total.

4.7. Bacterial growth quantification by qPCR

At 3 dpi leaf disc samples were collected to quantify bacterial growth by qPCR. Three leaf discs from two plants of each independent biological replicate were pooled in a 2 ml tube containing ca. 20 zirconium beads, flash-frozen with liquid nitrogen and stored at −80 °C for later DNA isolation. DNA extraction was carried out with the GeneJet plant genomic DNA Purification Kit (Thermo scientific). 350 μL of lysis buffer A were added to the frozen samples, which were then ground using the ball-mill as described above. The remaining steps followed according to the kits instructions yielding 100 μl final DNA solution. Subsequently, the DNA concentration was adjusted to 4ng/μL and about 20 ng of DNA were subjected to qPCR analysis. The DNA-based quantification of *Pst* growth in Arabidopsis tissue was carried out as described by Ross and somssich. (2016). Here the PerfeCta SYBR Green Fast Mix (Quantabio) was used and the primer for biomass analysis are given in supplementary table 2. The CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was programmed as followed: 3 min 95 °C; 50 × 10 s 95 °C, 10 s 62 °C, 10 s 72 °C, melting curve from 65 °C to 95 °C for the bacterial biomass determination and 3 min 95 °C; 50 × 10 s 95 °C, 20 s 56 °C, 20 s 72 °C, melting curve from 65 °C to 95 °C for measuring the plant biomass.

4.8. Meta-analysis of public expression data

For the meta-analysis with GENEVESTIGATOR (Hruz et al., 2008) in Figs. S2 and S3 only experiments were chosen, which were based on Arabidopsis WT plants. Based on their conserved co-regulation six flavonoid pathway genes (set A) were chosen to select 150 perturbation experiments in which these genes were 52 times up- and 98 times down-regulated. Some pathogenesis related experiments were included to address if there is also up-regulation of CHS in response to pathogens in Arabidopsis and one elicitor experiment with only weak effects on suppression of this pathway. Three more sets of pathogen defense-related genes were compared to the FPGs. The set B consisted of six known genes for pattern recognition, set C of six genes for defense signalling and set C of six gene involved in production of secondary metabolites for pathogen defense.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.06.026.

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