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The role of miRNAs in regulating the expression of flavonol pathway genes and its possible impact on the crosstalk between UV-B and flg22 signal cascades in *Arabidopsis thaliana*

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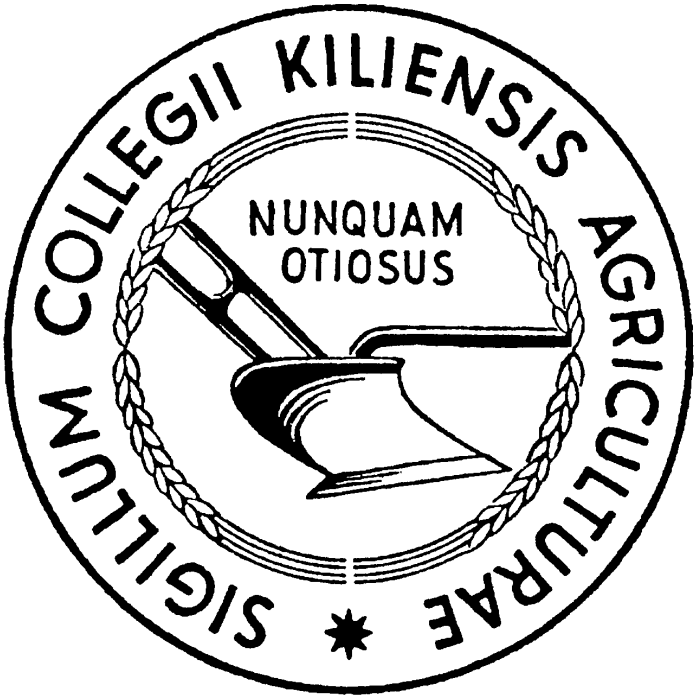

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List of Abbreviations

ABA	abscisic acid
AFB	auxin signaling F-box protein
AGO1	argonaute 1
AIM1	abscisic acid-induced myb 1
ANOVA	analysis of variance
ANS	anthocyanidin synthase
AP2	apetala 2
ARF	auxin response factor
ATAF	Arabidopsis transcription activation factor
BAK1	BRI1-associated kinase 1
bHLH	basic region helix-loop-helix
BIK1	botrytisinduced kinase 1
BOS1	botrytis susceptible 1
bp	base pair
°C	celsius
C4H	cinnamate 4-hydroxylase
CBF	C-repeat binding factors
cDNA	complementary DNA
CDPK	calcium-dependent protein kinase
cfu	colony forming unit
CHI	chalcone flavanone isomerase
CHS	chalcone synthase
cm	centimeter
Col-0	Columbia-0
COP1	constitutive photomorphogenic 1
Ct	threshold cycle
CUC	cup-shaped cotyledon
d	day
DCL1	dicer-like 1
DEG	differentially expressed gene
DFR	dihydroflavonol reductase
dpi	day post-inoculation
DREB	dehydration-responsive element-binding proteins
EAT	early activation tagged
ET	ethylene
ETI	effector triggered immunity
F3H	flavanone 3-hydroxylase
flg22	flagellin
FLS2	flagellin-sensing 2
FPG	flavonol pathway gene
GA	gibberellic acid
GAMYB	gibberellic acid MYB

GEO	Gene Expression Omnibus
GL2	glabrous 2
GO	gene ontology
GRAS	GAI-RGA-SCR
GRF	growth regulating factor
h	hour
H. schachtii	*Heterodera schachtii*
H3K9ac	histone 3 at lysine 9
HAM	hairy meristem
HAMPs	herbivore associated molecular patterns
HAT	histone acetylase
HD-ZIP III	class III homeodomain leucine zipper
HDAC	histone deacetylase
hpi	hour post-inoculation
HR	hypersensitive response
HY5	elongated hypocotyl 5
HYH	HY5 homolog
HYL1	hyponastic leaves 1
JA	jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
Ler-0	Landsberg-0
LRR	leucine rich repeat domain
LRRRLK	leucine-rich repeat receptor-like kinase
M	mol per liter
MAMP	microbe-associated molecular pattern
MAP2K/MKK/MEK	MAP kinase kinases
MAP3K/MAPKKK/MEKKs	MAP kinase kinase kinase
MAP4K	MAP kinase kinase kinase kinase
MAPK/MPK	mitogen-activated protein kinase
MFSN	miRNA functions based on functional similarity network
min	minute
MIR	miRNA gene
miRNA	microRNA
miTRATA	miRNA-truncation and tailing analysis
mRNA	messager RNA
MTI	molecular-pattern-triggered immunity
MYB	myeloblastosis oncogene
NAC	NAM, ATAF1, 2, and CUC2
NB	nucleotide binding domain
NCBI SRA	National Center of Biotechnology Information Sequence Read Archive
ncRNA	non-coding RNA
NLA	nitrogen limitation adaptation
NPR1	pathogenesis-related genes 1
Nramp6	natural resistance-associated macrophage protein 6

nt	nucleotide
ORF	open reading frame
P-SAMS	Plant Small RNA Maker Site
P1	pericarp color 1
PAMP	pathogen-associated molecular pattern
PAP1	production of anthocyanin pigment 1
PBL	PBS1-like
PCR	polymerase chain reaction
PFG	production of flavonol glycosides
phasiRNA	phased secondary siRNA
PHB	protein homeobox
PHV	phavoluta
PlanTE-MIR DB	plant TE related miRNA database
PMRD	Plant miRNA database
PMTED	Plant miRNA target expression database
PNRD	Plant non-coding RNA database
PPR	pentatricopeptide
PR	pathogen related
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PRRs	plant pattern recognition receptors
Pst	*Pseudomonas syringae* pv. *tomato*
PTGS	post-transcriptional gene silencing
PTI	PAMP triggered immunity
R-gene	resistance gene
RACE	rapid amplification of cDNA end
rasiRNA	repeat-associated small interfering RNA
RD26	*dehydration 26*
REV	revoluta
RISC	miRNA-induced silencing complex
RLK	receptor-like kinase
RNA Pol II	RNA polymerase II
ROS	reactive oxygen species
RRM	RNA recognition motif
rRNA	ribosomal RNA
RRTF1	redox-responsive transcription factor 1
RSV	*Rice Stripe Virus*
RT-qPCR	quantitative reverse transcription PCR
RTL	relative transcription levels
s	second
SA	salicylic acid
SAM	shoot apical meristem
SAR	systemic acquired resistance
SE	serrate

SID2	salicylic acid induction deficient 2
siRNA	small interfering RNA
SMZ	schlafmutze
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
SNZ	schnarchzapfen
SPL7	squamosa promoter binding protein-like 7
sRNA	small RNA
T-DNA	transfer DNA
TAPIR	target prediction for plant miRNA
TE	transposable element
TF	transcription factor
TIR1	transport inhibitor response 1
TOE	target of early activation tagged
ToLCNDV	*Tomato leaf curl New Delhi Virus*
ToLCV	*Tomato Leaf Curl Virus*
TPM	transcript per million
TPR	tetratricopeptide repeat
TRAM	transductive multi-label classification
tRNA	transfer RNA
tt	*transparent testa*
TuMV	*Turnip Mosaic Virus*
UFGT	UDP glucose-flavonoid 3-*O*-glucosyl transferase
UV-B	ultraviolet-B
UVR8	UV resistance locus 8
VSRs	viral suppressors of RNA silencing
WT	wild-type

Chapter I: General introduction

1 Plant defense mechanism in response to simultaneous abiotic and biotic stresses

Plants are confronted with various stress factors (abiotic and biotic) in their natural habitats and have to deal with multiple and complex interactions numerous environmental factors. Biotic stress factors include organisms such as symbionts, parasites, pathogens, herbivores and competitors, whereas abiotic stresses consist of parameters and resources, which determine plant growth like temperature, relative humidity, light, availability of water, mineral nutrients, and CO_2, as well as wind, ionizing radiation, or pollutants (Rejeb et al., 2014).

In the course of evolution, plants have evolved specific mechanisms allowing to adapt their environment and survive even under stressful conditions. In physiological view, exposure of plants to biotic and abiotic stress can induce a disruption/change in plant metabolism thus resulting in the reduction in plant fitness and ultimately in productivity (Suzuki et al., 2014). Abiotic stress is one of the most important factors with harmful impact on growth and cause severe losses in agriculture worldwide. The resulting reduction in plant growth can reach up to 50% in most plant species (Peleg and Blumwald, 2011). And, biotic stress constitutes an additional challenge to plants, capable of causing a severe damage in agriculture by pathogen or herbivore attack (Mordecai, 2011). Plants responses to these stresses are complex and involve numerous physiological, molecular, and cellular adaptions. A crucial step in plant adaptions is the timely perception of the stress in order to respond in an efficient and rapid manner. After recognition, the plants' constitutive basal defense mechanisms lead to an activation of complex signaling cascades of defense varying from one stress to another (Andreasson and Ellis, 2010; AbuQamar et al., 2009). Following exposure to abiotic and/or biotic stresses, specific ion channels and kinase cascades (Fraire-Velázquez et al., 2011) are activated, reactive oxygen species (ROS) (Nath et al., 2017), phytohormones like abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Großkinsky et al., 2016) are accumulated, and a reprogramming of the genetic machinery leads to adequate defense reactions and an increase in plant tolerance to minimize the biological damage caused by the stressful environment (Ramegowda and Senthil-Kumar, 2015; Ku et al., 2018).

1.1 Plant response to abiotic stresses

Plant abiotic stress includes all abiotic factors or stressors from the environment that can impose stress on a variety of species (Sulmon et al., 2015). These stresses encompass extreme levels of light (high and low), radiation (UV-B and UV-A), temperature (high and low, e.g., chilling, freezing), water (drought, flooding, and submergence), chemical factors (heavy metals and pH), salinity due to excessive Na^+, deficient or in excess of essential nutrients, gaseous pollutants (ozone, sulfur dioxide), mechanical factors and other less frequently occurring stressors.

Plants are rooted in the environment they grow in, and have to accommodate to the changing conditions brought about by a multitude of environmental factors. A huge challenge in plant abiotic stress research is to elucidate how plants perceive the different stressors, how the early signals are transduced within the plant, what is the diversity of response pathways elicited by these factors, and how they are genetically determined (Yoshida et al., 2014). Also for model plants and reference genotypes, the challenge remains to understand how signaling pathways have evolved within a species to program a suite of responses differing in signals and regulatory networks, and how genotypes are adapted to specific stressful environments. Many studies demonstrated the comparison of a few genotypes, tolerant and sensitive, for analysis of differential responses to a defined stress. Since these responses can be attributed to the differences in sets of genes in the genotypes, an understanding of the diversity in their signaling pathways offers opportunities to integrate diverse functional genomics, datasets of gene expression, metabolomics and stress physiological responses in the network of plant responses across genotypes.

Within the electromagnetic radiation spectrum, the UV radiation describes a spectral range between 200 and 400 nm, which borders on the visible range. The UV radiation is divided into three effective types: UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). Among them, UV-B is a crucial component of solar radiation to all terrestrial and aquatic plants, which were exposed during the early evolutionary phase of the earth. Zu et al. (2010) demonstrated there is a general consensus that UV-B induction generates physiological, biochemical, morphological and anatomical changes in the plants. According to the literature, the enhancement of UV-B radiation can affect the terrestrial plants at different functional levels

involving conformational changes and damages to different molecules such as DNA, proteins and lipids (Li et al., 2010). As a result, if damage to macromolecules, that is, DNA is not effectively repaired, and the UV-B effect will be translated to the biochemical level with the consequent alteration and/or impairment of the plant functionality (e.g., photosynthetic process, growth, yield). Even though it is clear that there is a wide range of both intra- and interspecific sensitivity to UV-B radiation (Gilbert et al., 2009), the plants through the evolution have acquired different protective strategies to avoid the harmful effects of UV-B radiation. The two major protective mechanisms are: (i) fielding through the production of soluble phenolics (e.g., flavonoids, anthocyanins, hydroxycinnamic acid derivatives), insoluble polyphenols (e.g., lignin), cell-wall bound UV-absorbing compounds (Csepregi et al., 2017; Csepregi et al., 2016; Machinandiarena et al., 2018), as well as by reflection of the UV-B radiation by epicuticular waxes and cuticular structures (Agati and Tattini, 2010); (ii) removal and direct reversion of the DNA lesions induced by UV-B radiation (Kimura et al., 2004).

The increase of secondary metabolites synthesis has been recognized as one of the most frequently observed plant response to UV-B irradiation (Bassman, 2004; Höll et al., 2019). Over the past two decades, considerable attention has been focused on the UV-B-induced biosynthesis of phenylpropanoid-derivative compounds, particularly flavonoids and hydroxycinnamic acid derivatives (Bassman, 2004). Although these compounds exhibit important interspecific differences induced by the UV-B irradiation, they are often derivatives of the flavonols quercetin and kaempferol (Buer et al., 2010). The flavonoids are ubiquitous molecules occurring in the vacuoles and cell walls of epidermal cells and in non-secretory and glandular trichomes. It has been assumed that they primarily have the function of attenuating the shorter solar wavelengths due to their high quantum efficiency (Burchard et al., 2000). In this regarding, the location of flavonoids in trichomes (Wei et al., 2019), cuticular wax layers (Fukuda et al., 2008), and epidermal cells (Burchard et al., 2000) may largely prevent that the UV-B radiation reaches sensitive targets within the plant.

The UV RESISTANCE LOCUS8 (UVR8) acts specifically to regulate the UV-B response, together with the expression of genes to establish the UV-B protection in plants (Jenkins, 2009). In addition, the UVR8 also mediates the expression of genes activated at low UV-B fluency level, showing

consistency with their involvement in the photomorphogenic UV-B responsive signaling pathway (Brown and Jenkins, 2008). No other component is known to act specifically in the photomorphogenic UV-B responses. The transcriptome analysis revealed that a set of approximately 70 genes are stimulated by UV-B irradiation under control of the UVR8. Among these several genes are known to have important roles in the UV-B protection mechanism, including those encoding principal enzymes of the flavonoid biosynthesis pathway, as well as DNA photolyases and enzymes involved in amelioration of the photooxidative damage (Jenkins, 2009). The Arabidopsis UVR8 mutant shows severe necrosis under exposure to UV-B levels found in the bright sunlight, whereas it is indistinguishable from the wild-type in the absence of UV-B irradiation (Brown and Jenkins, 2008). The UVR8 regulates the expression of both transcription factors (TFs) ELONGATED HYPOCOTYL5 (HY5) and HY5 HOMOLOG (HYH) at low UV-B fluency levels. The transcriptome analysis reports approximately the half of genes regulated by the UVR8 is also regulated by the TF HY5 (Brown et al., 2005). Further analysis suggests that the TFs HY5 and HYH may regulate all the genes of the UVR8 component, and therefore are pivotal downstream effectors of the UVR8-mediated signaling pathway. In fact, the HY5 is evidently a very crucial regulator of the UV-B responses because the HY5 mutant, similar to the UVR8 mutant, is very sensitive to UV-B exposure, while the HYH mutant is less sensitive, thus indicating that it has a subsidiary role in UV-B signal pathway (Brown and Jenkins, 2008). All these findings demonstrate that the UVR8 is a key regulator of the UV-B protection, helping to promote the survival of plants exposed to UV-B irradiation (Jenkins, 2009).

1.2 Plant response to biotic stresses

Biotic stresses cause plant damage by other living organisms such as bacteria, fungi, nematodes, viruses, insects, protists and viroids. The occurrence of new pathogen races and insect biotypes poses great threat to crop production (Sanghera et al., 2011). Phytopathogens account for about 15% losses in global food production, and are a major challenge in breeding resistant crops. Considering that genetic polymorphism is present in phytopathogenic agents and insect populations, changes in the climatic factors are considered to further influence on this polymorphism, consequently resulting in emergence of more aggressive strains or biotypes, which will substantially alter the established outcome of host-pathogen interactions (Anderson

et al., 2004a). Thus, disease or insect pest outbreaks might be expected even worsen by expanding to the areas they were not prevalent before (Ijaz and Khan, 2012).

Plant mechanisms of resistance to various pathogens and insect pests are known to involve an array of morphological, genetic, biochemical and molecular processes (Howe and Jander, 2008). These mechanisms may be expressed continuously as preformed resistance, or they may be inducible and deployed only after attack. Plant success in deploying these resistance mechanisms is an evolved ability to persist in stressful and variable environments (Nürnberger and Kemmerling, 2018). The recent realization that plant mechanisms of disease/insect resistance or susceptibility are mechanistically related to animal immunity has significantly reshaped our view of plant resistance mechanisms (Ausubel, 2005).

Plant innate immunity is divided into microbial-associated molecular-pattern-triggered immunity (MTI; also called PTI) and effector-triggered immunity (ETI). On the one hand, In MTI/PTI, innate immunity is defined by receptors for microbe-associated molecules, conserved mitogen associated protein kinase signaling cascades and the production of antimicrobial peptides/compounds (War et al., 2011). A common strategy employed by adapted pathogens is to secrete effector proteins that avoid or regulate PTI recognition. Therefore, the identification of plant pattern recognition receptors (PRRs) that sense pathogens' or insect pests' conserved molecules termed and the subsequent PTI is a typical example for plant-pathogen interaction studies (Monaghan and Zipfel, 2012). On the other hand, to counter stealth afforded by the microbial effectors, plants have evolved an intracellular surveillance involving polymorphic NB-LRR protein products encoded by resistance genes (R-genes), named after their characteristic feature of the presence of nucleotide binding (NB) and leucine-rich repeat (LRR) domains (Kiraly et al., 2007). This type of plant defence is referred to as ETI and is synonymous to pathogen race/host plant cultivar-specific plant disease resistance (Monaghan and Zipfel, 2012). ETI, which is the most successful means of controlling pathogens able to suppress or evade PTI, is a structural and functional basis of pathogen survival and evolutionary dynamics in their feeding mechanisms. It is now clear that effectors are determinants of pathogens' ability to suppress or evade the plant's arsenal targeted towards pathogen/herbivore associated molecular patterns (PAMPs/HAMPs) (Nürnberger and Kemmerling, 2018). ETI engages a compensatory mechanism

within the defense network to transcriptionally coordinate and increase the defense against pathogens. Although R-genes mediated resistance is generally not durable, ETI is now effectively deployed through pyramiding of several R-genes in the same cultivar, which boost resistance durability and spectrum (Onaga and Wydra, 2016).

Generally, PTI and ETI trigger similar defence responses in plants, but ETI is much faster and quantitatively stronger than PTI (Wei et al., 2013). ETI is often associated with a localized cell death termed the hypersensitive response (HR) that functions to restrict further spread of microbial attack (Kiraly et al., 2007; Dodds and Rathjen, 2010). Hence, the important feature of ETI is the ability to sense microbe-mediated modifications inferred on points of vulnerability in the host, whereas PTI is able to sense infectious-self and non-self. It is believed that by guarding against weak points or even setting up decoys to confuse invaders, ETI is an efficient defence system for more progressed infections (Malinovsky et al., 2014; Van der Hoorn and Jones, 2004), whereas PTI is important for non-host resistance and for basal immunity in susceptible host plant cultivars.

PTI is the first facet of active plant defence and can be considered as the primary driving force of plant-microbe interactions (Schwessinger and Zipfel, 2008). PAMPs occur throughout the pathogen classes, e.g. bacterial flagellin (*flg22*) and EF-Tu (*elf18*), fungal chitin (*CEBiP*) and mannans of yeast, xylanase (*LeEIX1/2*) and Oomycetes' heptaglucan (*HG*) (Dodds and Rathjen, 2010). The early responses induced by PAMPs occur within few minutes to hours and are varied, ranging from rapid ion fluxes across the plasma membrane, oxidative burst, activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) to local induction of defence-related genes or pathogen cell wall or cell membranes lyasing enzymes/peptides. Flagellin constitutes the main building block of bacterial flagellum, and is the best characterized PAMP in plants so far. A 22 amino acid (*flg22*) peptide-spanning region in the N-terminal part of flagellin of *Pseudomonas syringae* is sufficient to elicit the whole array of typical immune responses in plants (Felix et al., 1999). The PRR responsible for flagellin perception in the model plant *Arabidopsis thaliana* is the leucine-rich repeat receptor-like kinase (LRRRLK) *FLAGELLIN-SENSING 2* (*FLS2*). Functional *FLS2* homologs have been identified in other species, including tomato, grapevine, *Nicotiana benthamiana* and rice, suggesting that the receptors for

the *flg22* epitope of bacterial flagellin are evolutionarily ancient and conserved (Boller and Felix, 2009; Trdá et al., 2014). Despite evolutionary conservation, *FLS2* proteins from different plant species, such as tomato flagellin receptor (*LeFLS2*), grapevine (*VvFLS2*) and *Arabidopsis thaliana* (*AtFLS2*), still exhibit different perception specificities to elicitation determinants of flagellins (Trdá et al., 2014; Takai et al., 2008). This suggests that the domains found in *FLS* may have undergone some functional innovations that contribute to different perception specificities. Flagellin also seems to be recognized by other means in certain plant species. For instance, in rice, *flg22* epitope does not allow the activation of PRR, but flagellin induces cell death (Takai et al., 2008). Moreover, the glycosylation status of flagellin proteins is emerging as a determinant of recognizing adapted and non-adapted bacteria by *Solanaceae* plants, such as tobacco and tomato (Taguchi et al., 2006). Additionally, another flagellin, *flgII-28*, was identified in *Solanaceae* plant, though the corresponding PRR is yet to be identified (Cai et al., 2011). Both *flg22* and *flgII-28* are physically linked by a stretch of 33 amino acid residues, suggesting that both molecules are detected by the same receptor, *FLS2* (Clarke et al., 2013). The signaling pathways triggered in plant cells following *flg22* detection include rapid binding of FLS2 to *BRI1-associated kinase 1* (BAK1) by reciprocal transphosphorylation of their kinase domains (Chinchilla et al., 2007). The plasma membrane localized receptor-like cytoplasmic kinase *BOTRYTISINDUCED KINASE 1* (*BIK1*) and related *PBS1-LIKE* (*PBL*) kinases associate with *FLS2/BAK1* (Lu et al., 2010). The complex formed triggers multiple rapid phosphorylation events resulting in *BIK1* release. *BIK1* plays a central role in conveying signals from not only *FLS2* but also other PRRs, including *EFR*, *CERK1* and the DAMP receptor, *PEPR1/PEPR2*. The signal transduction downstream of *flg22* perception includes a Ca^{2+} burst, activation of *CDPKs* and *RbohD* required for the ROS burst and induction of *MAPK* cascades. These signaling cascades activate transcriptional reprogrammers such as the TFs *WRKY*, which are required for induction of defence gene expression (Ghareeb et al., 2011).

1.3 The crosstalk between abiotic stress and biotic stress signaling

From the perception of the stimulus/stress to the final response in cells, plants use various signaling pathways depending on the challenge(s). It seems that plants respond in a specific manner when they have to confront more than one stress simultaneously, and the response cannot be predicted based on the plants response to the individual single stresses (Atkinson and

Urwin, 2012). Research on multiple stresses has been trying to simulate natural conditions, but in the field, conditions are not controlled, and one stress can strongly influence the primary stress defense response of the plants (Fujita et al., 2006). Additional factors that can influence an interaction are the intensity of the stress and the plant species. Various interactions can take place between the defenses induced after perception of the stresses. They depend on the specific combination of stresses and even on the degree of simultaneity (Fraire-Velázquez et al., 2011; Rasmussen et al., 2013; Ramegowda et al., 2013). It is not clear whether simultaneous stresses are rather antagonistic, synergistic or additive, inducing more or less susceptibility to a specific kind of stress (Anderson et al., 2004b; Asselbergh et al., 2008). Combination of more than one stressor can have a negative and additive effect on plants, the second stress being the one that leads to a greater damage (Suleman et al., 2001). On the other hand, the combination of stresses can also lead to antagonistic responses in the plants (Ton et al., 2009; Yasuda et al., 2008).

Plants are able to manage simultaneous exposure to abiotic and biotic stress, and there is evidence for a link between the responses to these two stressful situations (Atkinson and Urwin, 2012; Wiese et al., 2004). Interestingly, one possible outcome of multiple stress exposure is that plants that are able to defend themselves facing one stress can become more resistant to other stresses. This phenomenon is called cross-tolerance, showing that plants own a powerful regulatory system that allows them to adapt quickly to a changing environment condition (Capiati et al., 2006; Suzuki et al., 2012). However, some plants confronted with each stress individually have also been reported to be more susceptible compared to a simultaneous exposure to two different stresses (Suzuki et al., 2014). In addition, certain environmental stresses have the possibility to predispose the plant in order to allow it to respond faster and in a resistant manner to additional challenges. Therefore, cross-tolerance between environmental and biotic stress may induce a positive effect and enhanced resistance in plants and have significant agricultural implications. Wounding, for instance, increases salt tolerance in tomato plants (Capiati et al., 2006). For instance, in tomato plants, localized infection by *Pseudomonas syringae* pv. *tomato* (*Pst*) induces systemic resistance to the herbivore insect *Helicoverpa zea* (Stout et al., 1999). The association between abiotic and biotic stress is also demonstrated by the reduced infection of tomato by *Botrytis cinerea* and *Oidium neolycopersici* following the application of drought stress (AbuQamar et al., 2009). In *Arabidopsis*, ozone exposure can induce resistance to virulent

Pseudomonas syringae strains (Sharma et al., 1996). Conversely, biotic stress can also interfere to boost the resistance to abiotic stress. This effect is visible when plants are under pathogen attack. Infection may cause stomatal closure to hinder pathogen entry and as a consequence water loss is reduced and leads to an enhancement of plant resistance under abiotic stress (Goel et al., 2008). Xu et al. (2008) showed that viral infection protects plants against drought stress. Interestingly, abiotic stress regulates the defense mechanisms at the site of pathogen infection as well as in systemic parts, thus ensuring an enhancement of the plant's innate immunity system (Yasuda et al.,2008). Likewise, osmotic and proton stress are inducers of resistance in barley against powdery mildew. This induced resistance depends on the formation of callose-containing papillae capable of blocking fungal growth (Wiese et al., 2004). Also, stress combination induces diverse signaling pathways, which share some components and common outputs (Atkinson and Urwin, 2012; Rasmussen et al., 2013). This might help plants to minimize energy costs and create a flexible signaling network (Jakab et al., 2005).

1.4 Signaling pathways induced by multiple stress responses

1.4.1 Mitogen-activated protein kinase cascades

Following perception and recognition of stress stimuli, mitogen-activated protein kinase (MAPK) cascades are activated. MAPK cascades have evolved to transduce environmental and developmental signals into adaptive and programmed responses. In a general model, stimulated plasma membrane receptors activate MAP kinase kinase kinases (MAP3Ks; also called MAPKKKs or MEKKs) or MAP kinase kinase kinase kinases (MAP4Ks). Sequential phosphorylations ensue as MAP3Ks activate downstream MAP kinase kinases (MAP2Ks; also called MKKs or MEKs) that in turn activate MAPKs. MAPKs then target various effector proteins in the cytoplasm or nucleus, which include other kinases, enzymes, or transcription factors (Cristina et al., 2010). They control the stress response pathways (Wurzinger et al., 2011). MAPKs are highly conserved in all eukaryotes and are responsive to signal transduction of diverse cellular processes under various abiotic and biotic stress responses, and certain kinases are involved in both stresses (Fujita et al., 2006). Since MAPKs are involved in different stress responses, they have an important role in the crosstalk of abiotic and biotic stress (Šamajová et al., 2013). In cotton, for instance, the kinase *GhMPK6a* negatively regulates both biotic and abiotic stress (Li et al., 2013). Moreover, MAPK

pathways activated by pathogen attack are mediated by SA, and the resulting expression of *PR* genes induces defense reactions (Xiong and Yang, 2003). In addition, MAPK such as MPK3, MPK4, and MPK6 also responded to various abiotic stresses (Gudesblat et al., 2007). MAPK cascades are involved in regulating the crosstalk between different stress responses (Andreasson and Ellis, 2010). MPK3 and MPK6 are essential for full primed defense responses (Beckers et al., 2009), therefore, these two kinases could be crucial for mediating tolerance to further stresses. In rice, overexpression of the *OsMPK5* gene and also kinase activity of OsMPK5 induced by ABA contributes to increased abiotic and biotic stress tolerance. *OsMPK5* seems to play a double role in the rice stress response, one as a positive regulator of resistance to the necrotrophic brown spot pathogen *Cochliobolus miyabeanus* and the second as a mediator of abiotic stress tolerance (Sharma et al., 2013). Furthermore, activation of *MPK1* and *MPK2* could confront with UV-B, wounding, and pathogens in order to increase their tolerance resistance in tomato plants (Holley et al., 2003). Also, MAPK signaling also interacts with ROS and ABA signaling pathways resulting in enhanced plant defense and induction of adaption to both abiotic and biotic stress (de Zelicourt et al., 2016).

1.4.2 Transcription factors and molecular responses in the crosstalk

Changes in gene expression occur after perception of a/biotic stress in plants and regulated by the action of transcription factors. The altered expression of certain genes is a key point in helping plants to set up an effective defensive state, and there is convincing evidence that many genes are multifunctional and able increase resistance in plants towards more than one stress (Jensen et al., 2008). The activity of such genes involved in defense is mediated by hormones like ABA, SA, JA and ET in plants. For example, the activity of the *BOTRYTIS SUSCEPTIBLE1* (*BOS1*) gene is regulated by both ABA and JA signaling pathways and induces resistance to both osmotic stress and necrotrophic pathogens, and *bos1* mutant plants are more susceptible to both stresses (Mengiste et al., 2003). In Arabidopsis, the TF *MYB96* plays an important role in protection under pathogen infection by mediating the molecular link between both ABA induced by drought stress and SA expressed following pathogen infection (Seo et al., 2010). Additionally, abscisic acid-induced myb 1 (AIM1) in tomato responds positively to the combination of abiotic stress and infection with *Botrytis cinerea* (AbuQamar et al., 2009) and *OsMAPK5*, which has kinase activity,

is a positive regulator of the response to drought, salt, and cold stresses and disease resistance in rice (Xiong and Yang, 2003).

Interestingly, many PATHOGENESIS RELATED (*PR*) genes are also upregulated upon exposure of a plant to abiotic stress ensuring disease resistance. PR proteins are also crucial for plant resistance against pathogens, and their expression is strongly upregulated when plants are infected (Seo et al., 2010). Over-expression of certain TFs in plants confronted with cold stress and pathogen infection (*Pst* DC3000) activates cold-responsive PR genes, thereby conferring protection against both stressors (Seo and Park, 2010). The upregulation of some TFs after treatment to abiotic stress leads to an accumulation of PR proteins. The TFs including C-repeat Binding Factors (*CBF*), Dehydration-Responsive Element-Binding proteins (*DREB*) and No Apical meristem ATAF and Cup-Shaped Cotyledon (*NAC*) have been extensively studied as players of the primary abiotic stress signaling pathways ensuring resistance under stress (Tran et al., 2010). Among them, *CBF* is induced under cold stress together with a set of PR proteins (Snider et al., 2000). Transgenic Arabidopsis overexpressing the TF *NAC* family member *NTL6*, which is induced by cold stress, boost their defense response against pathogen attack by promoting an upregulation of the *PR1* gene (Seo et al., 2010). Moreover, Tsutsui et al. (2009) showed that the TF *DREB* could regulate the response of cross-tolerance between abiotic and biotic stress insuring the resistance of response to cold and pathogen in Arabidopsis. Recently, it has been proposed that the WHIRLY1 protein and *REDOX-RESPONSIVE TRANSCRIPTION FACTOR1* (*RRTF1*) could participate in the traffic of communication between plastids and the nucleus (Foyer et al., 2014). WHIRLY1 perceives the redox changes in the plastid and carries the information to the nucleus in a nonexpressor of pathogenesis-related genes 1 (NPR1)-independent manner. The authors propose this protein as an ideal component in retrograde signaling that will lead to acclimation and adaption to new stresses. In the same way, *RRTF1*, which is induced by biotic and abiotic stresses, could be priming distant leaves to defend themselves against further stresses.

2 microRNA in plant

MicroRNAs (miRNAs), 20-24 nucleotides (nt) RNAs abundant in plants and animals, are a class of endogenous small noncoding RNAs that negatively regulate gene expression. They are produced

through a multistep process including transcription, precursor processing, methylation, and assembly of miRNA-induced silencing complex (miRISC). On the basis of sequence complementarity, miRNAs direct target mRNA cleavage, translational repression and DNA methylation. Recently, progress has been made on identification and functional of miRNAs in response to developmental and environmental cues, including that many miRNAs have previously unrecognized roles in regulating plant-environment interactions and cross-kingdom gene regulation as well (Song et al., 2019).

2.1 Plant microRNA biogenesis

miRNA biogenesis in plants, like that in animals, begins with the transcription of miRNA genes (MIRs) into primary miRNAs (pri-miRNAs) by RNA polymerase II (RNA Pol II) (Kim et al., 2010; Wang et al., 2018a). Most MIRs, which are usually located in intergenic regions, are independent transcription units. While only a few MIRs, located in the intronic sequences of protein-coding genes, can be cotranscribed with their host genes (Rajagopalan et al., 2006; Yan et al., 2012; Yang et al., 2012). The MIR genes are transcribed by RNA Pol II to generate single-stranded hairpin-containing primary transcripts (pri-miRNA). The pri-miRNA is then cleaved, in the nucleus, by Dicer-like 1 (DCL1), in association with hyponastic leaves 1 (HYL1) and serrate (SE), to produce a precursor miRNA (pre-miRNA). The pre-miRNA is, in turn, cleaved by DCL1 and its cofactors, thus generating a duplex composed of the mature miRNA and its complementary strand. Many other protein factors contribute to miRNA precursor processing through phospho-regulation, RNA splicing and other unknown molecular mechanisms. The miRNA duplex is then actively transported from the nucleus to the cytosol through interaction with the hasty (HST) exportin. One of the two strands of the duplex is then loaded onto the argonaute 1 (AGO1) protein, the main constituent of the multiprotein RNA-induced silencing complex (RISC). During RISC loading, one strand of the small RNA duplex is selected as the guide strand and incorporated into AGO1 to form a functional RISC, whereas the other strand is removed and degraded. The AGO1-associated strand guides the RISC to target mRNAs by sequence complementarity, resulting in target cleavage or the inhibition of protein synthesis (Zhu, 2008; Yu et al., 2017).

Pairing between most plant miRNAs and their target mRNAs results in cleavage of their target miRNAs within the region of pairing, yielding 5’ and 3’ cleavage products (Addo-Quaye et al.,

2008). The PIWI domain of AGO proteins, which forms a fold analogous to RNase H, constitutes the catalytic center (Yuan et al., 2005). Arabidopsis AGO1, AGO2, AGO4, AGO7 and AGO10 have the cleavage activity (Carbonell et al., 2012). Most 5’ and 3’ cleavage products are degraded by exonucleases. The cleavage products of transcripts targeted by 22-nt miRNAs, instead of being degraded, are stabilized by SUPPRESSOR OF GENE SILENCING 3 and converted by RNA-DEPENDENT RNA POLYMERASE 6 into double-stranded RNAs. The double-stranded RNAs are subsequently generated by DCL4 into 21-nt phased secondary siRNAs (phasiRNAs), which can, like miRNAs, guide AGO1 to cleave their target mRNAs (Fei et al., 2013).

miRNAs can also suppress gene expression through translational repression. Whereas target mRNA cleavage results in reduction in the levels of target mRNAs, translational inhibition leads to reduced accumulation of proteins translated from target mRNAs. The occurrence of both target mRNA cleavage and translational repression leads to a disproportional decrease in protein levels versus mRNA levels. In plants, most miRNAs and their target mRNAs have near-perfect sequence complementarity.

Even though, studies on miRNA-dependent DNA methylation is limited. Some studies provide compelling evidence that miRNAs are capable of directing DNA methylation. Bao et al. (2004) and Wu et al. (2010) demonstrated that directing DNA methylation is another mode of miRNA action, which is not at the posttranscriptional level, but at the transcriptional level, adding further complexity to miRNA-mediated regulation of gene expression. The major steps of miRNA biogenesis and actions and factors involved in these steps are illustrated in Figure 1.

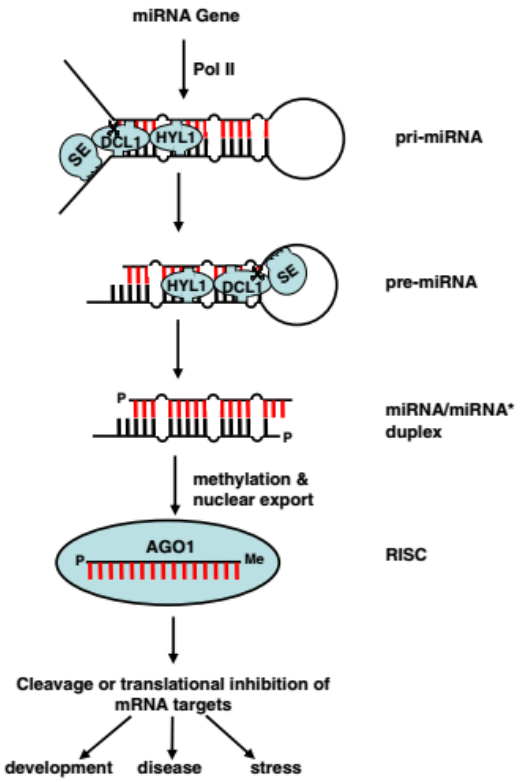

Figure 1 miRNA biogenesis and function in plants. Primary miRNA transcript is processed by the RNase III enzyme DCL1 (containing two double-stranded RNA-binding domains) and its associated RNA-binding cofactors HYL1 (containing two double-stranded RNA-binding domains) and SE (a C2H2-type zinc finger) to generate a miRNA, which is then methylated, exported to the cytoplasm and incorporated into the Agonaute 1 (AGO1)-containing RNA-induced silencing complex (RISC) to silence mRNA targets important for development, diseases, and stress responses. (Source: Zhu, 2008)

2.2 Computational Identification and target prediction of plant miRNAs

To identify miRNAs and their targets in plants, both experimental and computational approaches have been applied so far. Identification of miRNAs in plants started in early 2000s with direct-cloning approaches (Llave et al., 2002; Reinhart et al., 2002). The advent of high throughput/deep sequencing greatly promoted the miRNA research resulting in exponential growth in the number of plant miRNAs, not only identified but also functionally annotated (Jagadeeswaran et al., 2010; Rosewick et al., 2013). Several databases to archive miRNAs and their functional annotation such as miRBase have been established (Table 1).

miRBase (http://www.mirbase.org/) represents a comprehensive database with searchable online repository of published miRNA sequences and associated annotation. This database was started in 2002 with its first release included just 5 miRNAs from one plant species (*Arabidopsis*

thaliana), whereas its latest version (Release 22, October 2018) contains 38,589 entries representing hairpin precursor miRNAs, expressing 48,885 mature miRNA products in 271 species (Kozomara et al., 2019).

miRNEST represents a wide-ranging database of animal, plant and virus miRNAs (Szcześniak and Makalowska, 2014; Szcześniak et al., 2011; http://mirnest.amu.edu.pl). It provides miRNA data on the basis of computational predictions and high-throughput sequencing. It's current version (miRNEST 2.0), which contains miRNAs from 522 animal and plant species as well as 22 viruses. Interestingly, this database also includes degradome data of 2,041 entries.

Plant miRNA database (PMRD, http://bioinformatics.cau.edu.cn/PMRD/) provides information about plant-miRNA sequences as well as their targets (Zhang et al., 2009a). It contains the sequence information, secondary structure, target genes, expression profiles and a genome browser. PMRD contains more than 8,400 miRNA entries from >120 model plants as well as major crops including *Arabidopsis thaliana*, rice (*Oryza sativa*), wheat (*Triticum aestivum*), soybean (*Glyciene max*) and maize (*Zea mays*), besides providing predicted target-genes and interaction-site in the database. An updated version of PMRD has been launched as PNRD (Plant Non-coding RNA Database) with greater number of entries and plant species as well as broad spectrum RNA types (Yi et al., 2014). There are total 25,739 entries of non-coding RNA (ncRNA) including lncRNAs, tRNA, rRNA, tasiRNA, snRNA, and snoRNA from around 150 plant species, especially food crops. It offers various search and analysis tools for the user such as ncRNA keyword search, for example ID search, target search, and toolkits for predicting online novel miRNAs and for calculating coding potential along with the availability of BLAST tools (http://structuralbiology.cau.edu.cn/PNRD/index.php).

Plant miRNA Target Expression Database (PMTED, http://pmted.agrinome.org/) is a plant-specific miRNA database, useful to study miRNA functions by inferring their target gene expression profiles among the large amount of existing microarray data. It contains tools to search miRNA targets, retrieve expression data and the user can find out differentially expressed genes (Sun et al., 2013).

The TAPIR (target prediction for plant miRNAs) webserver (http://bioinformatics.psb.ugent.be/webtools/tapir/) predicts targets for plant miRNAs, with two

available modes, the first “Fast” mode using the FASTA search engine while the second “Precise” mode using the RNA-hybrid search engine (Bonnet et al., 2010).

miRPlant is an integrated tool for identification of plant miRNA from RNA sequencing data (An et al., 2014, http://www.australianprostatecentre.org/research/software/mirplant). miRPlant works on the strategies specifically developed to identify hairpin excision regions and hairpin structure filtering for plants. Interestingly, it does not need third party tools, rather, it uses a graphic user interface for input and output of the data, and the display of recognized miRNA with RNAseq reads is done with the help of a hairpin diagram.

PlantMirnaT developed by Rhee et al. (2015) is a miRNA and mRNA integrated analysis system via utilizing the sequencing data effectively. Its major features include a short read mapping tool, and an algorithm which takes into consideration the miRNA expression and distribution in target mRNAs (Rhee et al., 2015; https://sites.google.com/site/biohealthinformaticslab/resources).

miTRATA (miRNA-Truncation and Tailing Analysis) is a web-tool for truncation and tailing analysis of miRNA, and utilizes the miRBase (Release 22, October 2018) and useful to analyze 3’ modifications of miRNAs (Patel et al. 2016). In recent years, thorough miRNA biogenesis has revealed more complex features and secondary structures of their precursors. Consequently, Evers et al. (2015) proposed a freely available public access tool “miRA” (https://github.com/mhuttner/miRA) for identification of plant-miRNA precursors, which is adaptable to heterogeneous and complex precursor populations. Interestingly, Meng et al. (2016) developed a tool to predict the functions of plant miRNAs on the basis of their functional similarity network through application of transductive multi-label classification. Transposable elements (TEs) have been recognized for their prominent roles in determining non-coding regions including miRNAs of the genomes (Gim et al., 2014). In order to develop a plant TE related miRNA database (PlanTE-MIR DB) was proposed very recently by Lorenzetti et al. (2016). The authors identified more than 150 miRNAs overlapping TEs in 10 plant genomes. This pubic database is hosted at http://bioinfo-tool.cp.utfpr.edu.br/plantemirdb/.

It is noteworthy to mention that in spite of numerous computational tools to identify plant-miRNAs and their target prediction, there is a lack of curated databases of stress related miRNAs. Zhang et al. (2013) advocated for a need to construct a cohesive database system for data deposit

and further applications of plant abiotic stress related miRNAs and accordingly developed PASmiR, which allows the users to retrieve miRNA-stress regulatory entries by keyword-search such as plant species and type of stress (Zhang et al., 2013). Similarly, Remita et al. (2016) proposed a web-based server/database "WMP" dedicated to wheat miRNAs, particularly stress-responsive miRNAs and is available at: http://wheat.bioinfo.uqam.ca.

Table 1 A summarized list of major tools available for plant miRNAs, their target identification/prediction and repositories.

Name of the database/resource /repository/tool	Description	Web link	References
TAPIR	Target prediction for Plant miRs	http://bioinformatics.psb.ugent.be/webtools/tapir/	Bonnet et al., 2010
miRTarBase	The experimentally validated miR-target interactions database	http://mirtarbase.mbc.nctu.edu.tw/index.php	Hsu et al., 2011
PMRD	Plant miRNA Database	http://bioinformatics.cau.edu.cn/PMRD/	Zhang et al., 2009a
miRanalyzer	miR detection and analysis tool for next-generation sequencing experiments	http://bioinfo5.ugr.es/miRanalyzer/miRanalyzer.php	Hackenberg et al., 2011
PmiRKB	Plant miR Knowledge Base. Four major functional modules, SNPs, Pri-miRs, MiR-Tar and Self-reg, are provided	http://bis.zju.edu.cn/pmirkb/	Meng et al., 2011
miRDeep-P	A computational tool for analyzing the miR transcriptome in plants	http://faculty.virginia.edu/lilab/miRDP/	Yang and Li, 2011
C-mii	A tool for plant miR and target identification	http://www.biotec.or.th/isl/c-mii	Numnark et al., 2012
Semirna	Searching for plant miRNAs using target sequences	http://www.bioinfocabd.upo.es/semirna/	Muñoz-Mérida et al., 2012
mirTool	A comprehensive web server providing detailed annotation information for known miRs and predicting novel miRs that have not been characterized before	http://centre.bioinformatics.zj.cn/mirtools/	Wu et al., 2013
PASmiR	A literature-curated database for miR molecular regulation in plant response to abiotic stress		Zhang et al., 2013
miRBase	Searchable database of published miR sequences and annotation	http://www.mirbase.org	Kozomara and Griffiths-Jones, 2013

miRPlant	An Integrated Tool for Identification of Plant MiR from RNA Sequencing Data	http://www.australianprostatecentre.org/research/software/mirplant	An et al., 2014
MTide	An integrated tool for the identification of miR-target interaction in plants	http://bis.zju.edu.cn/MTide/	Zhang et al., 2014
PNRD	It is an updated version of PMRD	http://structuralbiology.cau.edu.cn/PNRD/index.php	Yi et al., 2014
PlantMirnaT	A miRNA-mRNA integrated analysis system	https://sites.google.com/site/biohealthinformaticslab/resources	Rhee et al., 2015
miRA	Plant miRNA identification tool especially for organisms without existing miRNA annotation. It is also useful for identifying species-specific miRNAs	https://github.com/mhuttner/miRA	Evers et al., 2015
miPEPs	MiRNAs Encode Peptides is a tool for functional analysis of plant miRNA family members		Couzigou et al., 2015
sRNAtoolbox	A set of tools for expression profiling and analysis of sRNA bench results	http://bioinfo5.ugr.es/srnatoolbox	Rueda et al., 2015
miRge	A fast multiplexed method of processing sRNA-sequence data to determine miRNA entropy and identify differential production of miRNA isomiRs	http://atlas.pathology.jhu.edu/baras/miRge.html	Baras et al., 2015
BioVLAB-MMIA-NGS	MiRNA and mRNA integrated analysis using high-throughput sequencing data coupled with bioinformatics tools.	http://epigenomics.snu.ac.kr/biovlab_mmia_ngs/	Chae et al., 2015
DMD	A dietary miRNA database from 15 dietary plant and animal species	http://sbbi.unl.edu/dmd/	Chiang et al., 2015
WMP	Database for abiotic stress responsive miRNAs in wheat	http://wheat.bioinfo.uqam.ca	Remita et al., 2016
miTRATA	A tool for miRNA truncation and tailing analysis	https://wasabi.dbi.udel.edu/~apps/ta/	Patel et al., 2016
MFSN	A tool for prediction of plant miRNA functions based on functional similarity network (MFSN) through application of transductive multi-label classification (TRAM) to the MFSN		Meng et al., 2016
PlanTE-MIR	Database for transposable element-related plant microRNAs	http://bioinfo-tool.cp.utfpr.edu.br/plantemirdb/	Lorenzetti et al., 2016

P-SAMS	A Plant Small RNA Maker Site (P-SAMS) is a web tool for artificial miRNAs and synthetic trans-acting small interfering RNAs	http://p-sams.carringtonlab.org	Fahlgren et al., 2016

2.3 Plant-derived miRNA in regulating plant response to a/biotic stress

A/biotic stresses cause up- or down-regulation of miRNAs or expression of new miRNAs in plants. A set of miRNAs has been identified, which are responsively expressed under various biotic and abiotic stress conditions, including nutrient deficiency (Liang et al., 2015), drought (Ferdous et al., 2015), cold (Zeng et al., 2018), heat (Zhao et al., 2016), salinity (Parmer and Shaw, 2018), UV-B radiation (Zhou et al., 2007; Sunitha et al., 2019) and mechanical stress (Fal et al., 2016) as well as fungal, bacterial, viral and nematode infections (Islam et al., 2018).

2.3.1 Plant-derived miRNA in regulating plant response to abiotic stress

Ultraviolet-B: Ultraviolet-B (UV-B) irradiation causes molecular damage at the DNA, RNA levels along with hyper accumulation of ROS (Zhou et al., 2007). In a study on Arabidopsis, 21 putative light-responsive miRNAs belonging to 11 different miRNA families-miR156, miR159, miR160, miR165/166, miR167, miR169, miR170, miR172, miR393, miR398 and miR401 were reported (Zhou et al., 2007). Some members of the same miRNA families, that were predicted to be upregulated by UV-B radiation in Arabidopsis (miR156, miR160, miR165/166, miR167, miR398 and miR168) were however found to be upregulated by UV-B radiation in *Populus tremula*. The three miRNA families of miR159, miR169 and miR393 were up regulated in Arabidopsis but down regulated in *P. tremula*. Recently, Sunitha et al. (2019) demonstrated that co-expression patterns of the upregulated miRNAs miR156, miR482, miR530, and miR828 with cognate target gene expressions in response to high-fluency UV-B in grapevine berry. These data suggest species-specific response of plants to UV-B irradiation stress (Jia et al., 2009).

Cold Stress: MiR319c is believed to be a biomarker for plant response to cold stress. miR319c regulates the mRNA levels of TCP transcription factors (Palatnik et al., 2007), while miR397 targets the laccase family members LAC2, LAC4 and LAC17, which are important in the lignification process (Bosch et al., 2011). The upregulation of miRNAs upon cold stress, including miR393,

miR397b, miR402 and miR319c of Arabidopsis was first reported by Sunkar and Zhu (2004). Many miRNAs were found to be significantly induced e.g. miR165/166, miR169, miR172, miR393, miR396, miR397 and miR408 while some other miRNAs including miR156/157, miR159/319, miR164, miR394 and miR398 were only transiently regulated in response to cold stress (Zhou et al., 2008). miR397 and miR169 were found to up regulated in Arabidopsis (Sunkar and Zhu, 2004; Liu et al., 2008), Populus (Lu and Huang, 2008) and Brachypodium (Zhang et al., 2009b) while miR172 was up regulated in Arabidopsis and Brachypodium. In Populus, miR168ab and miR477ab were up regulated while miR156g-j, miR475ab and miR476a were found to be down regulated (Lu and Huang, 2008). Moreover, 18 miRNAs, including miR156k, miR166k, miR166m, miR167abc, miR168b, miR169e, miR169f, miR169h, miR171a, miR535, miR319ab, miR1884b, miR444a.1, miR1850, miR1868, miR1320, miR1435 and miR1876 were identified in rice in response to cold stress (Lv et al., 2010). More recently, in Brassica rapa, eight conserved (miR166h-3p-1, miR398b-3p, miR398b-3p-1, miR408d, miR156a-5p, miR396h, miR845a-1, miR166u) and two novel miRNAs (Bra-novel-miR3153-5p and Bra-novel-miR3172-5p) were differentially expressed in leaves of 'Longyou 7' under cold stress (Zeng et al., 2018).

Heat Stress: Sudden increase in temperature (heat shock) leads to denaturation of proteins (enzymes) and interferes with cellular machineries to repair proteins and membranes. In response to heat stress, differential miRNA expression was observed in wheat. Among the 32 miRNA families found in wheat, nine were conserved heat responsive miRNAs. Among others, miR172 was down regulated and miRNAs-miR156, miR159, miR160, miR166, miR168, miR169, miR393 and miR827 were up regulated in plants in response to heat stress (Xin et al., 2010). Moreover, miR398-CSD/CCS-HSF, miR396-WRKY6 and miR159-GAMYB pathways regulate plant heat tolerance (Zhao et al., 2016).

Salinity Stress: Salinity is the most serious abiotic stress. High salinity interferes with the plants' ability to uptake water causing similar conditions as drought (Munns, 2005). In rice, three salt-inducible members of the miR169 family-miR169g, miR169n and miR169o and miR393 were found (Zhao et al., 2009; Gao et al., 2011), which specifically cleaves the NF-YA gene transcripts. Moreover, in Arabidopsis, miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394, miR396 and miR397 were found to be up regulated while miR398 was

down regulated (Liu et al., 2008). In *P. vulgaris*, miRS1 and miR159.2 were found to be highly upregulated (Arenas-Huertero et al., 2009). In *P. trichocarpa*, miR530a, miR1445, miR1446a-e, miR1447 and miR171l-n were down regulated whereas miR482.2 and miR1450 were up regulated (Lu and Huang, 2008). A microarray experiment on salt-tolerant and salt-sensitive *Zea mays* identified 98 miRNAs from 27 families. miR156, miR164, miR167 and miR396 families were reported to be downregulated, while miR162, miR168, miR395 and miR474 families were up regulated in salt-shocked maize roots (Ding et al., 2009). The various other important miRNAs involved in salinity stress, in different plant species are summarized by Parmer and Shaw (2018).

Drought Stress: Drought, one of the most dominant abiotic stresses, was caused due to shortage in precipitation, soil water deficiency and excess of evaporation (Shukla et al., 2008). Plants tolerate drought conditions by enhanced water uptake, reduced water loss and by other mechanisms (Bartels and Sunkar, 2005). Recent studies on *Triticum dicoccoides* (Kantar et al., 2011), cowpea (Barrera-Figueroa et al., 2011), soybean (Kulcheski et al., 2011), *Phaseolus vulgaris* (Arenas-Huertero et al., 2009) and tobacco (Frazier et al., 2011) have identified differential expression patterns of miRNAs in response to drought stress. In Arabidopsis, miR396, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158 and miR169 were found to be downregulated in response to drought (Liu et al., 2008) where miR393, miR319, and miR397 were up regulated (Sunkar and Zhu, 2004). Induction of miR393 is linked to drought stress, in which it decreases the TIR1 levels, a positive regulator of plant growth and development, hence resulting in retardation of plant growth. In rice, miR169g, miR171a and miR393 were all induced in response to dehydration (Zhou et al., 2010; Jian et al., 2010). The result of a study of genome wide profiling on drought-stressed rice found that 16 miRNAs (miR156, miR159, miR168, miR170, miR171, miR172, miR319, miR396, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088 and miR1126) were down regulated and 14 miRNAs (miR159, miR169, miR171, miR319, miR395, miR474, miR845, miR851, miR854, miR896, miR901, miR903, miR1026 and miR1125) were up regulated in response to drought stress (Zhou et al., 2010). In Populus, miR171l-n, miR1445, miR1446a-e, miR1444a, miR1450, miR482.2, miR530a, miR827, miR1448 and miR1447 were identified to be drought-responsive miRNAs (Lu et al., 2008). In *P. vulgaris*, miRS1, miR1514a and miR2119 were found to be moderately up regulated whereas miR159.2, miR393 and miR2118 were found to be highly up regulated (Arenas-Huertero et al., 2009), while

in *Medicago truncatula*, miR169 was found to be down regulated only in roots, where miR398ab and miR408 were strongly up regulated (Trindade et al., 2010). From the drought-tolerant wild emmer wheat (*Triticum turgidumssp. dicoccoides*) 13 differentially regulated miRNAs were identified, including miR1867, miR896, miR398, miR528, miR474, miR1450, miR396, miR1881, miR894, miR156, miR1432, miR166 and miR171 (Kantar et al., 2011). The various other important miRNAs involved in drought stress, in different plant species are summarized by Ferdous et al. (2015).

2.3.2 Plant-derived miRNA in regulating plant response to biotic stress

Bacterial infection: Nearly all plants and animals have PRRs, which are able to perceive PAMPs as signal and activate host defense mechanisms (innate immunity) against bacterial infection. In Arabidopsis, miR393, induced by a bacterial PAMP was the first miRNA showing a vital role in PTI by negatively regulating the auxin signaling pathway (Navarro et al., 2006). Using small RNA-expression profiling on Arabidopsis infected by leaves collected at 1 and 3 h post-inoculation (hpi) with *Pst DC3000 hrcC*, Fahlgren et al. (2007) identified three miRNAs (miR160, miR167 and miR393) which were highly induced except for one (miR825) that was downregulated. The role of miRNAs in plant basal defense was further supported by the finding that Arabidopsis miRNA-deficient mutants *dcl1* and *hen1* showed enhanced growth of the bacterium *Pst DC3000 hrcC* and several bacteria strains that are non-pathogenic to Arabidopsis (Navarro et al., 2008). Although many miRNAs are induced to repress negative regulators of plant defense, some are however depressed, which can help strengthen plants against pathogens. In Arabidopsis, miR398b and miR773 proved to be downregulated by flg22, resulting in enhancement of callose deposition and plant resistance against bacteria (Stork et al., 2010). Upon bacterial infection, miR400 is downregulated (Park et al., 2014; Zhang et al., 2011), leading to enhanced expression of *PENTATRICOPEPTIDE REPEAT1/2* genes, both of which encode mitochondria-localized pentatricopeptide repeats and likely contribute to PTI by controlling ROS metabolism (Torres and Dangl, 2005). Moreover, miR398 in Arabidopsis was downregulated by infection with Pst either carrying *avrRpm1* and Pst *avrRpt2* but was unaffected by *Pst DC3000* infiltration (Jagadeeswaran et al., 2009).

Viral infection: In rice, miRNAs, including miR160, miR166, miR167, miR171, and miR396 family of miRNAs, were found to accumulated in response to *Rice stripe virus* (RSV, a negative sense and ambisense RNA virus) infection (Du et al., 2011), which implies that miRNAs also involved in developing viral resistance against viral infection in plants possibly by targeting viral suppressors of RNA silencing (VSRs). In order to circumvent plant host defense mechanism (RNA silencing), plant viruses activate their counter-defense mechanism by expressing VSRs (Voinnet, 2005). In addition, *Turnip mosaic virus* (TuMV)-encoded RNA silencing repressor P1/HC-Pro has been found to have link with miR171. In Arabidopsis, P1/HC-Pro induced miR156 and miR164 was reported (Zhou and Luo, 2013). A novel miRNA, brassica-miR1885 was found to be induced by TuMV infection (He et al., 2008). In *Tomato Leaf Curl Virus* (ToLCV) miR159 was found up regulated while miR164 and miR171 were reported down regulated (Naqvi et al., 2008). In a study, the miR159/319 and miR172 were found as potential biomarkers for Tomato leaf curl New Delhi virus (ToLCNDV) infection (Naqvi et al., 2010). The role of individual miRNAs in response to viral infection has not yet been reported.

Fungal infection: Upon plant-fungal interaction, plant's miRNAs along with their targets are either upregulated or downregulated modulating the disease establishment (Zhao et al., 2012; Gupta et al., 2012). For instance, miR2118 have been predicted to target TIR-NBS-LRR in cotton infected with *Verticillium dahliae* (Yin et al., 2012), while pbe-SR23 and pbe-SR3 have been predicted to target TIR-LRR in *Populus* upon infection with *Dothiorella gregaria* (Chen et al., 2012). Campo et al. (2013) have shown Osa-miR7695 mediated negative regulation of natural resistance-associated macrophage protein 6 (OsNramp6) in disease resistance and over-expression of Osa-miR7695 in rice has resulted in resistance to blast fungus. miR396 has been predicted to target a set of transcription factors GROWTH-REGULATING FACTOR (GRF) family to regulate the resistance to infection by both necrotrophic fungi, *Plectosphaerella cucumerina* and *Botrytis cinerea*, and hemibiotrophic fungi, *Fusarium oxysporum* f. sp. *conglutinans* and *Colletotrichum higginsianum* (Soto-Suárez et al., 2017). Additionally, in rice, miR164a was downregulated by the *M. oryzae* infection and consequently its target TF *OsNAC60* was depressed, leading to enhanced defense response against *M. oryzae* (Wang et al., 2018b). Shen et al. (2014) identified 62 oilseed rape miRNAs responsive to *Verticillium longisporum* infection. Upon *Verticillium* infection, miR160, miR167, miR390 and miR164 are downregulated. While, miR1885 was also reported to be highly

upregulated in the infected roots in *B. napus*. The validated target of miR1885 encodes a TIR-NBSLRR R protein (Bra027889). More recently, Salvador-Guirao et al. (2018) demonstrated that rice plants with activated MIR166k-166h expression exhibited resistance to infection by the fungal pathogens *M. oryzae* and *Fusarium fujikuroi*, the causal agents of the rice blast and bakanae disease, respectively.

Nematode infection: A number of miRNAs displaying changes in expression in root cells in response to nematode infection have recently been identified in various plant species (Jaubert-Possamai et al., 2019). Sequencing identified 30 mature differentially expressed miRNAs in Arabidopsis syncytia induced by *Heterodera schachtii* (*H. schachtii*) at 4 and 7 dpi (Hewezi et al., 2008). Tian et al. (2017) identified 60 miRNAs in soybean from 25 miRNA families as differentially expressed miRNAs relative to uninfected roots in susceptible and/or resistant cultivars and validated the expression of most of these miRNAs by qPCR. A recent analysis of syncytia from tomato plants infected with *Globodera rostochiensis*, performed at 3, 7, and 10 dpi, identified between 200 and 300 miRNAs at each stage as differentially expressed miRNAs (Koter et al., 2018). In Arabidopsis, miR396 was repressed at the onset of syncytium formation in roots infested with *H. schachtii* and upregulated at later stages, whereas its target transcription factors GRF1, GRF3, and GRF8, displayed the opposite pattern (Hewezi et al., 2012). Recently, Noon et al. (2019) reported that a repression of the miR396 family associated with an upregulation of soybean GRF genes was observed in soybean syncytia induced by *Heterodera glycines* at 8 dpi. A combination of 5' RLM-RACE and a reporter gene approach demonstrated that the GRF6 and GRF9 genes were targeted by miR396 in syncytia, indicating the miR396/GRF module is essential for *Heterodera glycines* infection, and this role is conserved in Arabidopsis and soybean (Hewezi et al., 2016). Furthermore, they demonstrated an inverse correlation in the expression of the conserved miR827 and its known target NLA (nitrogen limitation adaptation) during syncytium development in Arabidopsis (Hewezi et al., 2016). In addition, a role for the miR858/MYB83 module has been established in Arabidopsis syncytia induced by *H. schachtii*, in which an inverse correlation of transcript levels was observed between miR858 and its target MYB83 at 7, 10, and 14 dpi (Piya et al., 2017). Modulation of the expression of these genes through gain- and loss-of-function approaches altered the Arabidopsis response to nematode infection, demonstrating a role for this module in syncytium formation.

3 Secondary metabolite

It is well known that plants respond actively to stress by producing phytoalexins and other metabolites. Flavonoids are representative plant secondary metabolites. In the model plant *Arabidopsis thaliana*, at least 54 flavonoid molecules (35 flavonols, 11 anthocyanins and 8 proanthocyanidins) are found. In general, flavonoids are sub-classified into several families including flavonol, flavone, flavanone, flavan-3-ol, isoflavone and anthocyanidin according to the structure of and the modifications to the A, B and C rings which constitute their structural chemical (Panche et al., 2016).

3.1 Biosynthetic pathway of flavonoid

The biosynthesis of flavonoids in plants has been extensively characterized (Tohge et al., 2017; Petrussa et al., 2013). Flavonoids are synthesized via the phenylpropanoid and polyketide pathway, which starts with the condensation of one molecule of CoA-ester of cinnamic acid or derivatives such as coumaric or ferulic acid, and three molecules of malonyl-CoA, yielding a naringenin chalcone as major product. This reaction is carried out by the enzyme chalcone synthase (CHS). The chalcone is isomerised to a flavanone by the enzyme chalcone flavanone isomerase (CHI). From these central intermediates, the pathway diverges into several branches, each resulting in a different class of flavonoids. Flavanone 3-hydroxylase (F3H) catalyzes the stereospecific 3β-hydroxylation of (2S)-flavanones to dihydroflavonols. For the biosynthesis of anthocyanins, dihydroflavonol reductase (DFR) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanins), which are converted to anthocyanidins by anthocyanidin synthase (ANS). The formation of glucosides is catalyzed by UDP glucose-flavonoid 3-*O*-glucosyl transferase (UFGT), which stabilizes the anthocyanidins by 3-*O*-glucosylation (Bohm, 1998). An overview of the flavonoid biosynthetic pathway is presented in Figure 2. Flavonoids play an important role in plant defense, and CHS as the gatekeeper of flavonoid biosynthesis plays an important role in regulating the pathway. In fact, *CHS* gene expression is influenced by many stress and environmental factors such as UV, wounding or pathogen attack (Gläßgen et al., 1998; Ito et al., 1997).

3.2 Regulation of CHS gene expression

CHS is the first committed enzyme in the biosynthesis of all flavonoids. This enzyme belongs to a family of type III polyketide synthase, which is one of the general polyketide synthases and forms a catalyticallyactive single polypeptide (Austin and Noel, 2003). CHS catalyzes the so-called Claisen-ester condensation concomitant with CO_2 liberation from malonyl-CoA and acyl-thioester (i.e., *p*-coumaroyl-CoA). Actually CHS catalyzes the formation of a triketide intermediate from pcoumaroyl-CoA and three molecules of malonyl-CoA; then spontaneous cyclization of the triketide intermediate results in the formation of naringenin chalcone (Austin and Noel, 2003). There are three CHS-like genes forming a small gene family in Arabidopsis. Among them, *AtCHS* (*TT4*, At5g13930) has been identified as the gene participating in the synthesis of flavonoids, and thus the mutant of this gene is designated as *transparent testa* (*tt*) *4* since it lacks proanthocyanidin formation in the seed coat (Shirley et al., 1995). Two other genes, *PKSA/LAP6* (At1g02050) and *LAP5/PSKB* (At4g34850), are known to encode hydroxyalkyl-α-pyrone synthases which are essential for pollen development and the biosynthesis of sporopollenin, the constituent of exine in the outer pollen wall (Kim et al., 2010). In Arabidopsis, parsley and snapdragon only a single copy of the *CHS* gene has been identified. In most angiosperms CHS has been shown to be encoded by a multigene family, such as in petunia (violet 30) (Koes et al., 1987), morning glories (Ipomoea) (Durbin et al., 2000), Gerbera (Meng et al., 2004), leguminous plants (Aoki et al., 2000) and *Cannabis sativa* (Sanchez, 2008).

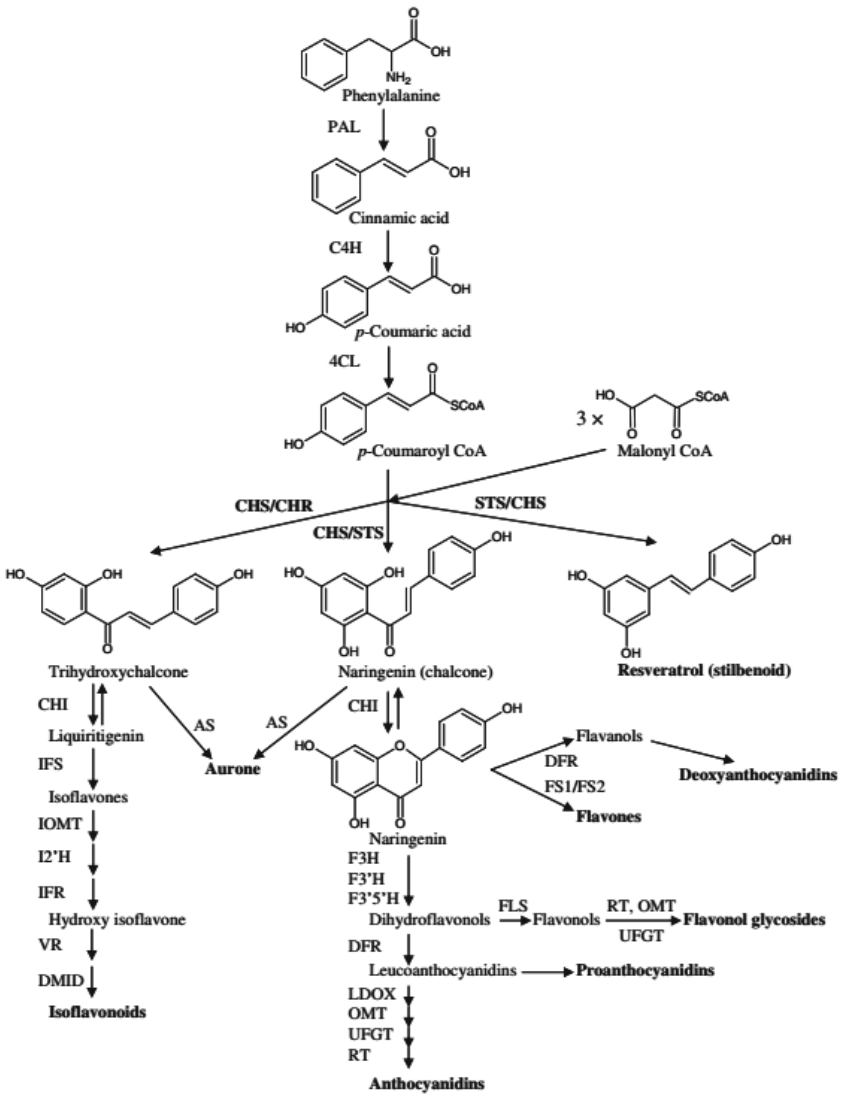

Figure 2 Flavonoid biosynthetic pathway. ANS anthocyanidin synthase; AS aureusidin synthase; C4H cinnamate-4-hydroxylase; CHR chalcone reductase; DFR dihydroflavonol 4-reductase; DMID 7,2'-dihydroxy, 4'-methoxyisoflavanol dehydratase; F3H flavanone 3-hydroxylase; F3'H flavonoid 3' hydroxylase; F3'5'H flavonoid 3'5 hydroxylase; FS1/FS2 flavone synthase; I2'H isoflavone 2'-hydroxylase; IFR isoflavone reductase; IFS isoflavone synthase; IOMT isoflavone O-methyltransferase; LCR leucoanthocyanidin reductase; LDOX leucoanthocyanidin dioxygenase; OMT O-methyltransferase; PAL phenylalanine ammonialyase; RT rhamnosyl transferase; UFGT UDP flavonoid glucosyl transferase; VR vestitone reductase; STS stilbene synthase; FLS flavanol synthase. (Winkel-Shirley, 1999; Yamaguchi et al., 1999; KEGG pathways) (Source: Dao et al., 2011)

Many studies have shown that the *CHS* gene is constitutively expressed in flowers, but also its expression can be induced by light or UV light and in response to phytopathogens, elicitors or wounding in different parts of the plant, resulting in enhanced production of flavonoids (Koes et

al., 1987; Ryder et al., 1984, 1987; Bell et al., 1986; Burbulis et al., 1996). The *CHS* expression is also regulated by the circadian clock (Thain et al., 2002).

The level of *CHS* gene expression is reflected by the level of the *CHS* transcripts in plant cells. The *CHS* promoter was studied extensively in *Phaseolus vulgaris*, *Antirrhinum*, *Arabidopsis* and parsley (Dixon et al., 1994; Faktor, 1997; Feinbaum et al., 1991; Lipphardt et al., 1988). Studies on the *CHS* promoter in *Arabidopsis thaliana* and many other species have led to the identification of several consensus *cis*-elements such as the G-box (GACGTG) and/or H-box (CCTACC) that are important for transcriptional activation of *CHS* in response to abiotic elicitation or environmental stimuli (Schulze-Lefert et al., 1989; Staiger et al., 1989; Van der Meer et al., 1990; Harrison et al., 1991; Loake et al., 1992; Hartmann et al., 1998). The *cis*-elements found in the defined minimal Arabidopsis *CHS* promoter are bound by basic region helix-loop-helix (bHLH) and R2R3-MYB-type TFs (Hartmann et al., 2005). Several R2R3-MYB and bHLH TFs were found to interact with the WD40-containing protein TRANSPARENT TESTA GLABRA1, to form a regulatory complex controlling multiple enzymatic steps of flavonoid biosynthetic pathway (Broun, 2005).

3.3 CHS activity in plant resistance

In nature plants are exposed to a variety of biotic and abiotic stresses. Flavonoids have diverse biological functions in plant growth, development, and environmental adaptation, including UV protection, antioxidation, defense responses, and attraction of pollinators and animals for seed-dispersal (Winkel-Shirley, 2001; Muhlemann et al., 2018). During stress conditions, a plant is expressing a number of genes as part of its defense. Among these genes, CHS is quite commonly induced in different plant species under different forms of stress like UV, wounding, herbivory and microbial pathogens resulting in the production of compounds that have e.g. antimicrobial activity (phytoalexins), insecticidal activity, and antioxidant activity or quench UV light directly or indirectly.

Phenolic compounds like flavonoids strongly absorb UV light and thus are able to protect plants from DNA damage caused by UV. Anthocyanins belong to a class of flavonoids that accumulate in leaves and stems as plant sunscreen in response to light intensity (Leyva et al., 1995). Expression of CHS genes is known to be regulated by light through a photoreceptor-mediated mechanism (Koes et al., 1989). In several cases, it was found that the photoregulated production of flavonoids

is at least in part due to the transcriptional induction of CHS (Feinbaum and Ausubel, 1988; Taylor and Briggs, 1990). Examination of CHS expression in parsley cell culture suggested that a UV-B light receptor, a blue light receptor and phytochrome may all play a role in light-induced CHS expression (Ohl et al., 1989). High intensity light and UV-A were found to regulate expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants (Feinbaum et al., 1991). High-intensity light treatment of Arabidopsis plants for 24 h caused a 50-fold increase in CHS enzyme activity and an accumulation of visibly detectable levels of anthocyanin pigments in the vegetative structures of these plants (Feinbaum and Ausubel, 1988).

4 **Aim and outline of the thesis**

In nature plants are often simultaneously challenged by different stress factors. The abiotic stress UV-B irradiation induces the production of UV-protective flavonols, but their accumulation is attenuated by biotic stress, e.g. by treatment with pathogen elicitors (flg22). This suppression has been shown to occur via suppression of flavonol pathway genes (FPGs) enabling the plant to direct its secondary metabolism to a more efficient pathogen defense response. In this study, we employed Arabidopsis plants to unravel the molecular mechanism underlying this signal crosstalk.

Experiments based on Arabidopsis cell cultures treated with water (control), flg22 (a 22 amino acid peptide from bacterial flagella as a MAMP), UV-B (to enhance FPG expression) and co-treated with UV-B/flg22 (suppressing the UV-B-induced FPGs), showed that this kind of “crosstalk” could be investigated in this model plant. Chalcone synthase (CHS) was induced by UV-B but suppressed when flg22 was additionally applied (Schenke et al., 2011; Schenke et al., 2014; Schenke and Cai, 2014). There are already indications that this suppression is mediated by transcription factor (TF) regulation. Two MYB TFs, the positive regulator MYB12 (UV-B upregulated and flg22 suppressed) and the negative regulator MYB4 (activated by UV-B, but much faster by flg22) have been implicated in the regulation of the FPGs (Figure 3). However, it is still unknown if these TFs are the crucial players in this crosstalk or just co-regulated with the FPGs. Moreover, since miRNA acts as an important post-transcriptional regulator in many processes of plant, it is reasonable to assume that miRNAs constitute a crucial regulatory layer in the crosstalk of plant responses to biotic and abiotic stress. Therefore, the overall aim of this thesis is to explore the role of miRNAs

in regulating the expression of flavonol pathway genes and its possible impact on the crosstalk between UV-B and flg22 signal cascades in Arabidopsis.

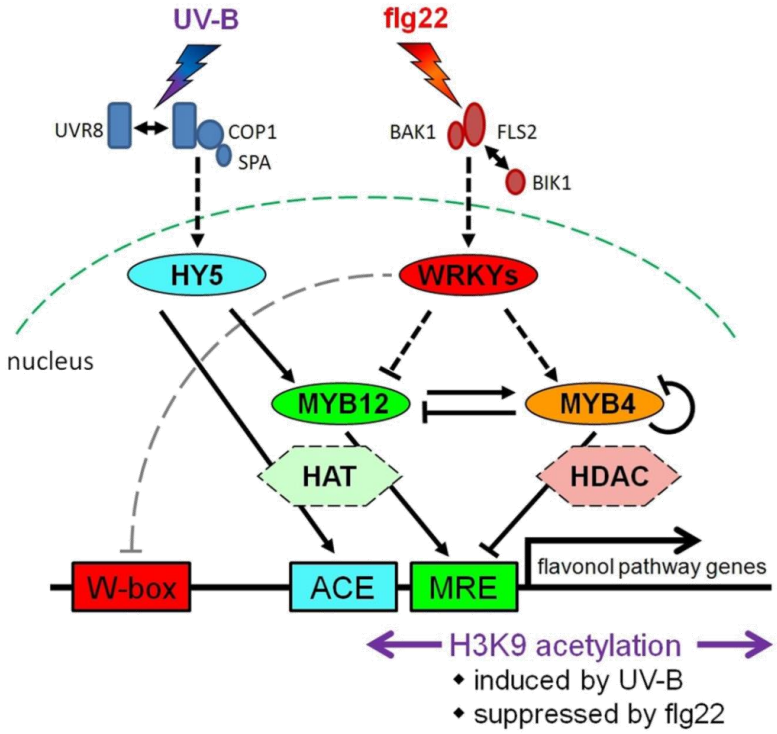

Figure 3 Working model displaying the interaction of transcription factors in regulation of the flavonol pathway genes (FPGs) in Arabidopsis cell cultures. The suppressive effect of flg22-induced signaling (MTI) on UV-B-activated FPG expression may be mediated by WRKY TFs via downregulating the positive regulator MYB12 and/or upregulating the negative regulator MYB4. The two MYB TFs are good candidates to recruit histone acetylase (HAT) or histone deacetylase (HDAC) activity, respectively, thereby being responsible for changes in H3K9 acetylation at the FPG loci.

To this end, four working programs were followed in this thesis:

1) Establishment of Arabidopsis in planta system for studying the crosstalk between flg22 and UV-B induced flavonol signaling pathways;
2) RNAseq- and small RNAseq-based analysis for identification of key miRNAs involved in the crosstalk of flg22 and UV-B induced signaling pathways;
3) Identification, validation and functional characterization candidate miRNAs and target genes and
4) Investigation on the mode of action of the miRNA-target interaction and its impact on the crosstalk between flg22 and UV-B induced signaling pathways.

The present dissertation contains four chapters. These are:

Chapter I: General introduction

Chapter II: Investigation of the crosstalk between the flg22 and the UV-B induced flavonol pathway in *Arabidopsis thaliana* seedlings

Chapter III: Identification and characterization of microRNAs involved in the crosstalk between flg22 and UV-B induced signal cascades in *Arabidopsis thaliana*

Chapter IV: miR858 and miR828 are master regulators for UV-B- and flg22-signal crosstalk in *Arabidopsis thaliana*

Chapter V: General discussion

5 Reference

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Chapter II: Investigation of the crosstalk between the flg22 and the UV-B induced flavonol pathway in *Arabidopsis thaliana* seedlings

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1 Abstract

In cell culture, we have shown that flavonol metabolite accumulation depends on expression of the Arabidopsis flavonol pathway genes (FPGs), which are upregulated by UV-B irradiation but repressed by the bacterial elicitor flg22 during MAMP Triggered Immunity (MTI). The suppression of flavonoid production during MTI is believed to allow the plant focusing its metabolism on the pathogen defense by directing phenylalanine resources from UV-B protective flavonol production towards production of phytoalexins and cell wall fortification by lignin incorporation during MTI. Here we extend our observations made initially in cell cultures by describing that this kind of signal crosstalk between UV-B and flg22 is also functional in planta. We demonstrate that such signal crosstalk is fully functional in Arabidopsis in planta. However, we observed some differences in the expression patterns of MYB transcriptions factors (TFs) as compared to data from the cell culture system. Our data suggest that in planta the TF MYB111 might play a more dominant role than the TF MYB12, which was strongly regulated in cell cultures. Thus we can present an updated working model how this crosstalk might function. We believe that this system based on seedlings of the model plant *Arabidopsis thaliana* constitutes a valuable platform for further dissection of the underlying molecular mechanism, e.g. by deploying gain/loss-of-function of candidate genes.

2 Introduction

In their environment plants are continuously exposed to various stresses of abiotic and biotic nature. In case of UV-B radiation, plants defend themselves for example with the production of sinapoylmalate and flavonoids, which function as natural sunscreens (Li et al., 1993; Landry et al., 1995; Meissner et al., 2008). Since more than two decades it is known that UV-B-induced flavonol pathway genes (FPGs) are downregulated when concomitant elicitation by microbe-associated molecular pattern (MAMPs) occurs (Lozoya et al., 1991; Gläßgen et al., 1998). This induces the so-called MAMP triggered immunity (MTI), a plant defense response against pathogens, reprogramming the secondary metabolism to produce anti-microbial phytoalexins and lignification to prevent the pathogen spreading within the host tissue (Schenke and Cai, 2014). Because all flavonoids, lignin and the phytoalexin scopoletin are derived from the same precursor phenylalanine a suppression of UV-B induced flavonoids is suitable to provide more resources for the pathogen defense. Experiments based on Arabidopsis cell cultures treated with water (control), flg22 (a 22 amino acid peptide from bacterial flagella as a MAMP), UV-B (to enhance FPG expression) and co-treated with UV-B/flg22 (suppressing the UV-B induced FPGs), showed that this kind of “crosstalk” could be investigated in this model plant. Comparing UV-B and UV-B/flg22 co-treatment showed striking correlation between acetylation of histone 3 at lysine 9 (H3K9ac) and gene expression of several FPGs additional to chalcone synthase (CHS) protein and flavonoid metabolite (e.g. quercetin- and kaempferol-derivatives) accumulation, all being induced by UV-B but suppressed when flg22 was additionally applied (Schenke et al., 2011; Schenke et al., 2014). There are already indications that this suppression is mediated by transcription factor (TF) regulation. Two MYB TFs, the positive regulator MYB12 (UV-B upregulated and flg22 suppressed) and the negative regulator MYB4 (activated by UV-B, but much faster by flg22) have been implicated in the regulation of the FPGs (Schenke and Cai, 2014). However, it is still unknown if these TFs are the crucial players in this crosstalk or just co-regulated with the FPGs. In order to address this question and get some genetic evidence we had to establish first a seedling based system allowing us to test mutant plants for impaired crosstalk. Here we describe this system as a prerequisite step to analyze homozygous mutants in this crosstalk between abiotic and biotic stress responses.

3 Materials and methods

3.1 Plant treatment

9 seedlings of Col-0 (WT) or *fls2* (SALK_062054) were grown together on a single Jiffy (Jiffy-7 Peat Pellets, Jiffy Products International AS, Norway); and after 6 weeks transferred to darkness in order to completely suppress *CHS* mRNA levels. After 2 days in darkness 1 pot was harvested as the dark control (D) and 2 pots were sprayed with water, while the remaining 2 pots were sprayed with 1 mM flg22 (Figure 1). To let flg22 taking effect the sprayed plants were incubated for another hour in darkness and were then exposed to UV-B or VIS-light as control for 4 h. Thus, each biological replicate consists of the 5 treatments dark control (D), Water / VIS-light control (C), flg22 treatment (F), UV-B treatment (U) and the co-treatment flg22 (UV-B (F/U). UV-B treatment was conducted as described before and UV-B levels were attenuated to natural levels by a glass plate or completely filtered by 2 sheets of polyester plastic foil as also described in Schenke et al. (2011). We decided to implement a dark period, because we wanted to ensure completely depleted CHS levels to get a strong induction effect by UV-B, which is necessary to observe later clear differences when mutants are compared to the wild-type plants. Also the cell cultures used in Schenke et al. (2011 and 2014) were kept in the dark until used for experiments, so both systems are easier comparable. Last, but not least the dark control allows observing the specific enhancing effect of UV-B on FPG expression, since UV-B has to be applied together with VIS light, which can also induce FPG expression alone.

In contrast to 1 µM flg22 treatment of cell cultures, treatment of whole seedlings proved to be more difficult. Even contact with flg22 solution by dipping was not practicable, so we decided to spray flg22. In order to achieve a clearly induced *CHS* suppression we had to use 1 mM flg22 for spraying (supplemental Figure 1). This appears very high. However, the actual concentration hitting the plant surface will not be that high. To rule out eventual side effects of such a high peptide concentration and to check if water can serve as an appropriate control we tested also 2 other flg22 preparations, which are supposed to be inactive. In comparison to active flg22 *from Pseudomonas sp.* (QRLSTGSRINSAKDDAAGLQIA) we applied *Agrobacterium sp.* flg22 (ARVSSGLRVGDASDNAAYWSIA) and *Sinorhizobium sp*. flg22 (AHVSSGLRVGQAADNAAYWSIA) and observed no negative effect compared to pure water (supplemental Figure 2), thus 1 mM flg22

can be used to spray seedlings. As an additional control for successful flg22 treatment we measured the expression of *FRK1*.

3.2 Harvest and RNA isolation

Of each treatment nine whole seedlings were harvested in a 2 ml tube containing ca. 20 zirconium beads (Zircosil 1.2-1.7 mm, Mühlmeier mahltechnik, Germany) for later RNA isolation, immediately flash-frozen in liquid nitrogen and stored at -80°C until further processing. Total RNA from seedlings was extracted with 1ml TRIzol® reagent (Thermo Fisher Scientific) according to the manufacturer's instructions using a Precellys® Evolution ballmill (bertin technologies) grinding for 3 x 20 sec at 6,800 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 new 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.

3.3 cDNA synthesis and qPCR

1 µg total RNA was treated with RNase-free DNaseI (Thermo Scientific) 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 supplier's 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: 10 min 95°C; 40 x 15 sec 95°C, 30 sec 59°C, 30 sec 72°C; 10 sec 95°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 (AT1G13320) as a reference gene, which is a gene found to be very stably expressed in many plants under almost all conditions (Schenke et al., 2011). Except for supplementary data, 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 1. Statistical analysis was carried out using a 2-Way ANOVA according to Minitab software (MINITAB, 2000).

3.4 Promoter analysis

The promoters of miR858 (AT1g71002), MYB11 (AT3g62610), MYB12 (AT2g47460) and MYB111 (AT5g49330) were analyzed using the "Highlight *cis*-elements" script developed by Sebastian Wolf (IPB). W-boxes were identified upon TTGAC(C|T), ACE elements upon CACGT and MRE upon (ACCNACC) sequences. The program is accessible under http://msbi.ipb-halle.de/cisHighlight/.

4 Results

Plants were treated as described in detail within the M&M part. Each biological replicate consists of 9 seedlings pooled for RNA isolation and includes the 5 treatments dark control (D), Water / VIS-light control (C), flg22 treatment (F), UV-B treatment (U) and the co-treatment flg22/UV-B (F/U). UV-B levels were attenuated to natural levels by a glass plate and completely filtered by polyester plastic foil as described in Schenke et al. (2011). Figure 1 gives an overview about the workflow to obtain material from one biological replicate.

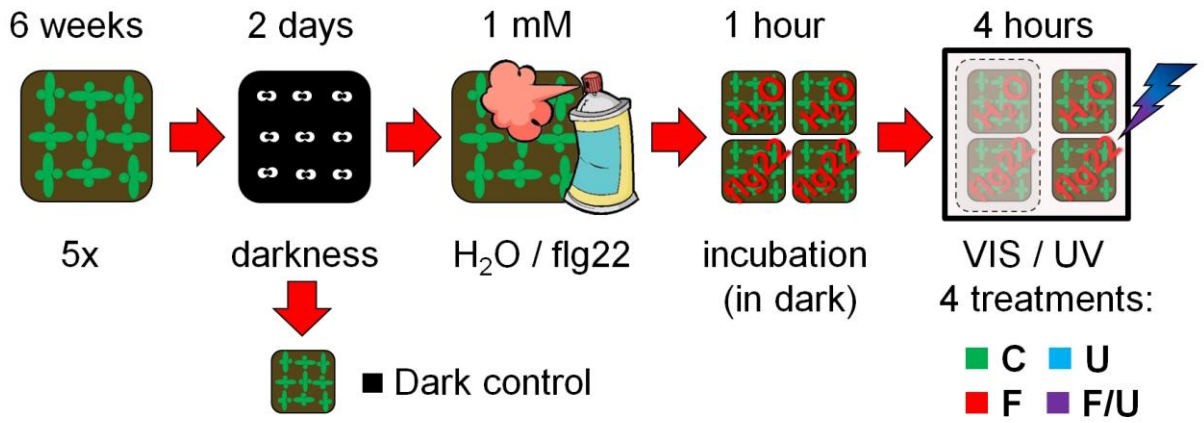


Figure 1 Flow-chart of the experimental setup. The seedlings were treated as indicated, finally flash-frozen in liquid nitrogen and stored at -80 °C before samples were subjected to RNA isolation.

We decided to spray flg22 onto the Arabidopsis seedlings, because dipping proved to be not reproducible (not shown) and found in preliminary tests that 1 mM flg22 peptide concentration is necessary for a clearly visible effect on chalcone synthase (CHS) expression (Figure S1). The CHS is the key-enzyme of the flavonoid pathway, being strongly induced by UV-B and suppressed by various biotic stress treatments, e.g. elicitation with the MAMP flg22. Therefore, this FPG is a good marker to analyze if the crosstalk takes place. Additionally, we checked FRK1 expression as a control for successful flg22 treatment. Though the flg22 concentration applied appears high, spraying means also that not every molecule comes into contact with the plant, so the real concentration on the plants surface is definitely lower. But to exclude any potential side effect of this concentration we decided to compare biologically active flg22 from pathogenic *Pseudomonas sp.* with versions based on *Agrobacterium sp.* and *Sinorhizobium sp*. sequences (Figure S2). These

had no different effect than the water control, so 1 mM flg22 is a suitable concentration for spraying and water can be used as an appropriate control.

Deploying these conditions, we could confirm that the down-regulation of flavonoid pathway genes (exemplified by CHS expression) during MTI elicited through flg22 is also functional in planta (Figure 2). Expression of the flg22-inducible marker gene FRK1 was checked to ensure efficient flg22-treatment.

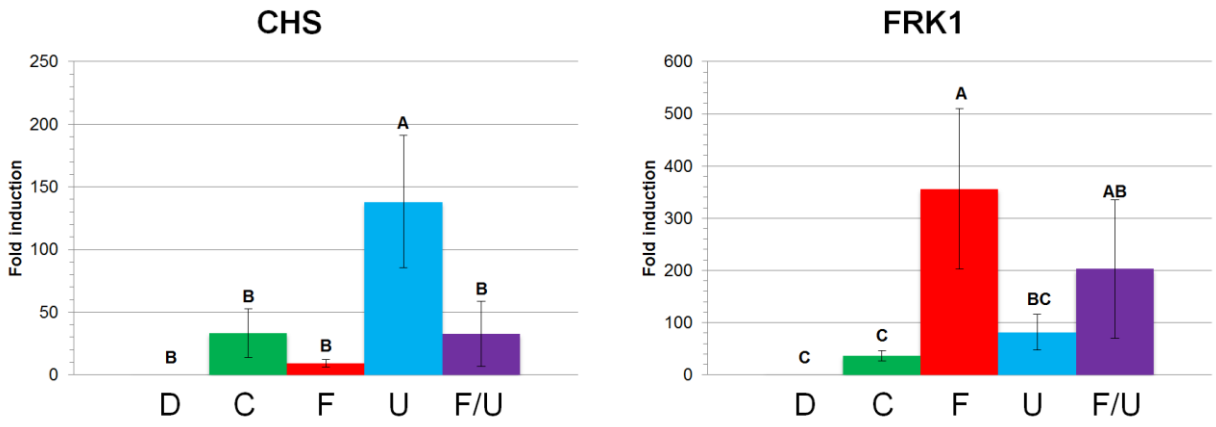


Figure 2 The crosstalk is functional in Col-0 wild-type plants. Expression analysis of the marker genes CHS and FRK1 was investigated after 4h in response to 5 different treatments: dark (0h control; D), water/VIS (C), flg22/VIS (F), UV-B/VIS (U) and both stresses simultaneously (F/U). Error bars represent standard deviation of triplicate experiments and statistical significance was checked by a two-way ANOVA.

In order to ensure our system is suitable to investigate loss-of-function mutants we treated homozygous *fls2* mutants, which lack the leucine-rich repeat serine/threonine protein kinase required for flg22 perception and signal transduction initiation. Any mutant impaired in flg22 or UV-B signaling should show disturbed the crosstalk between these two stresses by not suppressing the UV-B induced expression of CHS anymore. Indeed, we could show that the suppression of UV-B light induced CHS expression by flg22 co-treatment was observable in Col-0 wild-type seedlings (Figure 2), while it was blocked in the *fls2* background (Figure 3). In this mutant we could not observe any statistically significant differences between flg22 untreated and treated samples.

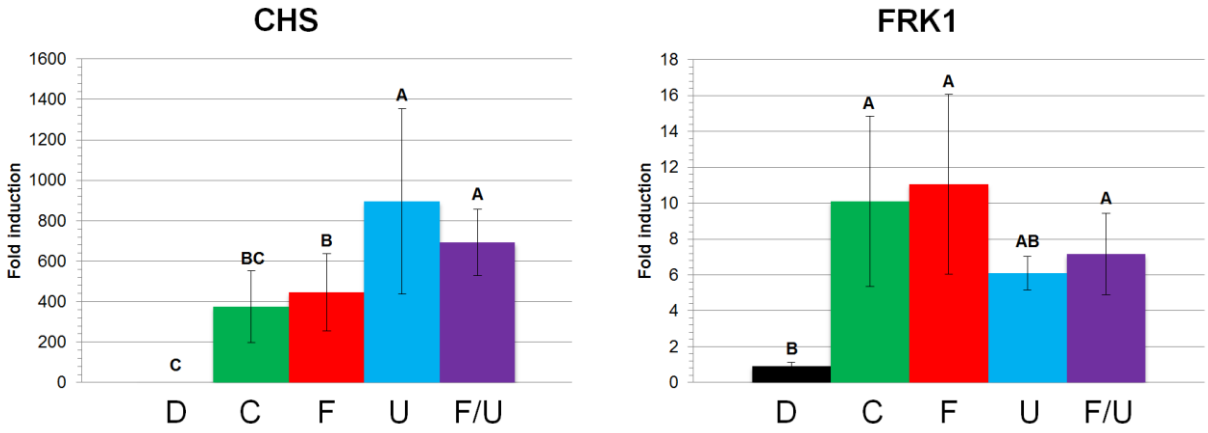


Figure 3 Mutant analysis deploying *fls2* plants. These plants lack the flg22 receptor and are thus impaired in launching MTI to suppress FPGs. Expression analysis of the marker genes CHS and FRK1 was investigated after 4h in response to 5 different treatments: dark (0h control; D), water/VIS (C), flg22/VIS (F), UV-B/VIS (U) and both stresses simultaneously (F/U). Error bars represent standard deviation of triplicate experiments and statistical significance was checked by a two-way ANOVA.

Since we observed antagonistic regulation of MYB TFs in the cell culture system we checked the expression of several TF implicated in the production of flavonoids (Jin et al., 2000; Stracke et al., 2007). While the expression pattern of the negative regulator MYB4, being upregulated by light and enhanced by flg22, and the three positive regulating MYBs 11, 12 and 111, being light (UV-B-induced and negatively affected by flg22, could be generally confirmed, we observed that in planta Myb111 was much stronger expressed than MYB12, which was the prominent UV-B upregulated MYB in cell cultures (Figure 4) (Schenke et al., 2011). As expected, no differences between flg22 untreated and treated samples could be observed in the *fls2* background.

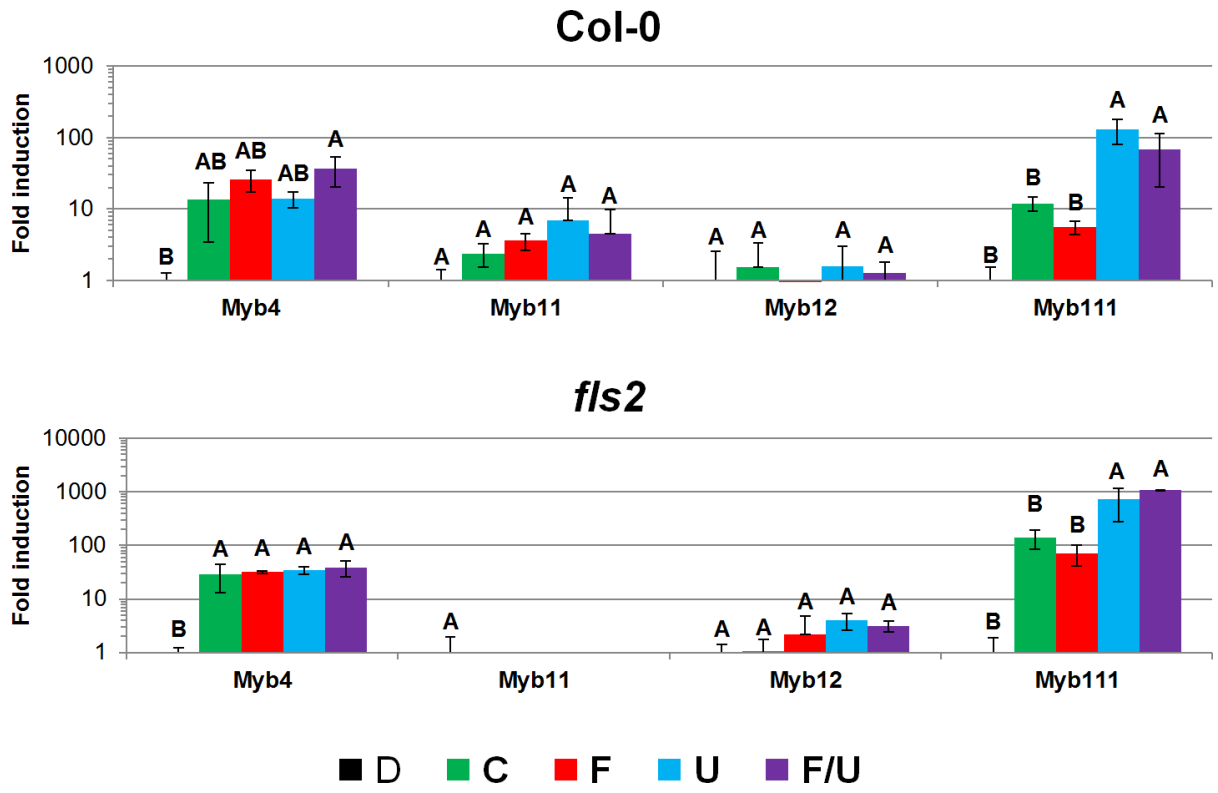


Figure 4 Expression analysis of MYB TFs potentially involved in the crosstalk. Expression was investigated after 4h in response to 5 different treatments: dark (0h control; D), water/VIS (C), flg22/VIS (F), UV-B/VIS (U) and both stresses simultaneously (F/U). Error bars represent standard deviation of triplicate experiments and statistical significance was checked by a two-way ANOVA.

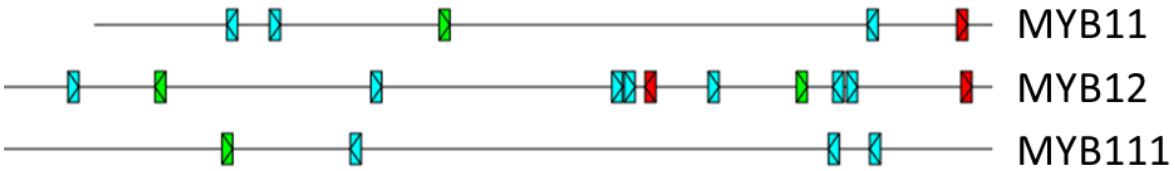
5 Discussion

Here we report an experimental system enabling the investigation of the crosstalk between UV-B and flg22-induced MTI in mutant plants to dissect the underlying molecular mechanisms (Figure 1). We could show that this crosstalk is fully functional in planta leading to flg22-induced suppression of UV-B-induced flavonol pathway genes (FPGs) exemplified by CHS expression analysis (Figure 2) and that this reaction is attenuated in mutant plants exemplified by the flg22-insensitive *fls2* KO plants (Figure 3). Now we should be able to investigate Arabidopsis loss-of-function mutants for impaired crosstalk to confirm and/or extend our model. Actually it would be sufficient to compare only the UV-B single treatment with the co-treatment with flg22 for this purpose. Since there is no statistical significance for the observation that UV-B somehow could negatively affect flg22-induced *FRK1* expression (Figure 2 and Figure 3), we cannot conclude that there might be reciprocal crosstalk between these two stress treatments.

Several MYB TFs have been implicated in the regulation of FPGs and flavonol content. One negative regulator MYB4 (Jin et al., 2000) and three MYB TFs (Stracke et al., 2007) appear to play a major role. The latter TFs show a distinct spatial expression pattern, being either expressed in distinct spots across the whole seedling (MYB11), in roots (MYB12) or the cotyledons (MYB111) (Stracke et al., 2007). Because we observed a much stronger regulation of MYB111 compared to MYB12 or MYB11 (Figure 4) and considering that this could be explained by the spatial expression pattern described by Stracke et al. (2007), we conclude that the cell cultures used in our former studies were derived from root material and that these cells could memorize their origin stably over several years and show thus a dominant regulation of MYB12. The expression of MYB12 in seedlings was not as strong as expected, probably due to the fact that the roots are not accessible to UV-B and flg22 as compared to leaf tissue and the bigger leaf portion compared to roots from whole seedlings. However, regulation of MYB111 is not as well investigated than MYB12. For example, binding of HY5 to the MYB111 promoter has not been proved experimentally, though there are the corresponding ACE *cis*-elements (Figure 5a). Furthermore, the previously proposed direct regulation by WRKYs can't be uphold, since the MYB111 promoter does not contain any W-Boxes required for WRKY binding (Schenke et al., 2011). Thus, there must be an additional layer of regulation and recently flavonoids have been reported to be regulated by a miRNA (miR858)

(Sharma et al., 2016). This miR858 can target MYB11, MYB12 and MYB111 (Figure 5b) and could be theoretically regulated by flg22 signaling via WRKYs binding to a W-box in its promoter (Figure 5c). However, until now miR858 has not been reported among e.g. flg22-upregulated miRNAs, probably due to the stringent cut-off criteria applied by Li et al. (2010). Nevertheless, miRNAs have been often associated with post-transcriptional regulation of various TFs (Shen et al., 2014; Megraw et al., 2016), and could thus serve as an explanation how the TFs in flavonol production could be down-regulated during MTI (Figure 6).

5a



5b

```
miR858a ------------------------AAGGUCGAACAGACAACGAAA--------------------
miR858b ------------------------CAAGGUCGAACAGACAACGAA---------------------
MYB11   ...GTCAACAATTGCGAGCAATCTACCGGGAAGAACAGACAACGAAATAAAAAACTATTGGAAT...
MYB12   ...GTCACTAATCGCGGGTCATCTACCAGGGAGAACAGACAACGAAATAAAAAATTATTGGAAC...
MYB111  ...GTCACTTATTGCAACACATCTACCAGGAAGAACAGACAACGAAATTAAAAACTATTGGAAC...
```

5c

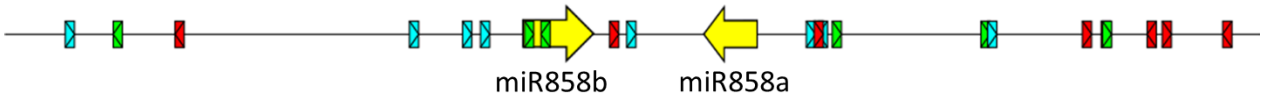


Figure 5 Potential involvement of a miRNA in regulation of the positive acting MYBs. A) MYB promoter comparison focusing on these *cis*-elements: ACE element: CACGT (binding of bZIP TFs, such as HY5), W-box: TTGAC(C|T) (binding site of WRKY TFs) and MRE elements: ACCNACC, which are potential MYB TF binding. Arrows in the boxes indicate sense or antisense direction. B) miR858 has the potential to target MYB11, MYB12 and MYB111. C) Analysis of the 6 kb genomic Arabidopsis miR858 locus indicating both miR858b and miR858a (yellow arrows) together with the three cis-elements.

With the description of this new system we are closer to natural conditions compared to the cell culture based system we used before and now it should be possible to test various factors which could contribute to the molecular mechanisms underlying this crosstalk. With an update of our model we summarize these regulatory possibilities, but not excluding that there might be also other TFs involved in this crosstalk (Figure 6).

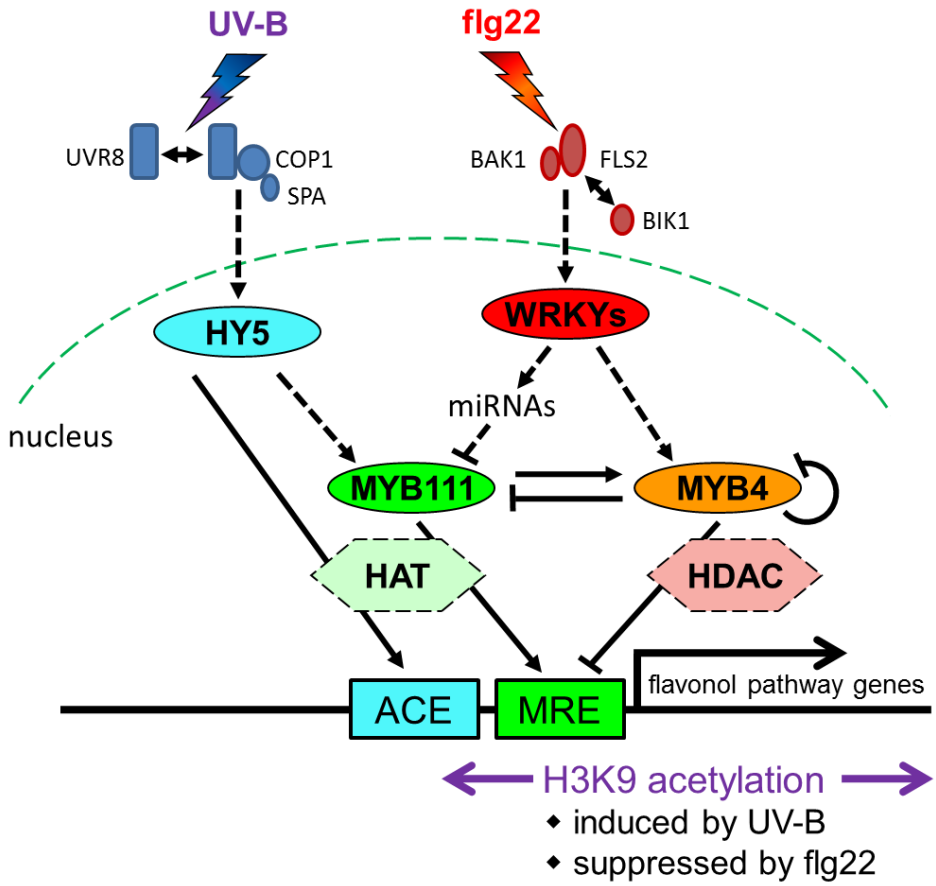


Figure 6 Updated model on the crosstalk between UV-B signaling and MTI in planta.

6 Reference

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8 Supplementary data

Table S1 Primer used for gene specific expression analysis by qRT-PCR

Gene	AGI code	Forward primer	Reverse primer	size
PP2A	AT1G13320	CAATGACGATGACGATGAGGTG	ATGCTCAACCAAGTCACTCTCC	208
CHS	AT5G13930	GTTCAAGCGCATGTGCGACAAG	GCCGCTTCTTTGCCTAGCTTA	165
FRK1	AT2G19190	GGTTGTAAGCCTCCTATAGTTCAC	TGAAATCTGACCGCTTCCTTCAAC	132
MYB4	AT4G38620	GCCACGTTGTTTCAAGTGCA	TCCATTGCTCATGTCACTCC	115
MYB11	AT3G62610	TTTGGGAACCAGGTGGTCAAC	TCTTCCAGGTCTACGCTTAGG	175
MYB12	AT2G47460	TCAGACCTCAAGCGTGGAAAC	TGGTAGATGACCCGCGATTAG	105
MYB111	AT5G49330	GGCAACAGATGGTCACTTATTG	GCTCCAGAAGACGATGAACAAG	182

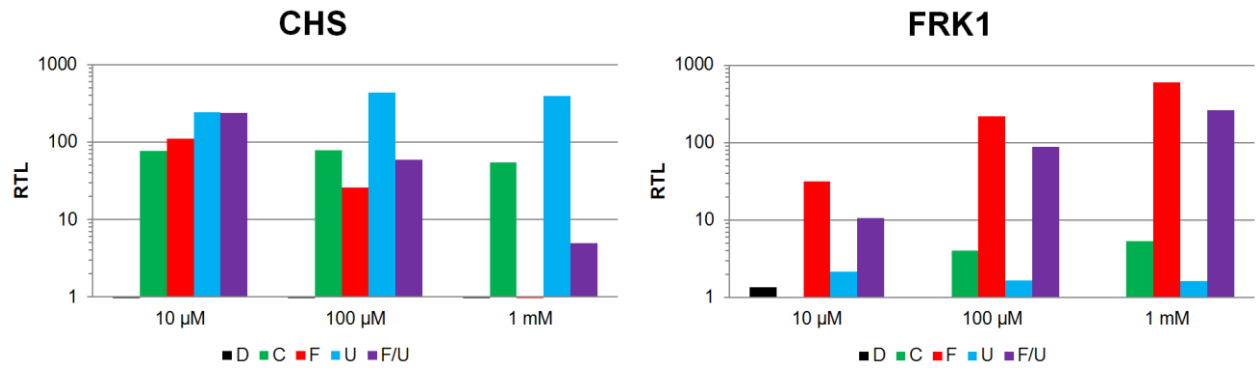

Figure S1 Preliminary test to investigate the necessary flg22 concentration to efficiently suppress the expression of the UV-B-inducible marker gene CHS and induce expression of the highly flg22-responsive FRK1.

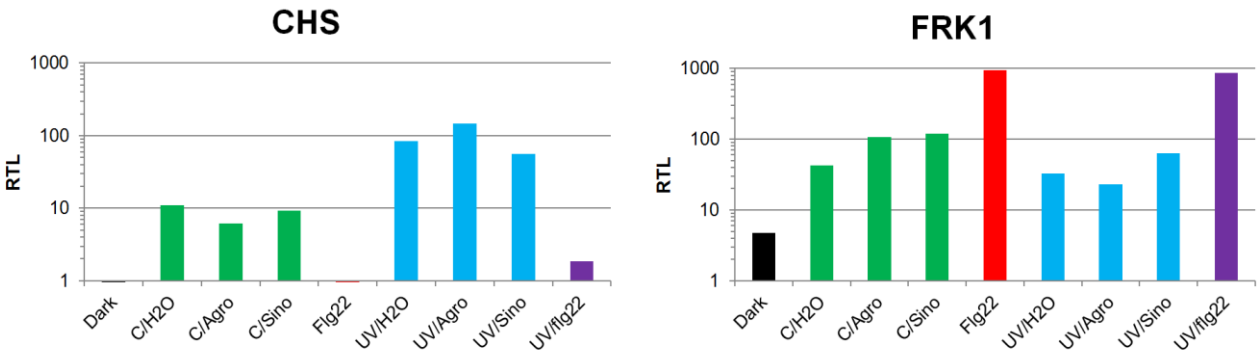

Figure S2 Preliminary test to investigate the general effect of high peptide concentration on the expression of the marker genes CHS (for successful UV-B induction and suppression by flg22; A) and FRK1 (for successful elicitation with the MAMP flg22; B).

Chapter III: Identification and characterization of microRNAs involved in the crosstalk between flg22 and UV-B induced signal cascades in *Arabidopsis thaliana*

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Submitted to *Plant, Cell & Environment*

1 Abstract

Plants are confronted with various abiotic and biotic stress factors in their natural habitats, and have evolved sophisticated and multifaced mechanisms to defense themselves. In our previous works, we have demonstrated the crosstalk between flg22 and UV-B-induced signal cascades in plants, in which the expression of Arabidopsis flavonol pathway genes (FPGs) are upregulated by UV-B irradiation but simultaneously repressed by the bacterial elicitor flg22 during MAMP Triggered Immunity (PTI). Although several transcription factors proved to be involved in the crosstalk, the underlying regulatory mechanism remains largely unsolved. By deep sequencing we identified 217 miRNAs representing 204 conserved and 13 novel miRNAs involved in the crosstalk. Among them, e.g. 106 miRNAs were upregulated by flg22 and downregulated by UV-B. Furthermore, a set of specific interactions between miRNAs and their targets were confirmed showing reciprocal changes in their expression levels, simultaneously. As revealed by GO and KEGG analysis *in silico*, predicted miRNA target genes participate in a series of plant biological and molecular processes as well secondary metabolism pathways. Two modulations of miRNA-target interactions were obtained in the crosstalk. The first one consisting of miR158, miR165, miR166, miR167, miR168, miR172, miR391, miR393, miR447, miR824, miR828, miR846 and miR858, which were all repressed by UV-B irradiation, while upregulated by flg22, and the second one constitute miR159, miR164, miR171 and miR822, being repressed by flg22 and upregulated by UV-B. Both miRNA sets display converse regulation with the change in transcript levels of their targets. Furthermore, we demonstrated that knockdown of miR858 (Group I) in Arabidopsis goes along with increased the chalcone synthase (CHS) expression while its overexpression results in its depression. Vice versa, knockout of miR164b (Group II) depresses the CHS gene expression while

its overexpression strongly elevated the CHS gene expression, providing the first genetic evidence that miRNAs identified in this study constitute an additional layer in regulating the crosstalk between flg22 and UV-B induced signaling cascades.

2 Introduction

Plants are continuously exposed to various abiotic and biotic stresses and have evolved sophisticated and multifaced mechanisms to defense themselves. From UV-B radiation, plants protect themselves with the production of sinapoylmalate and flavonoids by enhancing flavonol pathways gene (FPGs) expression (Meissner et al., 2008). Many reports demonstrated that the UV-B-induced flavonol pathway gene expression is abolished when a concomitant elicitation by microbe-associated molecular pattern (MAMPs) occurs (Serrano et al., 2012; Schenke et al., 2019). MAMPs induce the so-called MAMP triggered immunity (MTI), a plant defense response against pathogens by reprogramming the secondary metabolism to produce e.g. anti-microbial phytoalexins and lignification. Because all flavonoids, lignin and the phyoalexin or scopoletin are derived from the same precursor phenylalanine, it is believed that the suppression of UV-B induced flavonoids releases more resources benefiting plant pathogen defense (Schenke et al., 2011). This kind of “crosstalk” has been intensively investigated in Arabidopsis cell system and recently demonstrated in Arabidopsis whole plants (Zhou et al., 2017). Comparing UV-B-, flg22-treatment and UV-B/flg22 co-treatment reveals a striking correlation between the acetylation of histone 3 at lysine 9 (H3K9ac) and the gene expression of several FPGs in addition to chalcone synthase (CHS) and several flavonoid metabolites (e.g. quercetin- and kaempferol-derivatives) accumulation, which are all induced by UV-B but suppressed when flg22 was additionally applied, suggesting a complex mechanism governing the flg22/UV-B signaling crosstalk (Schenke et al., 2014).

Small RNA (sRNA) post-transcriptionally regulates many biological pathways in animals and plants (Li et al., 2017). As a major class of sRNAs, microRNAs (miRNAs) are endogenous ∼22 nt RNAs, generated from intronic or intergenic regions by RNA polymerase II, processed into pre-miRNAs by the Dicer-like 1 (DCL1)/HYPONASTIC LEAVES1 (HYL1)/SERRATE (SE) protein and then diced as a miRNA-miRNA* duplex. One selected strand of the duplex is incorporated in the argonaute (AGO) protein to execute their function in regulating gene expression and networks (Naqvi et al., 2012). In most cases, miRNAs target transcription factors by targeting mRNAs for cleavage or translational inhibition, leading to reprogram of gene expression and networks, so called post-transcriptional gene silencing (PTGS) (Pu et al., 2019).

Increasing data demonstrate the vital role of miRNAs by reprogramming a broad range of physiological processes. In plants, in addition to normal plant growth and development, the expression of many miRNAs is notably regulated by various biotic and abiotic stress factors. It is therefore widely believed that miRNAs form an additional regulatory layer to fine-tune gene expression and networks to develop adaptive responses to unfavorable conditions (Wang and Chen, 2019; Yang et al., 2019). It has been demonstrated that flg22 alone is able to induce the accumulation of miR393 in Arabidopsis contributing to plant defence response against bacterial infection by negatively regulating the mRNA levels of F-box auxin receptors, transport inhibitor response 1 (TIR1), auxin signaling F-box protein 2 (AFB2) and AFB3 (Navarro et al., 2006). Moreover, flg22 also induces miR160a accumulation and represses its target genes ARF16 and ARF17 to activate or repress transcription of primary auxin-response genes (Hagen and Guilfoyle, 2002). Several miRNAs including miR160, miR167, miR393, miR396, and miR824 that were enriched in flg22-treated AGO1 had been found to accumulate in plants after treatment with the DC3000 *hrcC*$^{-}$ strain, which lacks a functional type III secretion apparatus (Fahlgren et al., 2007; Li et al., 2010). In similar, miR156 and miR164, which were induced by infection with the virus TYMV p69, were also induced in transgenic Arabidopsis plants expressing the viral silencing suppressor P1/HC-Pro (Kasschau et al., 2003).

UV radiation causes damage at the DNA, RNA levels, and also induces the expression of miRNAs in plants. Zhou et al. (2007) has reported 21 UV-B-responsive miRNAs from 11 different miRNA families of miR156, miR159, miR160, miR165/166, miR167, miR169, miR170, miR172, miR393, miR398 and miR401 in Arabidopsis. In *Populus tremula*, 24 UV-B responsive miRNAs (13 upregulated and 11 downregulated) were identified (Jia et al., 2009). In Arabidopsis, Cryptochrome1 and Cryptochrome2 mediate the expression of miR172 after blue light stimulation in a CONSTANS-independent manner to regulate photoperiodic flowering time (Jung et al., 2007). A novel wheat miRNA, named Tae-miR6000 was reported by Wang et al. (2013) expressed after UV-B treatments, in addition to 6 miRNAs which were highly responsive to UV-B irradiation. Of these miR159, miR167a and miR171 were upregulated, and the remaining three (miR164, miR395 and miR156) were downregulated. Zhang et al. (2014) reported that miR408 is coordinately regulated by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE7 (SPL7) and ELONGATED HYPOCOTYL5 (HY5) in response to light. In grapevine berry (*Vitis vinifera* L.), a high-

fluency UV-B increased miR530 expression, while decreased miR403 abundances, thereby coordinating the post-transcriptional gene silencing activities (Sunitha et al., 2018). In similar, in maize leaves, 17 miRNAs were UV-B-responsive, from these 7 (osa-miR1429, miR444, miR166, miR164, ppt-miR533a, miR398 and miR165) were upregulated, while 10 (miR172, miR171, miR396, osa-miR1858a, ppt-miR896, miR399, miR395, miR529, ppt-miR903 and miR156) depressed (Casati, 2013). Nevertheless, UV-B-induced plant miRNA-target interactions are poorly understood, so far.

Interestingly, a subset of several transcription factors had been identified to be implicated in the regulation of the FPGs from previous studies (Schenke et al., 2011; Schenke et al., 2014; Schenke and Cai, 2014), from which some are targets of miRNAs. For instance, MYB75 and MYB11/MYB12/MYB111 are targeted by miR828 and miR858, respectively (Wang et al., 2016; Yang et al., 2013), indicative of the involvement of miRNAs in the crosstalk of the flg22 and UV-B signaling pathways (Zhou et al., 2017).

High-throughput sequencing technologies provide a powerful tool for disclosure of a large number of small RNA species, identification of new miRNAs or the expression profiles responsive to various stress factors (Zhu et al., 2008; Shen et al., 2014). To explore the role of miRNAs in the crosstalk between flg22 and UV-B induced signaling pathways, small RNAseq along with RNAseq experiments were employed with Arabidopsis seedling treated by flg22, UV-B and co-treated by flg22 and UV-B, respectively (Zhou et al., 2017). Here, we report identification of 217 differentially expressed nonredundant miRNA precursors, including 204 conserved and 13 novel miRNAs. From the conserved miRNAs 22 were upregulated by flg22 and downregulated by UV-B as well as 12 were downregulated by flg22 and upregulated by UV-B. Bioinformatics analysis and experimental data identified a subset of candidate miRNAs and their targets, which participate in diverse biological processes and metabolism pathways, including the flavonol biosynthesis pathway as expected. Our data provide for the first time the evidence that plant-derived miRNAs constitute a crucial regulatory layer governing the crosstalk of flg22- and UV-B signaling pathways, in which e.g. the modulation of miR858-MYB111, miR828-MYB75 and miR164b-NACs may act as a pivotal mechanism. A possible model of action is discussed.

3 Materials and methods

3.1 Plant materials

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used in this study. miR858 mutants (*STTM858* and miR858 overexpressing lines) and knockdown mutant *STTM828* were kindly provided by Dr. Huiyong Zhang and Dr. Guiliang Tang (Addgene plasmid # 84157; http://n2t.net/addgene:84157; RRID: Addgene_84157), respectively. Arabidopsis of miR164 knockout- and overexpressing- line were purchased from NASC.

3.2 Experiment design

Plant treatment followed the strategy described by Zhou et al. (2017). Nine seedlings of Arabidopsis plants were grown together on a single Jiffy (Jiffy-7 Peat Pellets, Jiffy Products International AS, Norway); and after 5 weeks transferred to darkness in order to completely suppress CHS mRNA levels. After 2 days in darkness, all plants were ready to be treated. Half pots were selected for spraying with HPLC-water, while the remaining pots were sprayed with 1 mM flg22 solution. To let flg22 taking effect, the sprayed plants were incubated for 1 hour in darkness and were then exposed to UV-B or VIS-light as control for 4 hours. UV-B treatment was conducted as described before and UV-B levels were attenuated to natural levels by a glass plate or completely filtered by 2 sheets of polyester plastic foil. Thus, each biological replicate consists of the four treatments water/VIS-light control (CK), flg22 treatment (flg22), UV-B treatment (UV-B) and the co-treatment flg22/UV-B (F/U) (Figure S1). Of each treatment, nine whole seedlings were harvested in a 2 ml tube containing ca. 20 zirconium beads (Zircosil 1.2-1.7 mm, Mühlmeier mahltechnik, Germany) for later RNA isolation, immediately flash-frozen in liquid nitrogen and stored at -80 °C until further processing.

3.3 RNA isolation and sRNA/RNA sequencing

Total RNA from Arabidopsis seedlings was extracted with TRIzol® reagent (Thermo Fisher Scientific) according to the manufacturer's instructions as described (Zhou et al., 2017). The samples were grinded using a Precellys® Evolution ballmill (bertin technologies) at 6,800 rpm for 3 x 20 sec. 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 new 1.5 ml tube and incubated on ice 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. The RNA pellet was dried in the air stream and dissolved in 50 µl DEPC-water. The quality and concentration of total RNA was controlled by gel electrophoresis and use of NanoVue Plus Spectrophotometer (GE Healthcare Life Science). Total RNA samples of three independent biological replications were pooled and used for sRNA- and mRNA-sequencing (Novogene Co., Ltd., Hong-Kong, China).

3.4 Data analysis

Small miRNA- and mRNA-seq data processing

All small RNA reads, referred to as raw reads, were processed to remove adaptors, low-quality tags and contaminants. Clean reads were then mapped to the *Arabidopsis thaliana* genome. Only reads with perfect genomic matches were processed for further miRNA and small interfering RNA (siRNA) annotation. After removing unexpected sRNA, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and degraded mRNA by aligning clean reads to GenBank, Rfam (10.1), *Arabidopsis thaliana* mRNA database, the sRNA sequences were subject to secondary hairpin structure prediction using Mireap (http://sourceforge.net/projects/mireap/). The resulting structures with <18 kcal/mol free energy, <300 nt spaces between miRNA and its complementary sequence miRNA*, and >16 nt matched nucleotides but <4 nt bulge of miRNA and miRNA* were retained as miRNA candidates. The conservation status of the candidates was analyzed by comparison with all plant miRNAs recorded in miRBase (release 22). All miRNA sequence data obtained are available from Gene Expression Omnibus (http:// www.ncbi.nlm.nih.gov/geo/).

Forward/reverse clean reads of mRNA-seq were obtained by Illumina sequencing from Novogene Co., Ltd. (Hong-Kong, China). FPKM values for all samples were calculated using the open-source software tools HISAT and StringTie with default settings, as described by Pertea et al. (2016) with the TAIR 10 genome/annotation of Arabidopsis (Lamesch et al., 2011).

Identification and enrichment analysis of miRNA target genes

Targets of each candidate miRNA were predicted using web server psRNATarget (http://plantgrn.noble.org/psRNATarget/; Dai and Zhao, 2011). *Arabidopsis thaliana* transcripts were mainly included in the prediction. The predicted targets with default parameters were processed in Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.jp/kegg/) which were performed using BLAST2GO (http://www.blast2go.com/b2ghome; Conesa and Götz, 2008). The bioinformatics pipeline is summarized in Figure S2.

3.5 Transcript analysis of miRNAs and target genes

Mature miRNA expression analysis was performed by stem-loop RT-qPCR, as described by Chen et al. (2005). For cDNA synthesis of miRNAs, 200 ng of RNA from each sample was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Germany) with *U6* snRNA as the internal control. The PCR consisted of an initial 3-min denaturation step at 94 °C followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The amplifications use the corresponding miRNA sequence as the sense primer and the stem-loop universal primer as the antisense primer. The PCR product was checked on 12% polyacrylamide/urea gel and defined using an ultra low range DNA ladder (Thermo Scientific, Germany).

Quantitative RT-PCR (RT-qPCR) reactions were performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) using the following conditions: 10 min 95 °C; 40 x 15 sec 95 °C, 30 sec 59 °C, 30 sec 72 °C; 10 sec 95 °C, melting curve from 65 °C to 95 °C. For cDNA synthesis of predicted targets, 1 µg of total RNA was treated with RNase-free DNase I (Thermo Scientific, Germany) of each sample was used. Normalization of the expression levels was carried out using *Actin2* as internal reference gene. The relative fold changes in expression of miRNAs and related genes were calculated using the comparative threshold cycle (Ct) method (Livak and Schmittgen, 2001). Each data point is based on three independent biological replicates measured as two or three technical replicates each. The sequences of the gene-specific primer used in this study are given in Supplementary Table S1. Statistical analysis was carried out using a 2-way ANOVA or Student's *T*-test according to Minitab software (MINITAB, 2000).

3.6 Data deposition

The data reported in this paper have been deposited in the National Center of Biotechnology Information Sequence Read Archive (NCBI SRA) Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo).

4 Results

4.1 Construction of small RNA libraries and analysis of small RNAseq data

To identify sRNAs involved in the crosstalk between flg22 and UV-B signal pathways, four sRNA libraries were constructed with Arabidopsis seedlings treated by mock, flg22, UV-B and F/U co-treatment, respectively, and sequenced by Illumina sequencing. After adaptor trimming, the numbers of sequence reads and unique sequence signatures from the raw data were calculated and then mapped to the Arabidopsis genome. In total, 1,075,792 (3,436,725 unique), 15,300,647 (3,950,534 unique), 12,156 959 (3,692,878 unique) and 13,511,026 (3,898,775 unique) reads 18-44 nt in size were obtained from the CK, flg22, UV-B and F/U, respectively (Table 1). The length of the mapped sequences ranged from 19 to 30 nucleotides (Figure S3). In all libraries, a major proportion of the sRNAs were of size 21-24 nt, most of which were 24-nt sRNAs, indicating that they are rich in sequence diversity. From the reads, 45.4%, 44.3%, 48.6% and 41.5% of unique reads of the respective four treatments could be mapped to the *Arabidopsis thaliana* genome, respectively. After removing tRNAs, rRNAs, snRNAs, snoRNAs, repeat-associated small interfering RNAs (rasiRNAs) and degraded mRNAs, other reads were referred to as endogenous sRNAs for further analysis. As expected, a dominant nucleotide preference for uracil (U) at the 5’ first position of 22-nt sRNAs (93.9%, 94.5%, 93.7% and 93.9% for CK, flg22, UV-B and F/U, respectively) and cytosine (C) at the end position of 22-nt sRNAs (44.1%, 44.3%, 44.9% and 44.0% for each treatment, respectively) was observed in all sRNA populations (Figure S4). Consistent with the earlier reports, there were two major peaks at 21, 23 and 24 nucleotides in the total sequence reads of all four libraries. When the unique sequence signatures were examined, the patterns of all four libraries were nearly identical (Figure S3). The 21, 23 and 24-nucleotide small RNAs were dominant in either their reads or unique sequences.

Table 1 Small RNAs from *Arabidopsis thaliana* seedlings

	Mock		flg22		UV-B		F/U	
	Unique	Abundant	Unique	Abundant	Unique	Abundant	Unique	Abundant
Total number of reads	3,436,725	10,757,992	3,950,534	15,300,647	3,692,878	12,156,959	3,898,772	13,511,026
Mapped to *Arabidopsis thaliana* genome	1,561,022 (45.4%)	8,387,091 (78.0%)	1,751,181 (44.3%)	12,124,402 (79.2%)	1,729,352 (48.6%)	9,701,375 (79.8%)	1,616,978 (41.5%)	10,178,890 (75.3%)

4.2 Identification of miRNAs and expression analysis

To identify miRNAs from the small RNAseq data, we first employed the whole genome of *Arabidopsis thaliana* for miRNA prediction. In this way, we identified 217 nonredundant miRNA precursors from all four libraries, which are able to form a perfect hairpin structure as expected by miRNA or miRNA*. In the next step, we compared these with known plant miRNAs in the miRBase (release 22), and identified 204 conserved and 13 novel miRNAs (Table S2), from which highly expressed (>15 reads in one library) are listed in Table 2 (99 conserved and 4 novel miRNAs). To confirm the expression of predicted miRNAs (Table S2), 31 conserved and 4 novel highly expressed miRNA sequences were selected for stem-loop reverse transcription PCR analysis of mature miRNAs or for quantitative reverse transcription (RT)-PCR of primary transcript of miRNAs. Results summarized in Figure S5 demonstrate the expression of 31 conserved miRNA sequences found in all treated and control Arabidopsis seedlings except for 4 novel miRNAs that did not show RT-PCR products (Figure S5). Therefore, we only focused on the conserved miRNAs in this study.

Table 2 Highly expressed conserved and novel miRNAs identified from the small RNAseq data

miRNA family	miRNA	Mature sequence	Read number			
			CK	flg22	UV-B	F/U
miR156	ath-miR156a-3p	GCTCACTGCTCTTTCTGTCAGA	479	603	423	391
	ath-miR156a-5p	TGACAGAAGAGAGTGAGCAC	6280	11147	7565	5221
	ath-miR156b-3p	TGCTCACCTCTCTTTCTGTCAGT	705	977	762	855
	ath-miR156c-3p	GCTCACTGCTCTATCTGTCAGA	1184	1477	1167	1147
miR157	ath-miR157ab-3p	GCTCTCTAGCCTTCTGTCATC	955	1594	1084	746
	ath-miR157a-5p	GCTCTCTATACTTCTGTCACC	3363	5337	4270	3490
miR158	ath-miR158a-3p	TCCCAAATGTAGACAAAGCA	232655	345880	277841	229842
	ath-miR158a-5p	CTTTGTCTACAATTTTGGAAA	248	288	336	186

	ath-miR158b	CCCCAAATGTAGACAAAGCA	1452	2220	1716	1424
miR159	ath-miR159a	TTTGGATTGAAGGGAGCTCTA	152684	227108	192650	151198
	ath-miR159b-3p	TTTGGATTGAAGGGAGCTCTT	106569	149049	125667	120137
	ath-miR159c	TTTGGATTGAAGGGAGCTCCT	9125.00	12670	9212	9472.00
miR160	ath-miR160a-5p	TGCCTGGCTCCCTGTATG-CCA	548.00	882	697	471.00
miR161	ath-miR161.1	TGAAAGTGACTACATCGGGGT	36397	50986	44375	39475
miR162	ath-miR162a-3p	TCGATAAACCTCTGCATCCAG	5389	7332	5857	4700
miR163	ath-miR163	TTGAAGAGGACTTGGAACTTCGAT	11375	16430	12602	17927
miR164	ath-miR164a	TGGAGAAGCAGGGCACGTGCA	619	1076	897	616.00
	ath-miR164b	TGGAGAAGCAGGGCACGTGCG	310	480	464	300.00
miR165	ath-miR165a-3p	TCGGACCAGGCTTCATCCCCC	12654	18869	14713	10395
miR166	ath-miR166a-3p	TCGGACCAGGCTTCATTCCCC	53825	73284	65183	52098
	ath-miR166a-5p	GGACTGTTGTCTGGCTCGAGG	1020	1104	1417	910
	ath-miR166c	TCGGACCAGGCTTCATTCCCC	1829	2047	2229	1553
miR167	ath-miR167a-3p	GATCATGTTCGCAGTTTCACC	1690	2064	2152	1858
	ath-miR167b	TGAAGCTGCCAGCATGATCTA	381	450	567	315
	ath-miR167a-5p	TGAAGCTGCCAGCATGATCTA	998	16765	12718	9265
	ath-miR167d	TGAAGCTGCCAGCATGATCTGG	3239	5213	3999	3274
miR168	ath-miR168a-3p	CCCGCCTTGCATCAACTGAAT	1538	3300	1808	2413
	ath-miR168a-5p	TCGCTTGGTGCAGGTCGGGAA	3367	8255	4811	5641
	ath-miR168b-3p	CCCGTCTTGTATCAACTGAAT	434	962	490	652
miR169	ath-miR169f-3p	GCAAGTTGACCTTGGCTCTGC	100	869	130	689
	ath-miR169g-3p	TCCGGCAAGTTGACCTTGGCT	220	544	210	409
miR170	ath-miR170-5p	TATTGGCCTGGTTCACTCAGA	713	890	811	721
miR171	ath-miR171a-3p	TGATTGAGCCGCGCCAATATC	1059	1203	1103	982
miR172	ath-miR172a	AGAATCTTGATGATGCTGCAT	8029	10356	11191	7595
	ath-miR172c	AGAATCTTGATGATGCTGCAG	677	747	837	526
miR173	ath-miR173-5p	TTCGCTTGCAGAGAGAAATCAC	2516	3955	3399	3091
miR1888	ath-miR1888a	TAAGTTAAGATTTGTGAAGAA	57	114	61	69
miR319	ath-miR319a	TTGGACTGAAGGGAGCTCCCT	10374	11187	11660	9067
	ath-miR319c	TTGGACTGAAGGGAGCTCCTT	15486	16783	15648	16457
miR390	ath-miR390a-5p	AAGCTCAGGAGGGATAGCGCC	1094	1342	1455	1097
miR391	ath-miR391-3p	ACGGTATCTCTCCTACGTAGC	358	835	704	677
miR3932	ath-miR3932b-5p	TTTGACGTGCTCGATCTGCTC	6041	8531	6900	6604
miR393	ath-miR393b-3p	ATCATGCGATCTCTTTGGATT	1110	1718	1427	861
miR394	ath-miR394a	TTGGCATTCTGTCCACCTCC	919	1531	1157	886
miR396	ath-miR396a-3p	GTTCAATAAAGCTGTGGGAAG	3070	3048	3536	2096
	ath-miR396a-5p	TTCCACAGCTTTCTTGAACTG	8539	14707	11105	9956
	ath-miR396b-3p	GCTCAAGAAAGCTGTGGGAAA	618	711	740	410
	ath-miR396b-5p	TTCCACAGCTTTCTTGAACTT	20650	33923	24215	19554
miR398	ath-miR398b-3p	TGTGTTCTCAGGTCACCCCTG	804	1869	2153	1073
	ath-miR398b-5p	AGGGTTGATATGAGAACACAC	1339	1531	2077	1156
miR399	ath-miR399b	TGCCAAAGGAGAGTTGCCCTG	17	51	25	30
miR400	ath-miR400	TATGAGAGTATTATAAGTCAC	198	270	214	215

miR403	ath-miR403-3p	TTAGATTCACGCACAAACTCG	13196	20744	16573	14912
	ath-miR403-5p	TGTTTTGTGCTTGAATCTAATT	578	831	569	572
miR408	ath-miR408-3p	ATGCACTGCCTCTTCCCTGGC	344	690	544	510
miR447	ath-miR447a.2-3p	TATGGAAGAAATTGTAGTATT	2474	2857	2751	3076
	ath-miR447a-3p	TATGGAAGAAATTGTAGTATT	523	722	594	526
miR472	ath-miR472-3p	TTTTTCCTACTCCGCCCATACC	421	522	428	526
	ath-miR472-5p	ATGGTCGAAGTAGGCAAAATC	261	321	330	426
miR5026	ath-miR5026	ACTCATAAGATCGTGACACGT	2258	3002	2293	2545
miR5595	ath-miR5595a	ACATATGATCTGCATCTTTGC	21	26	18	28
miR5642	ath-miR5642a	TCTCGCGCTTGTACGGCTTT	199	233	237	216
miR5651	ath-miR5651	TTGTGCGGTTCAAATAGTAAC	59	100	69	57
miR5653	ath-miR5653	TGGGTTGAGTTGAGTTGAGTTGGC	226	300	274	257
miR5654	ath-miR5654-3p	TGGAAGATGCTTTGGGATTTATT	196	272	221	167
miR5663	ath-miR5663-3p	TGAGAATGCAAATCCTTAGCT	293	453	406	325
	ath-miR5663-5p	AGCTAAGGATTTGCATTCTCA	294	464	412	332
miR5996	ath-miR5996	TGACATCCAGATAGAAGCTTTG	182	260	148	137
miR773	ath-miR773a	TTTGCTTCCAGCTTTTGTCTC	308	462	447	382
miR775	ath-miR775	TTCGATGTCTAGCAGTGCCA	488	632	598	558
miR781	ath-miR781a	TTAGAGTTTTCTGGATACTTA	718	846	712	694
miR8166	ath-miR8166	AGAGAGTGTAGAAAGTTTCTCA	26	63	46	49
miR822	ath-miR822-5p	TGCGGGAAGCATTTGCACATG	1365	1892	1879	1564
miR823	ath-miR823	TGGGTGGTGATCATATAAGAT	132	213	155	126
miR824	ath-miR824-3p	CCTTCTCATCGATGGTCTAGA	212	366	331	290
	ath-miR824-5p	TAGACCATTTGTGAGAAGGGA	190	309	315	188
miR825	ath-miR825	TTCTCAAGAAGGTGCATGAAC	376	542	427	299
miR826	ath-miR826a	TAGTCCGGTTTTGGATACGTG	35	59	40	55
miR827	ath-miR827	TTAGATGACCATCAACAAACT	244	345	348	423
miR828	ath-miR828	TCTTGCTTAAATGAGTATTCCA	12	13	17	12
miR829	ath-miR829-5p	ACTTTGAAGCTTTGATTTGAA	75	84	105	74
miR830	ath-miR830-3p	TAACTATTTTGAGAAGAAGTG	15	37	32	35
miR838	ath-miR838	TTTTCTTCTACTTCTTGCACA	134	184	129	132
miR841	ath-miR841a-3p	ATTTCTAGTGGGTCGTATTCA	51	75	41	56
	ath-miR841a-5p	TACGAGCCACTTGAAACTGAA	167	260	173	148
	ath-miR841b-3p	CAATTTCTAGTGGGTCGTATT	55	78	39	55
	ath-miR841b-5p	TACGAGCCACTGGAAACTGAA	41	79	34	33
miR843	ath-miR843	TTTAGGTCGAGCTTCATTGGA	185	275	187	203
miR844	ath-miR844-3p	TTATAAGCCATCTTACTAGTT	172	202	172	204
miR845	ath-miR845a	CGGCTCTGATACCAATTGATG	157	224	151	146
miR846	ath-miR846-3p	TTGAATTGAAGTGCTTGAATT	2864	4095	3042	3851
	ath-miR846-5p	CATTCAAGGACTTCTATTCAG	232	483	300	392
miR848	ath-miR848	TGACATGGGACTGCCTAAGCTA	79	153	116	110
miR850	ath-miR850	TAAGATCCGGACTACAACAAAG	61	87	63	75
miR858	ath-miR858a	TTTCGTTGTCTGTTCGACCTT	266	462	433	290
	ath-miR858b	TTCGTTGTCTGTTCGACCTTG	73	100	110	90

miR863	ath-miR863-5p	TTATGTCTTGTTGATCTCAAT	425	527	564	414
miR866	ath-miR866-5p	TCAAGGAACGGATTTTGTTAA	31	49	34	3
miR869	ath-miR869.2	TCTGGTGTTGAGATAGTTGAC	29	49	44	29
novel_1	novel_1	GTTTGATTCGTACACTTAGATTGT	659	795	723	876
novel_4	novel_4	TTCCTTCTGAAAACTAAAATT	15	29	24	18
novel_16	novel_16	GCTTACTCTCTCTCTGTCACC	26	51	40	21
novel_20	novel_20	AGTTAAGTTCATGTAGTTATAAGC	15	16	15	16

4.3 Identification of miRNAs responsive to flg22 and UV-B treatments

To identify miRNAs responsive to flg22 and UV-B treatments, the expression level of an individual miRNA was normalized by total reads in the corresponding library, giving the level of transcript per million (TPM) reads. miRNAs with > 5 TPM were chosen for further analysis and miRNAs with > 100% TMP increase or decrease by comparison were considered to be up- or downregulated, respectively (Hsieh et al., 2009). Following this, we identified 128, 129 and 121 miRNAs with altered abundances in the seedlings treated by flg22, UV-B and F/U, respectively. There are 75, 51 and 52 miRNAs upregulated while 53, 78 and 69 miRNAs downregulated by flg22, UV-B and F/U, respectively (Table S3-S5). We identified 22 miRNAs upregulated by flg22 and downregulated by UV-B (Table S6) as well as 12 miRNAs downregulated by flg22 and upregulated by UV-B (Table S7). Among them, several known miRNAs specifically responsive to flg22 or UV-B were given, e.g. miR172, miR393, miR824 for flg22 (Navarro et al., 2006; Li et al., 2010; Zou et al., 2018) and miR165, miR166, miR167 and miR172 for UV-B (Zhou et al., 2007; Jung et al., 2007). From these, 22 miRNAs clearly distinguished between flg22 and UV-B treatments in their patterns (Figure 1). For instance, the expression of miR172, miR447, miR824, miR846 and miR858 was remarkably elevated by flg22 treatment while suppressed by UV-B irradiation as well as by simultaneous induced by co-treatment with flg22/UV-B (Figure 1), by contrast, miR159ab, miR164b, miR171a and miR822 were downregulated by flg22 and upregulated by UV-B. These miRNAs expression patterns strongly suggesting their role in regulating the crosstalk. These 22 miRNAs were chosen as candidates for further analysis.

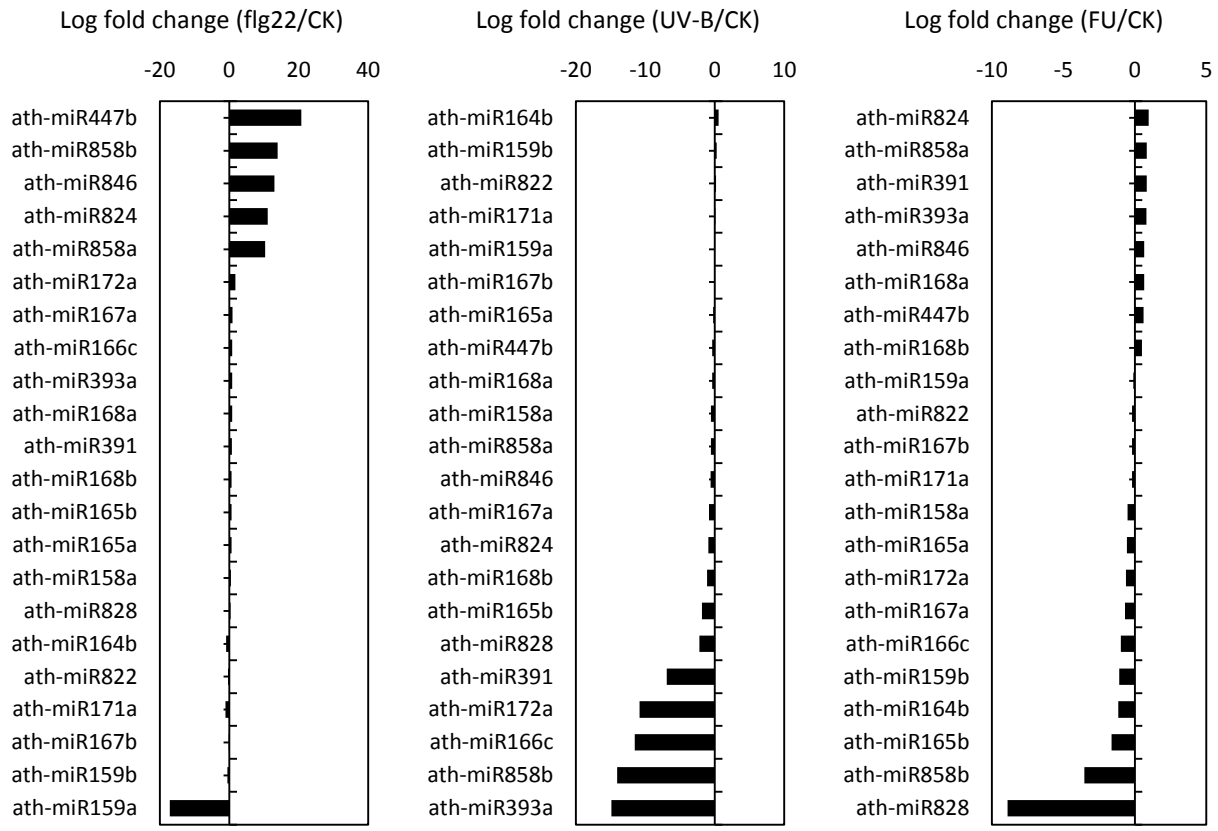


Figure 1 Differentially expressed miRNAs in libraries from flg22, UV-B and F/U, as compared with control. Fold change was calculated using formula: fold change = $\log_2$ (miRNA expression in treatment/miRNA expression in CK). miRNA expression in each library was normalized to obtain the expression of TPM. Formula for normalization: normalized expression = (actual miRNA count/total count of clean reads) × 1,000,000. If the expression level was equal to 0, it was changed to 0.01. If the expression level of miRNA was < 5 in both libraries, it was ignored in the differential expression analysis.

4.4 Prediction annotation of targets of differentially expressed miRNAs

Since the function of miRNAs is merely relying on the function of their targets and the target sites of plant miRNAs are dominantly in the open reading frames (ORFs) (Rhoades et al., 2002), we focused target prediction on coding sequences. For 22 differentially expressed candidate miRNAs, 555 targets were predicted (Table S8). In agreement with the literature, the majority of potential targets for the responsive miRNAs are transcription factors (Table 3). For example, miR158 targets pentatricopeptide (PPR) repeat-containing protein, miR828 and miR858 target MYB transcription factors. Similar results were obtained for miRNA families miR159 and miR172. Furthermore, we identified a subset of miRNA families, including miR164, miR166, miR171, miR393 and miR846, which target NAC domain containing protein, dicer-like (DCL), GRAS family transcription factor,

WRKY DNA-binding protein, tetratricopeptide repeat (TPR)-like superfamily protein, respectively, implying a multiple interactive mechanism underlying the crosstalk.

The predicted targets were used for the GO enrichment analysis, indicating that the genes targeted by responsive miRNAs are respectively involved in regulation of biological process, cellular component and molecular function processes (Table S9-S11). The significantly enriched GO terms ($P \leq 0.05$) were given in the biological process, cellular component and molecular function ontologies in UV-B-treated seedlings library, while no significant terms were found in the molecular function ontologies in flg22 and F/U-treated plants libraries (Table S9-S11). There were 9, 60 and 2 differentially expressed miRNAs upon treatment flg22, UV-B and F/U, respectively, enriched in the biological process ontology. The categories “cellular response to phosphate starvation” and “somatic embryogenesis” were significantly annotated in all treated samples. Additionally, “cellular process” was annotated at UV-B treatment (Table S10). In the molecular function ontology, 18 differentially expressed miRNAs could be enriched, with 8 and 8 significantly enriched GO terms upon UV-B treatment, including two enriched terms, nucleic acid binding transcription factor activity and transcription factor activity, sequence-specific DNA binding (Table S10). Proteins interact with each other to function in certain biological activities. Pathway analysis could be used to further understand the biological function of genes. The differentially expressed miRNAs sequences and their predicted targets were mapped to the reference canonical pathways in KEGG. Upon all treatments, the most common terms were “metabolic pathways” and “biosynthesis of secondary metabolites” (in red), which were the significantly enriched pathways (Table S12-S14).

Table 3 Predicted targets of conserved miRNAs in *Arabidopsis thaliana*

miRNA family	Function of predicted targets
ath-miR158	Pentatricopeptide (PPR) repeat-containing protein; tetratricopeptide repeat (TPR)-like superfamily protein; Dof-type zinc finger DNA-binding family protein
ath-miR159	Myb domain protein; myb-like HTH transcriptional regulator family protein; SET domain protein
ath-miR164	NAC domain containing protein
ath-miR165	Homeobox-leucine zipper family protein
ath-miR166	Dicer-like (DCL)
ath-miR167	Polyprenyltransferase; aldolase-type TIM barrel family protein
ath-miR168	Stabilizer of iron transporter sufd/Polynucleotidyl transferase; SET-domain containing protein lysine methyltransferase family protein
ath-miR171	GRAS family transcription factor; succinyl-coa ligase, alpha subunit
ath-miR172	Myb domain protein; target of early activation tagged (EAT); related to AP2.7; basic helix-loop-helix (bhlh) DNA-binding superfamily protein
ath-miR391	RNA recognition motif (RRM)-containing protein
ath-miR393	WRKY DNA-binding protein; auxin signaling F-box (AFB); basic helix-loop-helix (bhlh) DNA-binding superfamily protein
ath-miR447	Homeobox-leucine zipper protein family; BEL1-like homeodomain; CCT motif family protein
ath-miR824	AGAMOUS-like; protein kinase superfamily protein
ath-miR828	Production of anthocyanin pigment 1; myb domain protein; global transcription factor group
ath-miR846	Myrosinase-binding protein; mannose-binding lectin superfamily protein; tetratricopeptide repeat (TPR)-like superfamily protein; F-box and associated interaction domains-containing protein
ath-miR858	Myb domain protein; tetratricopeptide repeat (TPR)-like superfamily protein

4.5 Validation of responsive miRNA-target interactions

In total, 13 miRNA families (miR158, miR165, miR166, miR167, miR168, miR172, miR391, miR393, miR447, miR824, miR828, miR846, miR858) representing 15 candidate miRNAs and their potential target genes were selected for validation experiments (Figure 2A; Table S1). In addition, several miRNAs that were conversely upregulated by UV-B and downregulated by flg22 also included into the experiment, including miR159a, miR159b, miR164b, miR171a and miR822 (Figure 2B). Expression levels of all selected miRNAs and their target genes in respective treatments were simultaneously determined by RT-qPCR. As shown in Figure 2, the expression patterns of all analyzed miRNAs except for miR172a, miR391, miR393a, miR447b and miR824 were confirmed as those deduced from the small RNAseq data. This, together with reciprocal changes of transcript abundances of their targets strongly supports for their participation in the crosstalk (Figure 2A).

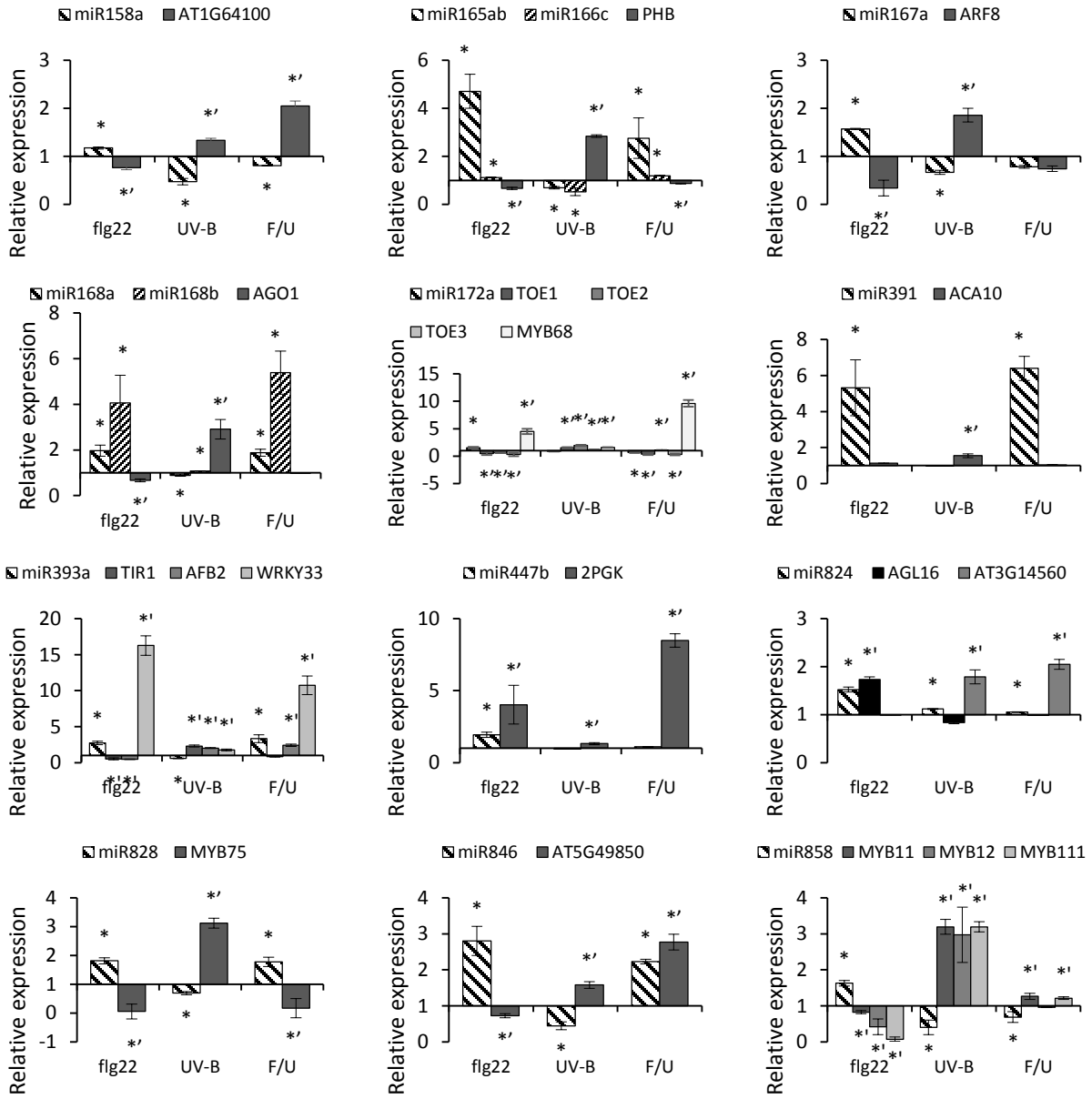
It has been reported that a gene can be targeted by multiple miRNAs (Krek et al., 2005; Lewis et al., 2005). In this study we found that both miR165ab and miR166c targeted PROTEIN HOMEOBOX (PHB) and that miR168a and miR168b targeted ARGONAUTE 1 (AGO1), showing reciprocal changes in their transcript abundances in different treatments. By contrast, miR172a, one miRNA could target members of TARGET OF EARLY ACTIVATION TAGGED (TOE) gene family, TOE1, TOE2 and TOE3, respectively. Moreover, miR858 and miR171a were able to target three MYB family members (MYB11, MYB12 and MYB111) and HAIRY MERISTEM (HAM) family (HAM1, HAM2 and HAM3), respectively. Also, multiple members of NAC gene family, NAC1, NAC2, NAC4 and AT3G12977 were all targeted by miR164b but except for NAC5, suggesting a possible selective targeting of miRNA on a multiple gene family in the crosstalk (Figure 2B).

4.6 miR858 and miR828 are key regulatory elements involved in the crosstalk

To get functional relevance of miRNA-target interactions in the crosstalk between flg22 and UV-B signaling cascades, the pairs of miR858-MYB111 and miR164b-NACs were chosen for further analysis. MYB111 proved to be functionally involved in the transcriptional regulation of the CHS expression (our RNAseq data not shown; Stracke et al., 2007; Stracke et al., 2010; Li et al., 2019). To ascertain the impact of expression levels of miR858 and miR164b on the CHS expression, we employed the short tandem target mimic *STTM858* and miR164b knockout (miR164b_KO) mutants and compared them with miR858- and miR164b-overexpression lines (miR858_OE and miR164b_OE). We found a strong suppression of miR858 up to 9.0-fold lower than in *STTM858* mutant plants compared with that in the wild-type Col-0, confirming the efficiency of our target mimicry construct in sequestering the activity of miR858, and a drastic increase in miR858 expression level up to 19.0-fold higher in miR858_OE lines than in the wild-type Col-0. Going along with those, the CHS transcript abundance was significantly reduced (1.3-fold) in miR858_OE line while increased (1.4-fold) in *STTM858* mutant plants, respectively, as compared with the control (Figure 3). In similar, a reverse change between miRNA and CHS expression levels was given in *STTM828* mutant plants (data not shown). Contrariwise, miR164b_KO drastically suppressed the CHS expression, while its miR164b_OE elevated the CHS expression. Taken together, these data provide the first genetic evidence for a functional involvement of miR858, miR828 and miR164b

in regulating the CHS expression, in which miR164b might be functional an antagonistically to the miR858 and miR828.

A



B

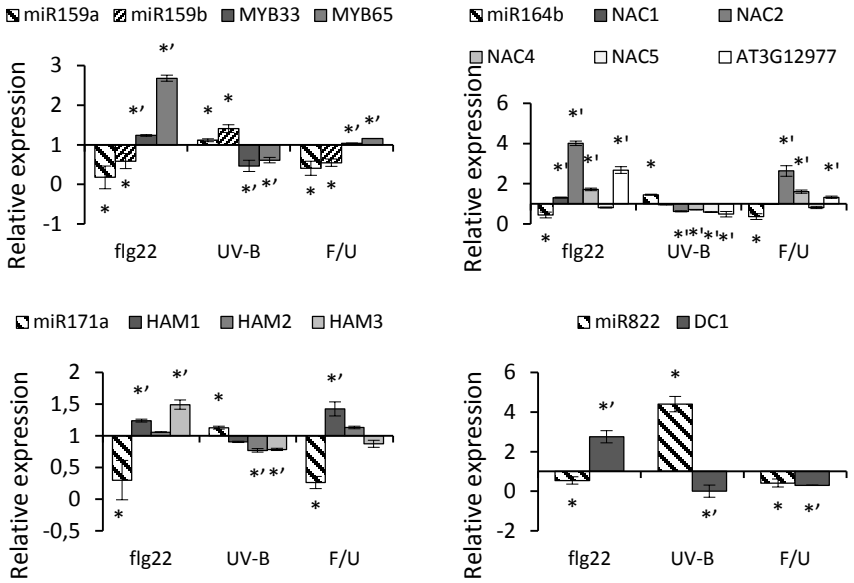


Figure 2 Quantitative RT-PCR analyses of the abundance of selected miRNAs. (A) miRNAs are upregulated by flg22 and downregulated by UV-B, as well as their potential target genes. (B) miRNAs are downregulated by flg22 and upregulated by UV-B, as well as their potential target genes. The relative expression level represents the $\log^{RQ}$ value based on the comparative Ct method (Livak and Schmittgen, 2001). Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of genes and miRNAs were carried out using Actin2 as internal reference gene. Statistically significant differences between the levels of CK and treatments transcripts were determined by using Student's T-test (*, $P < 0.05$ for miRNA expression; *', $P < 0.05$ for target expression).

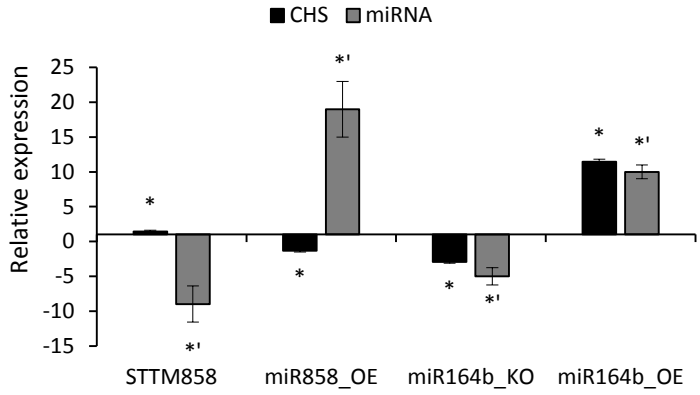


Figure 3 Quantitative-PCR analyses of the abundance of marker gene CHS and corresponding miRNAs in Arabidopsis *STTM858*, *miR858_OE*, *miR164b_KO* and *miR164b_OE*. The relative expression level represents the $\log^{RQ}$ value based on the comparative Ct method (Livak and Schmittgen, 2001). Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of genes and miRNAs were carried out using Actin2 and U6 as internal reference gene, respectively. Statistically significant differences between wild-type plant and miRNA mutant transcripts were determined by using Student's T-test (*, $P < 0.05$ for the CHS expression; *', $P < 0.05$ for the miRNA expression).

4.7 Presence of known *cis*-elements in the promoters of responsive miRNA genes

To understand the transcriptional regulation of miRNAs in response to flg22 and UV-B in general, we analyzed the promoters of 20 miRNAs above mentioned, aiming at identification of consensus TF binding sites by using the PlantCARE (Lescot et al., 2002). We identified a variety of known *cis*-elements characteristic of miRNA (Figure 4). They include for instance light responsive elements, such as MYC (CAATTG), G-box (TACGTG), GT-1 motif (GGTTAA), TCT-motif (TCTTAC), AE-box (AGAAACAA|TT) and GATA-motif (AAGATAAGATT) as well as *cis*-elements MYB (T|CAACCA, CAACAG), abscisic acid responsiveness ABRE (ACGTG), W box (TTGACC) and STRE (AGGGG) etc. (Table 4). Comparison of 15 miRNAs, which are upregulated by flg22 and downregulated by UV-B), retrieved prevalent *cis*-elements including MYB, ABRE, MYC, G-Box, GT1-motif and TCT-motif. Conspicuously almost all miRNAs have MYB motif and except for two all miRNAs contain ABRE and MYC. We found 12 miRNAs contain G-Box, GT1-motif and TCT-motif. Apparently, AE-box was dominantly involved in the upstream regions of miRNA genes that are upregulated by flg22 and downregulated by UV-B, while differing from this, miRNAs downregulated by flg22 and upregulated by UV-B all comprise GATA- or STRE-motif. Although most of these motifs have been well documented to be involved in plant response to diverse abiotic and biotic stresses, to gain insight into the transcriptional regulation of miRNAs simultaneously by flg22 and UV-B, further investigation on miRNA promoters are needed.

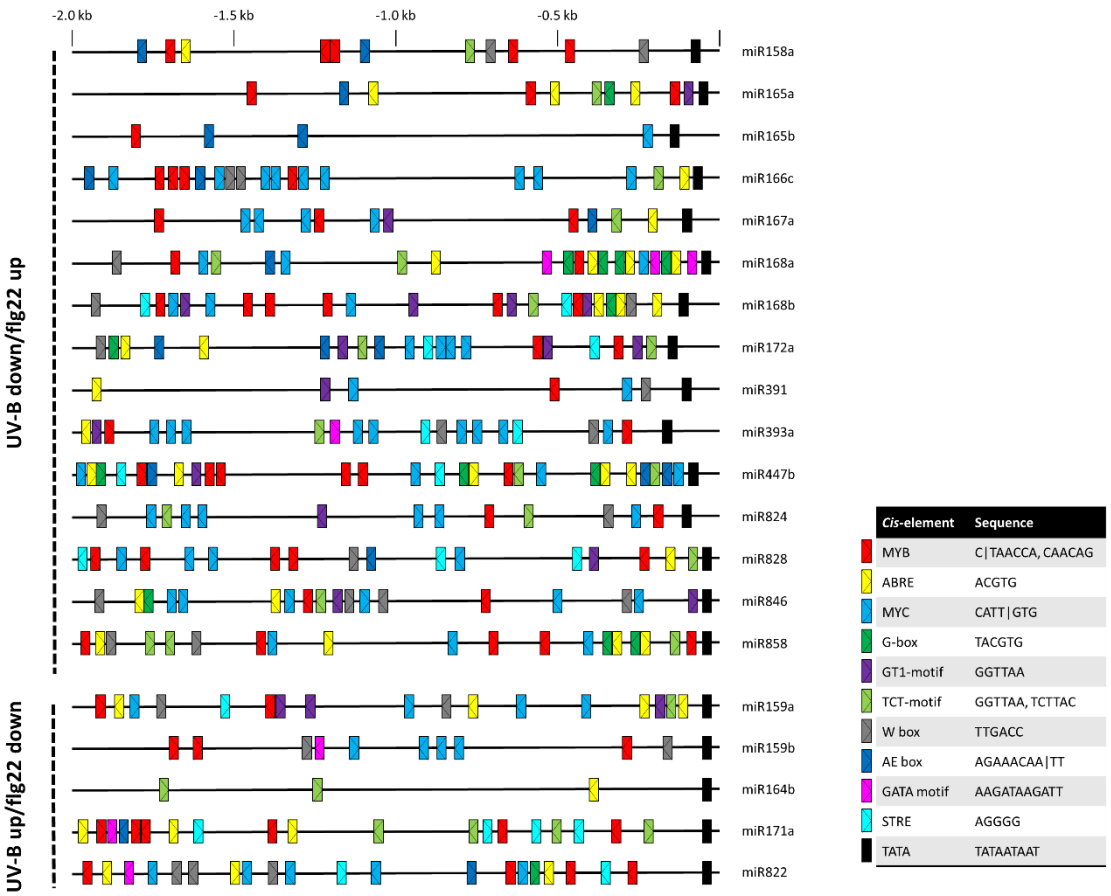


Figure 4 Motifs in the upstream regions of responsive miRNA genes that are predicted to be involved in the crosstalk between flg22 and UV-B in Arabidopsis.

Table 4 Motifs in the upstream regions of responsive miRNA genes that are predicted to be involved in the crosstalk between flg22 and UV-B in Arabidopsis

miRNA	*cis*-element	TATA-box	MYB	ABRE	MYC	G-Box	GT1-motif	TCT-motif	W box	AE-box	GATA-motif	STRE
upregulated by flg22 and downregulated by UV-B	miR158	1	5	1		1	1	1	2	2		
	miR165a	1	3	3		3	1	1		1		
	miR165b	1	1		1					2		
	miR166c	1	4	2	9	1		1	2	2		
	miR167a	1	3	1	4	1	2	1		1	1	
	miR168a	1	2	7	4	4	1	2	1	1	3	
	miR168b	1	6	4	3	2	4	1	2			3
	miR172a	1	2	2	4	1	3	2	1	3		2
	miR391	1	1	1	2	2	1		1		1	
	miR393a	1	2	1	9		1	1	2		1	2
	miR447b	1	3	4	3	3	1	1		2		1
	miR824a	1	2		6		1	2	2			
	miR828	1	5	1	4	1	1	1	1	1		3
	miR846	1	2	2	6	6	1		4			
	miR858	1	5	5	1	3	1	3	2	2		
downregulated by flg22 and upregulated by UV-B	miR159a	1	2	4	4	4	1	1	2			1
	miR159b	1	3		1		1		2		1	
	miR164b	1		1				2				
	miR167b	1	1	4	2	5			1			2
	miR171a	1	6	4	2	3		4		1	1	4
	miR822	1	4	3	5	4		1	3	1	1	1

5 Discussion

miRNAs are important regulators of gene expression at the post-transcriptional level and play vital roles in plant development and response to a/biotic stresses (Gleeson et al., 2014; Bertolini et al., 2013). Since so far, an integrated view of the transcriptional regulation in the crosstalk between flg22 and UV-B signal cascades is still lacking. Identification of miRNAs that are involved in this crosstalk is an essential step to assert the process. In this way, we focused on the identification and characterization of miRNAs from Arabidopsis whole seedlings treated by flg22, UV-B and F/U as well as by mock treatment through high-throughput sequencing. Since the function of miRNAs is merely relying on the function of their targets, potential targets of the miRNAs were *in silico* predicted and subsequently experimentally validated. The predicted targets were assigned to GO (Park et al., 2008) as well as KEGG classifications (Kanehisa et al., 2014) providing first insights into the network of miRNAs being involved in the crosstalk between flg22 and UV-B signal cascades in Arabidopsis. Our data support the idea that miRNAs constitute a crucial layer in regulating the crosstalk between flg22 and UV-B signal cascades.

Analysis of small RNAseq data identified 128, 129 and 121 miRNAs with altered abundance in Arabidopsis seedlings treated by flg22, UV-B and F/U, respectively, from which 75, 51 and 52 miRNAs were upregulated while 53, 78 and 69 were downregulated by flg22, UV-B and F/U, respectively. Identification of a large number of miRNAs responsive to flg22, UV-B and F/U treatments supports the idea that a multiple complex network of miRNAs constitutes a regulatory layer in the crosstalk of between flg22 and UV-B signal cascades. It has been reported that flg22 induces miR393 accumulation in Arabidopsis, which specifically targets TIR/AFB transcripts. The repression of TIR/AFB transcripts consequently downregulates auxin signaling pathway and enhances plant resistance to DC3000 bacteria (Navarro et al., 2006). In addition, miR158a, miR160a, miR167, miR169, miR391, and miR396 were induced while miR398b, miR156 and miR168 abundances are slightly reduced upon flg22 treatment (Navarro et al., 2006). In Arabidopsis, the expression of miR172b was also induced by flg22 treatment. MiR172b however regulates the transcription of the receptor FLS2 through TARGET OF EAT1 (TOE1) and TOE2 (Zou et al., 2018). Ultraviolet irradiation is one of the major abiotic factors restricting plant growth, development, and productivity. Several studies demonstrated that miRNAs functionally

participate in plant response to UV-B irradiation. For instance, miR396 was upregulated by UV-B in Arabidopsis leaves, and this induction was correlated with a decrease in the levels of its GRF targets (Casadevall et al., 2013). Zhou et al. (2007) reported 21 miRNAs of 11 miRNA families in Arabidopsis were upregulated under UV-B stress, including miR156/157, miR159/319, miR160, miR165/166, miR167, miR169, miR170/171, miR172, miR393, miR398, and miR401. Also, miR156, miR160, miR165/166, miR167, miR168 and miR398 were upregulated in *Populus tremula* by UV-B irradiation (Jia et al., 2009). Wang et al. (2013) isolated wheat miRNAs, from which miR159, miR167a and miR171 were upregulated while miR164, miR395 and miR156 were downregulated after UV-B treatment. The fact that miR158, miR159, miR167, miR171, miR172, miR391 and miR393 are also included in our datasets although with varied expression levels as reported except for miR165, miR166 as well as miR168 that was not upregulated by UV-B and downregulated by flg22, respectively, in our study (Figure 2). The discrepancy observed might be attributed to different experiment designs. Furthermore, it is of great interest and importance to further characterize miRNAs and their targets, which were newly identified to be regulated by flg22 or UV-B irradiation in this study, including miR447, miR824, miR828, miR846 and miR858, which were upregulated by flg22 and downregulated by UV-B, as well as miR164 and miR822, which were downregulated by flg22 and upregulated by UV-B (Figure 2). It is reasonable to believe that the understanding such miRNA-target interactions may shed more light on the multiple complex mechanism plants responses to a/biotic stresses.

The identification of 22 candidate miRNAs, which are clearly distinguished between flg22 and UV-B as well as F/U treatments in their expression levels provoked us to seek their possible involvement/role in the crosstalk. Since the function of miRNAs is merely relying on the function of their targets, we firstly predict targets of candidate miRNAs, and then characterized them *in silico* and finally determine the involvement of miRNA-target interactions in the crosstalk by simultaneously determining the changes in their expression levels in response to distinct treatments. In agreement with the literatures (Samad et al., 2017; Jangra et al., 2018), the majority of potential targets predicted are transcription factors, including several well studied transcription factors, like miR858-MYB11, -MYB12, -MYB111, as well as miR828-MYB75 (Sharmar et al., 2016; Yang et al., 2013), which proved to be involved in anthocyanin accumulation and biosynthesis, imply their role in the crosstalk. Simultaneous monitoring the expression levels of

miRNAs and their targets in response to distinct treatments reveals two modulations of miRNA-target interactions in the crosstalk between the flg22 and UV-B signal cascades. The first on consisting of miR158, miR165, miR166, miR167, miR168, miR172, miR391, miR393, miR447, miR824, miR828, miR846 and miR858, which were all repressed by UV-B irradiation, thus resulting in an increased transcript levels of the targets and the activation of CHS gene expression consequently, while they were all drastically upregulated by flg22, going along with the decrease in the transcript levels of the targets as well as CHS gene expression. In accordance with many reports that the UV-B-induced flavonol pathway gene expression was abolished when a concomitant elicitation by flg22 occurs (Serrano et al., 2012; Schenke et al., 2019), the flg22 effect on miRNA expression occurs also by co-treatment flg22/UV-B in our study. Thus, it is reasonable to believe that the flg22 triggered PTI plays a dominant role in reprogramming the secondary metabolism in the crosstalk between flg22 and UV-B induced signal cascades, which is partially relying on the modulation of miRNA-target interactions. There are already indications that this suppression is mediated by transcription factors (TFs) regulation. Two MYB TFs, the positive regulator MYB12 (UV-B upregulated and flg22 suppressed) and the negative regulator MYB4 (activated by UV-B, but much faster by flg22) have been implicated in the regulation of FPGs (Schenke and Cai, 2014). However, it is still unknown if these TFs are the crucial players in this crosstalk of just co-regulated with FPGs. In order to address this question and get some genetic evidence, we had to establish first a seedling based system allowing us to test mutant plants for impaired crosstalk. Here we describe this system as a prerequisite step to analyze homozygous mutants in this crosstalk between abiotic and biotic stress responses.

An antagonistic regulation of the FPGs and production of flavonoids (Jin et al., 2000; Stracke et al., 2007) mediated by the positive regulator MYB12 and negative regulator MYB4 was postulated by Schenke et al. (2011) and Zhou et al. (2017). In support of this, we could show that in the second group of miRNAs, their expression was vice versa upregulated by UV-B while repressed by flg22, supporting for their involvement in the crosstalk in an antagonistic manner (Zhou et al., 2017). To note the group II contains a few but prominent plant-derived miRNAs like miR159, miR164, miR171 and miR822, which target several well-known TFs involving plant response to a/biotic stresses (MYBs, NACs and HAMs). Thus, further characterization of their role in

modulation of the crosstalk between flg22 and UV-B signaling cascades is needed in order to gain more insights into the regulation of plant response to abiotic and biotic stresses.

The modulation of miRNA on CHS gene expression could be evidently demonstrated by investigation on Arabidopsis *STTM858* and *miR858_OE* as well as *miR164b_KO* and *miR164b_OE* mutant plants. We demonstrated that knockdown of miR858 (Group I) in Arabidopsis goes with increase of CHS gene expression while its overexpression results in depression of CHS gene expression. Vice versa, knockout of miR164b (Group II) depresses the CHS gene expression while its overexpression strongly elevated the CHS gene expression. Thus, we could provide the first genetic evidence that miRNAs identified in this study constitute an additional layer in regulating the crosstalk between flg22 and UV-B induced signaling cascades.

In support for many previous reports, we could show a large set of MYB TFs as direct targets of miRNAs identified in this study. MYB TFs represent a family of proteins that control many aspects of plant secondary metabolism, for instance the phenylpropanoid metabolism in Arabidopsis. In addition to MYB111 and MYB75, AtMYB66 are required for normal epidermal-cell patterning, and regulated the position-dependent expression of GL2 (GLABROUS 2) (Lee and Schiefelbein, 1999). Additionally, AtMYB91 was found to be a homologue of AmMYBPHAN from *A. majus* and ZmMYBRS2 from *Z. mays*. Moreover, AtMYB4 acts as a negative regulator of cinnamate 4-hydroxylase gene expression as well as negatively regulates several steps of phenylpropanoid metabolism in a dose-dependent way (Jin et al., 2000). The miRNA-MYB interactions are implicated in the regulation of FPGs and flavonol content (Sharma et al., 2016; Wang et al., 2016). For instance, MYB11, MYB12 and MYB111 are prominent targets of miR858 (Wang et al., 2016). Flavonoids have been reported to be regulated by miR858 affecting MYB11, MYB12 and MYB111 expression. In addition, miR858a is obviously able to enhance anthocyanin biosynthesis in Arabidopsis seedlings by repressing translation of MYBL2, the negative regulator of anthocyanin biosynthetic pathway (Sharma et al., 2016; Wang et al., 2016). Recently, Camargo-Ramírez et al. (2017) stated miR858 functioned as a negative regulator of Arabidopsis immunity by controlling accumulation of antifungal phenylpropanoid compounds. Also, miR828 seems to be functionally analog to miR858, which is downregulated by UV-B and upregulated by flg22. Recent studies indicated that miR828 was induced by sugar and attenuated in abscisic acid in Arabidopsis (Luo

et al., 2011) and wound inducible in sweet potato leaves (Lin et al., 2012). The sequence of miR828 was complementary to a region of an another MYB factor, MYB75, which caused a massive activation of phenylpropanoid biosynthetic genes and enhanced accumulation of lignin, hydroxycinnamic acid esters and purple anthocyanins (Yang et al., 2013). miR158 was induced by flg22 but repressed by UV-B (Figure 2A). miR158 targets numerous mRNAs coding for pentatricopeptide repeat proteins (PPR) (Allen et al., 2004). The PPR gene family was one of the largest families of putative RNA-binding proteins in plants containing more than 400 genes (Small and Peeters, 2000). It was predicted that the PPR family plays a central and broad role in regulating gene expression in organelles (Schmitz-Linneweber et al., 2005). One of the hallmark characteristics of the syncytium was the increasing number of organelles such as mitochondria (Jones, 1981). Nothing is known about gene expression and RNA processing in organelles during feeding site formation and this miRNA may be useful as novel investigative target in this context. It is worth mentioning that miR164 exhibited contrasting expression pattern in response to flg22 and UV-B. miR164 modulates plants development by controlling mRNA abundance of transcription factor NAC1 (Guo et al., 2005). NAC1 acts in transmitting auxin signals for Arabidopsis lateral root development (Xie et al., 2000). We could show a downregulation of miR164b accompanied by an upregulation of NAC1 in response to flg22, with a strong induction of miR164b followed by a reduction of NAC1 in response to UV-B (Figure 2B). We could show that NAC1, NAC2, NAC4 and AT3G12977 are targeted of miR164b except for NAC5. It is interesting to find out the mechanism governing such selective targeting on different target genes/members.

It is known that flavonoid biosynthesis is regulated by a variety of environmental and endogenous stimuli, including light, pathogen attack, several stresses and plant-growth regulators (Dixon and Paiva, 1995; Mol et al., 1996; Buer et al., 2006). In Arabidopsis, flavonol production was under transcriptional control of the R2R3-MYBs MYB11, MYB12 and MYB111 (Mehrtens et al., 2005; Stracke et al., 2007). In addition to miR828 and miR858, several miRNAs obtained from this study, including miR159, miR396, miR5654, miR838, miR844 and miR866 (Table 3) can also regulate different MYB TFs. This strongly implies for a multiple of flavonol pathway genes, which are regulated by miRNAs during the crosstalk between flg22 and UV-B in Arabidopsis.

Taken together, the data presented here provide the first evidence for the implication of miRNAs in the crosstalk between flg22 and UV-B signaling cascades, and a basis for further understanding the model of action underlying. In addition, it is also of great scientific and practical significance to address the role of miRNAs in regulating plant response to a/biotic stresses and its potential role for improving plant tolerance to changing environment, in general.

On the basis of our data resented in this study, we could propose a function model for miRNA-target interactions to illustrate how the miRNA and their targets are involved in regulation of the CHS-pathway via the crosstalk between flg22 and UV-B induced signal cascades in Arabidopsis (Figure 5). The model includes 13 miRNA families representing 15 miRNAs (Group I), which were upregulated by flg22 and downregulated by UV-B, while 4 miRNA families representing 5 miRNAs (Group II), which are downregulated by flg22 and upregulated by UV-B. Antagonistic effects of flg22 and UV-B on the expression of miRNAs in/between 2 groups as well as an antagonistic regulation of TFs between 2 groups are essential and constitute a multiple and complex regulatory layer in plant response to flg22 and UV-B induced signal cascades. A great challenge remains to dissect the mechanism how miRNAs are regulated in response to diverse environmental and endogenous stimuli, e.g. by flg22 and UV-B as well as their crosstalk in plants.

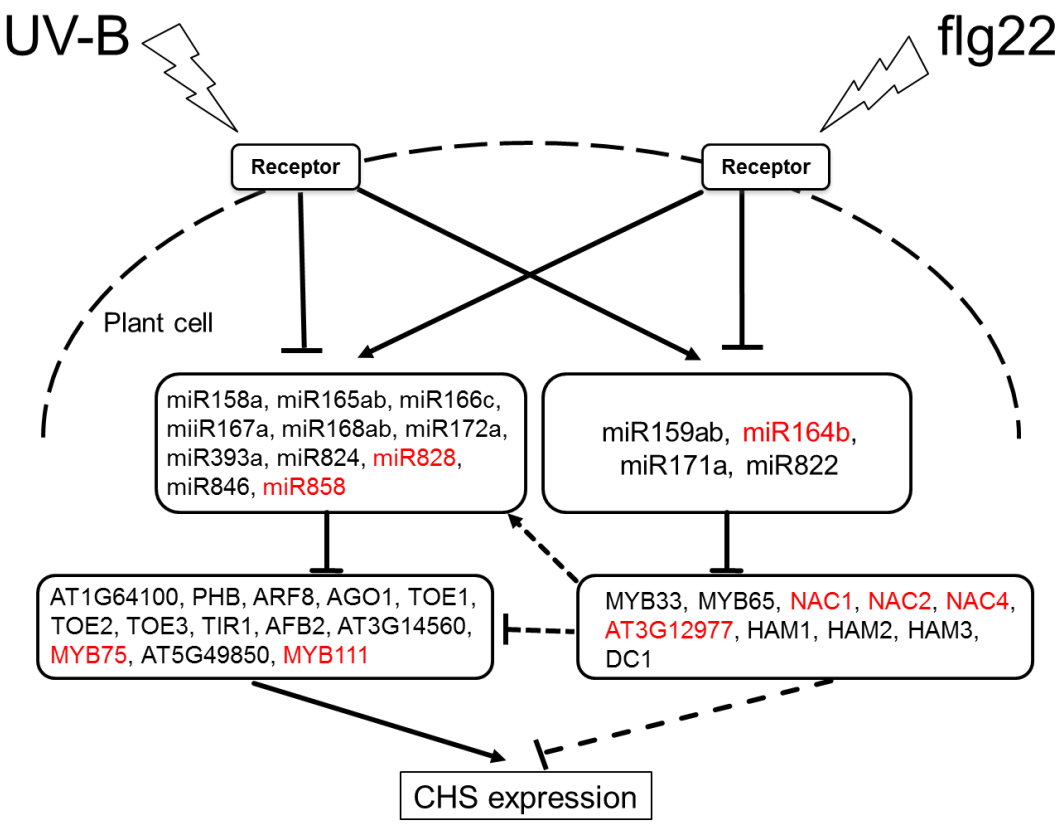


Figure 5 A functional Model for miRNA-target interaction involved in regulation of the crosstalk between flg22 and UV-B in Arabidopsis. The miRNAs regulate the TFs and finally FPGs. Antagonistic effects of flg22 and UV-B on the expression of miRNAs in/between 2 groups as well as an antagonistic regulation of TFs between 2 groups are essential in the crosstalk between flg22 and UV-B induced signal cascades and constitute a multiple and complex regulatory layer of CHS gene expression. Positive interactions are represented with arrows, and negative interactions with bars.

6 Conclusion

miRNAs are a class of non-coding endogenous small RNAs that play diverse and important roles in plant growth, development, and stress responses. Many evidences demonstrated that miRNAs are involved in plant response to biotic stress (flg22) and abiotic stress (UV-B), respectively. The majority of miRNAs targets are transcriptional factors. In this study, we report a comprehensive study on Arabidopsis miRNAs identified by using seedlings from three treatments (flg22, UV-B and F/U) through high-throughput sequencing and bioinformatics methods as well as validated by experiments. A population of small RNAs was characterized as conserved miRNAs with a few of novel miRNAs. The interactions between miRNAs and targets in response to distinct treatments were demonstrated by RT-qPCR analysis, strongly supporting for an indispensable role and function of miRNAs in the crosstalk between flg22 and UV-B signaling cascades. This study provides valuable data and information for further elucidation of the function and mechanisms of miRNAs in regulating plant responses to a/biotic stresses.

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8 Supplementary data

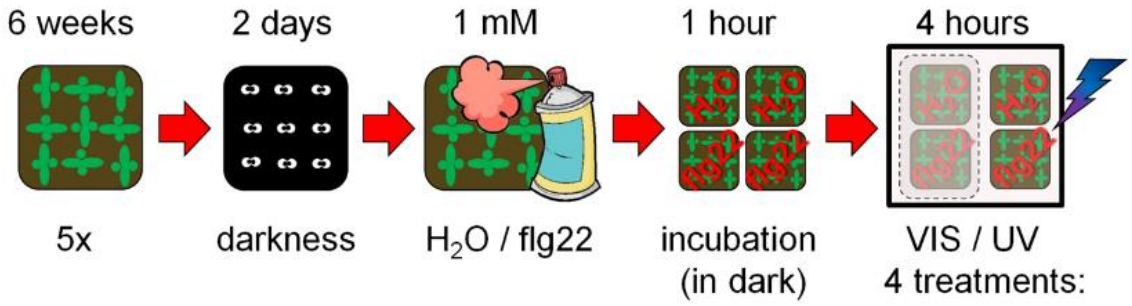


Figure S1 Flow-chart of the experimental setup. The seedlings were treated as indicated, finally flash-frozen in liquid nitrogen and stored at -80°C before samples were used for RNA isolation.

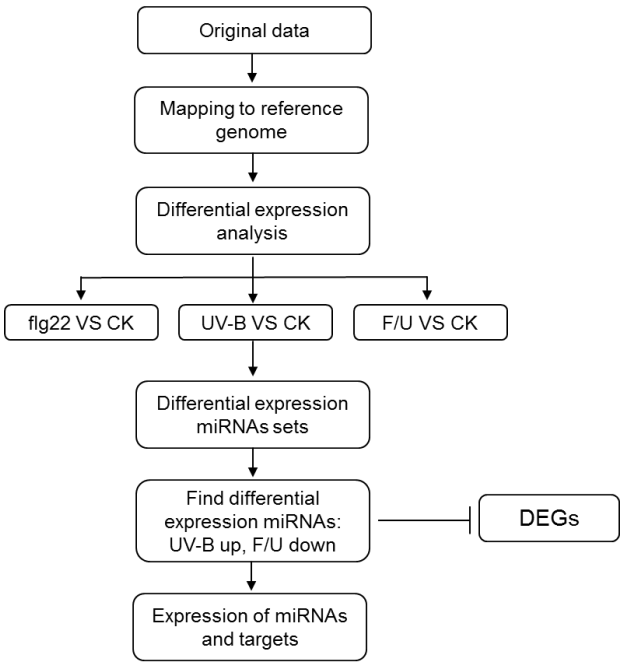


Figure S2 Bioinformatic analysis pipeline designed for this study.

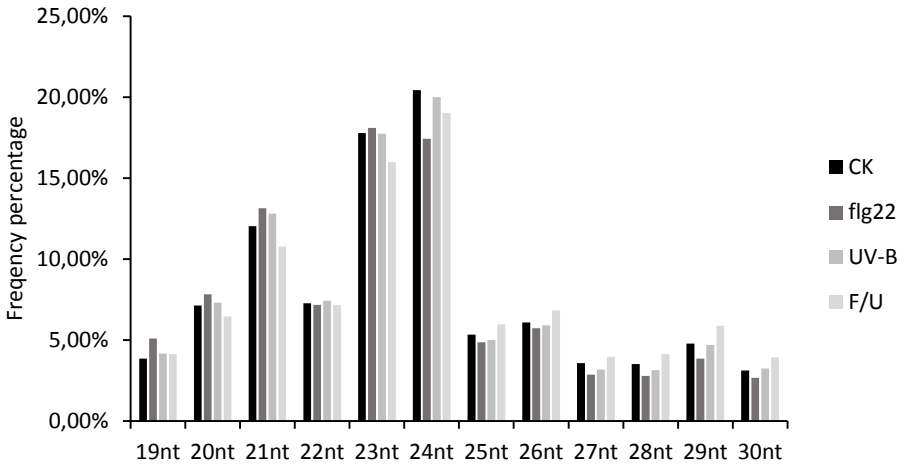


Figure S3 Distribution of total sequence reads in Arabidopsis thaliana small RNA libraries made from CK, flg22, UV-B and F/U treated seedlings.

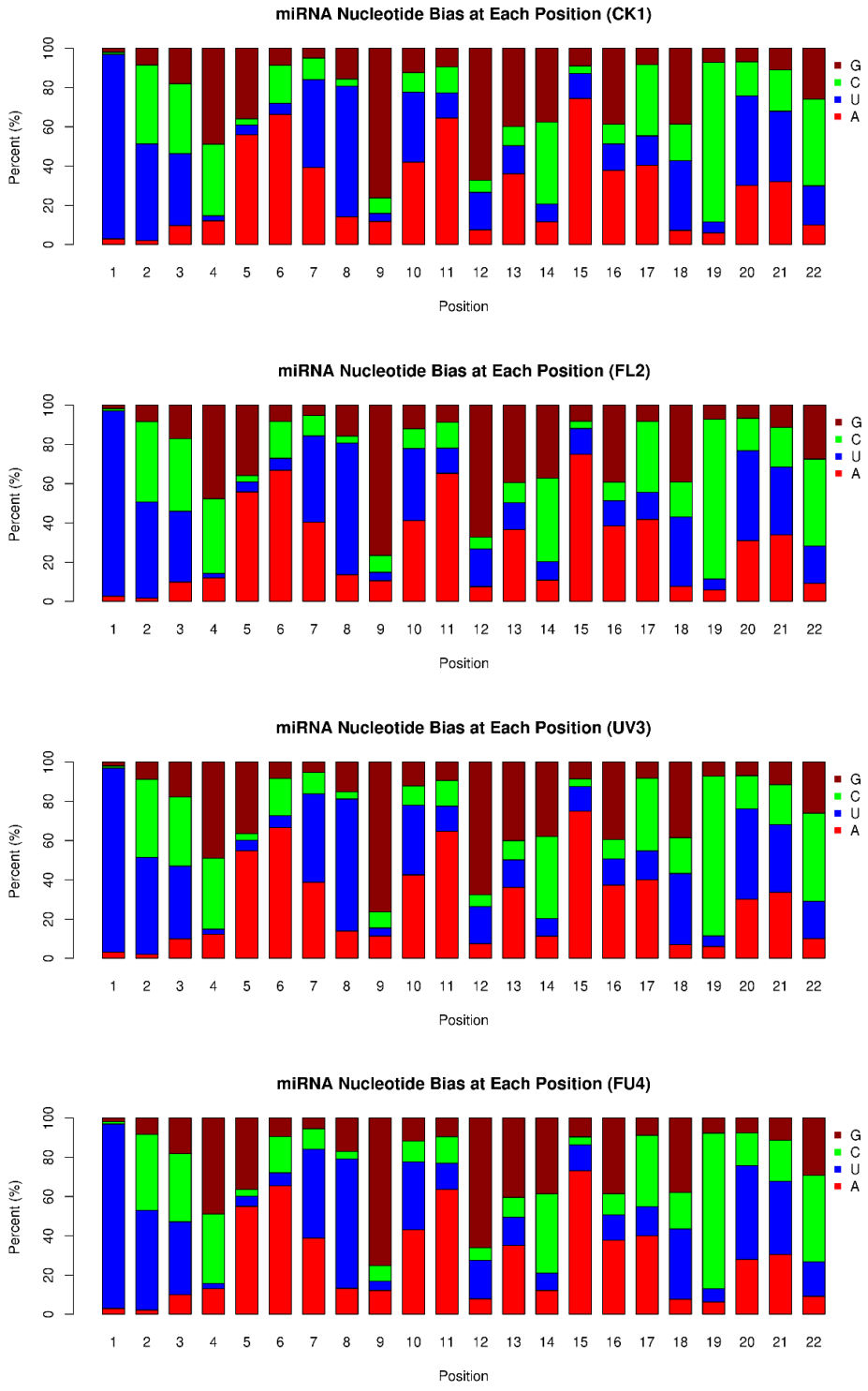


Figure S4 Nucleotide preferences at each position of the sequences that took as *Arabidopsis thaliana* small RNAs. Nucleotide preferences of four libraries (CK, flg22, UV-B and F/U) sequenced 22-nt non-redundant sequences.

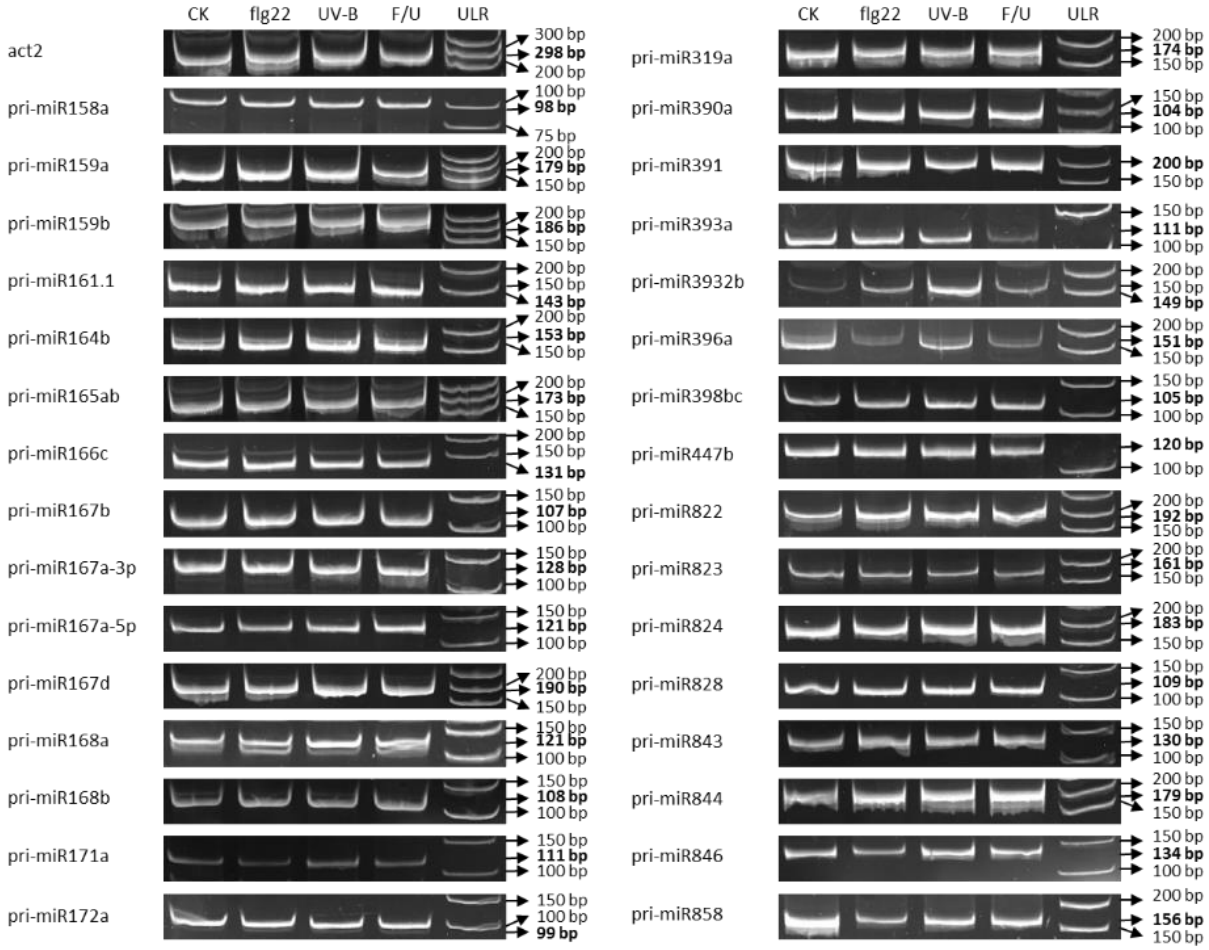


Figure S5 Validation of identified miRNAs by RT-qPCR. PAGE gel analyses of 31 small RNA sequences are shown. ULR, ultra low range DNA ladder.

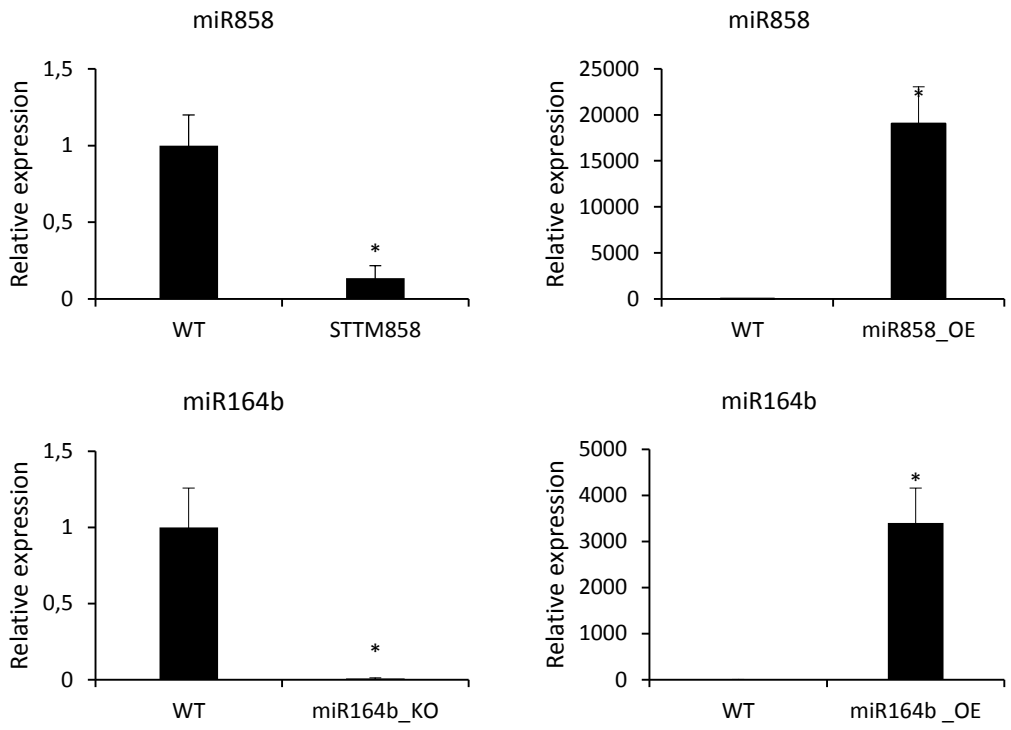


Figure S6 Expression validation of miRNA in *STTM858*, *miR858_OE*, *miR164b_KO* and *miR164b_OE* lines. The relative expression level represents the $\log^{RQ}$ value based on the comparative Ct method (Livak & Schmittgen, 2001). Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of genes and miRNAs were carried out using U6 as internal reference gene. Statistically significant differences between wild-type plant and STTM mutant transcripts were determined by using Student's T-test. (*, $P < 0.05$)

All Supplementary Tables are involved in CD, including:

Table S1 Primer used in this study

Table S2 All miRNAs involved in the crosstalk between flg22 and UV-B in *Arabidopsis thaliana*

Table S3 All differentially expressed miRNAs in libraries from flg22, compared with CK

Table S4 All differentially expressed miRNAs in libraries from UV-B, compared with CK

Table S5 All differentially expressed miRNAs in libraries from F/U, compared with CK

Table S6 Differentially expressed miRNAs upregulated by flg22 and downregulated by UV-B, compared with CK

Table S7 Differentially expressed miRNAs downregulated by flg22 and upregulated by UV-B, compared with CK

Table S8 Predicted potential target of all miRNAs involved in the crosstalk flg22 and UV-B in *Arabidopsis thaliana*

Table S9 Functional classification of different expressed miRNA targets of flg22 treatment with miRNA based on Gene Ontology (GO)

Table S10 Functional classification of different expressed miRNA targets of UV-B treatment with miRNA based on Gene Ontology (GO)

Table S11 Functional classification of different expressed miRNA targets of F/U treatment with miRNA based on Gene Ontology (GO)

Table S12 KEGG classification of miRNA targets of flg22 treatment

Table S13 KEGG classification of miRNA targets of UV-B treatment

Table S14 KEGG classification of miRNA targets of F/U treatment

Chapter IV: miR858 and miR828 are master regulators for UV-B- and flg22-signal crosstalk in *Arabidopsis thaliana*

Zheng Zhou, Dirk Schenke and Daguang Cai

In submission

1 Abstract

In nature plants are often simultaneously challenged by different stress factors. The abiotic stress UV-B irradiation induces the production of UV-protective flavonols, but their accumulation is attenuated by biotic stress, e.g. by treatment with pathogen elicitors (flg22). This suppression has been shown to occur via suppression of flavonol pathway genes (FPGs) enabling the plant to direct its secondary metabolism to a more efficient pathogen defense response. Identification of two highly conserved miRNAs (miR858 and miR828) being involved in the crosstalk and their targets MYB111 and MYB75 that proved to play an important role in regulation of FPGs and the flavonoid accumulation provoked us to assume that miR858-MYB111 and miR828-MYB75 interactions play an important role in the crosstalk. Here, we demonstrate that both miR858 and miR828 are regulated by UV-B and flg22 in a UVR8 and FLS2 receptors-dependent manner and relying on the respective signaling pathways. Comparison between miR858/MYB111-promoter-GUS and their transcript levels evidences that the MYB111 is regulated not only at the transcriptional level, but also suffered from post-transcriptional modification in response to the flg22 and UV-B challenges, in which miR858 acts as a determinant regulator. Following this, we conclude that the post-transcriptional regulation mediated by plant-derived miRNAs constitutes the crosstalk between the flg22 and UV-B induced signal cascades in Arabidopsis. This allows an extension of the crosstalk model between plant responses to biotic (flg22) and abiotic (UV-B) stresses in Arabidopsis.

2 Introduction

Plants are confronted with constant risk of infections by various stresses of abiotic and biotic in their natural habitats. In case of UV-B radiation, plants defend themselves for example with the production of sinapoylmalate and flavonoids, which function as natural sunscreens (Meissner et al., 2008). Since more than two decades, it is known that UV-B induced flavonol pathway genes (FPGs) are downregulated when concomitant elicitation by microbe-associated molecular pattern (MAMPs) occurs (Gläßgen et al., 1998). This induces the so-called MAMP-triggered immunity (MTI), a plant defense response against pathogens, reprogramming the secondary metabolism to produce anti-microbial phytoalexins and lignification to prevent the pathogen spreading within the host tissue (Schenke and Cai, 2014). Because all flavonoids, lignin and the phytoalexin scopoletin are derived from the same precursor phenylalanine, a suppression of UV-B induced flavonoids is suitable to provide more resources for the pathogen defense (Schenke et al., 2011). We have established an experimental system enabling the investigation of the crosstalk between UV-B and flg22-induced MTI in planta Arabidopsis. UV-B-induced FPGs are also suppressed by flg22 in Arabidopsis seedlings, demonstrating that this kind of “crosstalk” is fully functional in planta (Zhou et al., 2017).

The previous study revealed that several MYB transcription factors (TFs) have been implicated in the regulation of FPGs and flavonol content in this crosstalk in planta. One negative regulator MYB4 (Jin et al., 2000) and three positive regulating MYB TFs (Stracke et al., 2007) appear to play a major role. Strikingly, MYB111 presented a much stronger regulation by UV-B, compared to MYB12 and MYB11. The proposed direct regulation by WRKYs cannot be upheld, because the MYB111 promoter does not contain any W-Boxes required for WRKY binding (Supplemental Figure S1). This suggests that there must be an additional layer of regulation. MiRNAs are often associated with transcriptional and post-transcriptional regulation of various TFs (Yang et al., 2013; Shen et al., 2014; Megraw et al., 2016).

MicroRNA (miRNA), a class of endogenous small noncoding RNA with a size range of about 20-24 nucleotides, negatively regulate the expression of protein-coding genes by cleavage or suppressing translation (Jones-Rhoades et al., 2006). Accumulating evidence suggests that miRNAs have been characterized in plants as important factors involved in gene expression

regulation in multiple biological processes, such as development (Lelandais-Briere et al., 2010), phytohormone signaling (Liu and Chen, 2009), flowering and sex determination (Chuck et al., 2009), as well as adaption to biotic and abiotic stress conditions (Katiyar-Agarwal and Ji, 2010; Song et al., 2019). More than 180 Arabidopsis miRNA loci have been identified, representing nearly 80 miRNA families, many of which are important for plant development.

Arabidopsis miR393 was the first small RNA implicated in bacterial PAMP-triggered immunity (PTI) (Navarro et al., 2006). MicroRNA393 transcription was induced by the flagellin-derived PAMP peptide, flg22, to target mRNAs encoding the F-box auxin receptor transport inhibitor response 1 (TIR1) and related proteins. Moreover, using small RNA-expression profiling on Arabidopsis leaves collected at 1 and 3 h post-inoculation (hpi) with *Pst* DC3000 hrcC, Fahlgren et al. (2007) identified three miRNAs (miR160, miR167 and miR393) that were highly induced and one that was downregulated (miR825) after infection. Also in Arabidopsis, miR156 and miR164 were induced by infection with the virus TYMV p69 and were induced in transgenic Arabidopsis plants expressing the viral silencing suppressor P1/HC-Pro (Kasschau et al., 2003). Upon virus infection, new miRNAs (miR158 and miR1885) were specifically induced by turnip mosaic virus in *Brassica napes* (He et al., 2008).

In addition, miRNAs are also involved in the defense to abiotic stress. Several studies reported that, the TF coding genes No Apical Meristem (NAM-NAC) and cup-shaped cotyledon (CUC) that were regulated by the miR164 family in Arabidopsis, which were important in root and shoot development (Sieber et al., 2007; Raman et al., 2008). Furthermore, it is already known that hormone signaling and gene expression under miRNA control have deterministic roles in plant development (Liu and Chen, 2009; Liu et al., 2009). In Arabidopsis, miR159-targeting members of the gibberellic acid MYB (GAMYB) family regulated seed germination and anther formation (Reyes and Chua, 2007). The overexpression of miR159 and the inhibition of the MYB gene expression delayed flowering and caused male sterility (Millar and Gubler, 2005). In addition, to identify Arabidopsis miRNAs induced by UV-B radiation, a computational approach was used (Zhou et al., 2007). Of the 21 miRNAs belonging to 11 miRNA families identified in that study, the following were predicted to be regulated under UV-B stress: miR156/157, miR159/319, miR160, miR165/166, miR167, miR169, miR170/171, miR172, miR393, miR398, and miR401.

To identify miRNAs involved in the crosstalk, we deep-sequenced four small RNA libraries made from control, flg22, UV-B as well as flg22 and UV-B co-treated seedlings. In total 217 miRNAs representing 204 conserved and 13 novel miRNAs were identified, from which 22 miRNAs upregulated by flg22 and downregulated by UV-B. The abundance of two highly conserved miRNAs (miR858 and miR828) and their corresponding potential targets (MYB111 and MYB75) were validated. It is believed that the miR858-MYB111 and miR828-MYB75 interactions might act as key regulatory modules in the crosstalk between flg22 and UV-B signaling cascades.

MiR858 was initially identified in Arabidopsis (Rajagopalan et al., 2006), and later in apple (*Malus domestica*) (Xia et al., 2012) and cotton (*Gossypium hirsutum*) (Guan et al., 2014). Jia et al. (2015) reported that blockage of miR858 induces anthocyanin accumulation by modulating SlMYB48-like transcripts in tomato. Currently, Wang et al. (2016) demonstrated that miR858a enhanced anthocyanin biosynthesis in Arabidopsis seedlings by repressing translation of *MYBL2*, the negative regulator of anthocyanin biosynthetic pathway. MIR858a is a direct target of HY5 and displays light-responsive expression in an HY5-dependent manner. Moreover, flavonoids have been reported to be regulated by miR858 affecting MYB11, MYB12 and MYB111 expression (Sharma et al., 2016; Wang et al., 2016), which all negatively acting on flavonol production. Camargo-Ramírez et al. (2018) recently stated miR858 functioned as a negative regulator of Arabidopsis immunity by controlling accumulation of antifungal phenylpropanoid compounds.

Moreover, miR828 was first found to be expressed in Arabidopsis (Rajagopalan et al., 2006; Hsieh et al., 2009), and has also been discovered or predicted in other plant species, including grape (*Vitis vinifera*), alfalfa (*Medicago truncatula*), rice (*Oryza sativa*), poplar (*Populus trichocarpa*) and rape (*Brassica napes*) (Rajagopalan et al., 2006; Sunkar and Jagadeeswaran, 2008; Xia et al., 2012). Recent studies indicate that miR828 is induced by sugar and attenuated in abscisic acid in Arabidopsis (Luo et al., 2011) and wound inducible in sweet potato leaves (Lin et al., 2012). The sequence of miR828 is complementary to a region of another MYB factor, Myb75, which is involved in the anthocyanin biosynthesis pathway (Hsieh et al., 2009; Yang et al., 2013).

However, the involvement of the both miR858 and miR828 in regulation of UV-B and flg22 signal cascades and their crosstalk via targeting of the TFs MYB111 and MYB75 remains to be demonstrated. In order to address this, we investigated related miRNAs in respect of their

involvement in the crosstalk by small RNA-seq and RNA-seq to analyze the role of the miR858/miR828 in regulation of plant response to a/biotic stress factors. In this study, our data suggested that the working system we established before was further confirmed by mutant analysis. Moreover, miR858 and miR828 negatively control the crosstalk by repressing the expression of MYB111 and MYB75, respectively. MiR858 and miR828 appeared to be an important factor to regulate their potential targets MYB111 and MYB75 at various levels by promoter analysis. miR858 shows effects on plant-pathogen (e.g. *Pst* DC3000) interactions in the crosstalk between flg22 and UV-B signaling pathways. Overall, a possible functional model had been updated at transcriptional level.

3 Materials and methods

3.1 Plant materials

The UV RESISTANT LOCUS 8 (UVR8) mutant *uvr8*, which lacks 15 amino acids of receptor region in the Landsberg-0 (Ler-0) background was obtained from Jenkins (2014). UVR8 is the only UV-B specific photoreceptor identified (Jenkins, 2014; Rizzini et al., 2011). Homozygous transfer DNA (T-DNA) insertion mutants, including *hy5* (SALK_096651C), *fls2* (SALK_062054), *bak1* (At4g33430), *bik1* (At2g39660), *plc2* (At3g08510), *RbohD* (At5g47910), *mpk3* (At3g45640), *mpk6* (At2g43790), *myb4* (At4g38620), *myb11* (At2g47460), *myb12* (At2g47460), *myb111* (At5g49330, GK291D01), triple mutant *myb11/12/111* and *myb75* (At1g56650) in the Columbia-0 (Col-0) genetic background were obtained from Nottingham Arabidopsis Stock Centre (NASC). miRNA knockdown mutants (*STTM858* and *STTM828*) were kindly provided by Dr. Huiyong Zhang and Dr. Guiliang Tang (Addgene plasmid # 84157; http://n2t.net/addgene:84157; RRID: Addgene_84157), respectively. The miR164-overexpressing Arabidopsis line miR164 was purchased from NASC. To generate double mutant *uvr8×fls2* plants, *uvr8* homozygous plants were crossed with the *fls2* homozygous plants. F_3 generation plants homozygous for the *uvr8* and *fls2* mutation were identified for genotyping by PCR, using genomic DNA as template. Genomic DNA was extracted from leaf tissues with the Edward's method (Edwards et al., 1991). Primers used for genotyping are included in Supplemental Table S1.

3.2 Plant treatment

Plant treatment strategy described by Zhou et al. (2017). Nine seedlings of Arabidopsis plant were grown together on a single Jiffy (Jiffy-7 Peat Pellets, Jiffy Products International AS, Norway) and after 5 weeks transferred to darkness in order to completely suppress chalcone synthase (CHS) mRNA levels. After 2 days in darkness, all plants were ready to be treated. Half pots were selected for spraying with HPLC-water, while the remaining pots were sprayed with 1 mM flg22 solution. To let flg22 taking effect, the sprayed plants were incubated for 1 h in darkness and were then exposed to UV-B or VIS-light as control. Thus, each biological replicate consists of the four treatments water/VIS-light control (CK), flg22 treatment (flg22), UV-B treatment (UV-B) and the co-treatment flg22/UV-B (F/U). Of each treatment, nine whole seedlings were harvested in a 2 ml

tube for later RNA isolation, immediately flash-frozen in liquid nitrogen and stored at -80 °C until further processing.

3.3 RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted from seedlings using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 µg total RNA in 20 µl volume using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) at 42 °C in 1 h. Approximately 50 ng of the synthesized cDNA was used as a template for RT-qPCR reaction. RT-qPCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific). The mature miRNA sequence appended with two adenines on the 3' end was used as forward primer sequence to ensure correct binding of the primer to the poly(T) region of the mature miRNA cDNA and preclude potential binding to the miRNA precursor. The transcript of miRNA was quantified using a forward primer specific to miRNA precursor and the universal reverse primer. *U6* small nuclear RNA was used as an internal control for miRNA gene expression normalization. The PCR reactions were performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following program: 10 min 95 °C; 50 × 10 s 95 °C, 10 s 59 °C, 10 s 72 °C; 10 s 95 °C, melting curve from 65 °C to 95 °C. The PCR products were then exposed to a temperature ramp to generate the dissociation curves and determine amplification specificity. For the quantification of marker gene CHS and MYBs expression levels, real-time PCR was performed using the following program: 10 min 95 °C; 40 × 15 s 95 °C, 30 s 59 °C, 30 s 72 °C; 10 s 95 °C, melting curve from 65 °C to 95 °C. Normalization of the expression levels was carried out using *Actin2* as internal reference gene. Each data point is based on three independent biological replicates measured as two technical replicates each. Primers used for qPCR quantification assays are included in Supplemental Table S2.

3.4 RNA-Seq library preparation and data analysis

Nine seedlings of Arabidopsis wild-type plant Col-0 were grown together on a single Jiffy, and three biological samples of the whole seedlings of 5-week-old were harvested after treatments (mentioned above). Total RNA was isolated by Trizol reagent. Approximately 250 ng of mRNA was used for small RNA-seq and RNA-seq library preparation. Libraries were multiplexed and fed into HiSeq machines for sequencing, and then quality of the sequenced data was assessed. Low-

quality reads were trimmed, and uniquely mapped read was aligned to the Arabidopsis reference genome (TAIR10) using TopHat v2.0.14 (Trapnell et al., 2009). Number of reads assigned to individual genes were counted by HTSeq (Anders et al., 2015). Differentially expressed genes (DEGs) were determined by R package DESeq (Love et al., 2014) and adjusted *P* value cutoff of 0.05.

3.5 Promoter clone, plant transformation and GUS histochemical analysis

The promoter regions of MYBs (MYB111 and MYB75) and miRNAs (miR858 and miR828) were PCR amplified from Arabidopsis genomic DNA. The PCR products were cloned into the entry vector pDONR201 by BP reaction and then transferred into the destination vector pGWB433 by LR reaction to generate *pMYB111:GUS*, *pMYB75:GUS, pmiR858:GUS* and *pmiR828:GUS* construct. Constructs were sequenced and then transformed into the *Agrobacterium tumefaciens* strain GV3103, which were used to transform the flower buds of Arabidopsis wild-type Col-0 (Clough and Bent, 1998). Transgenic lines were selected on ½ MS medium plates containing 50 mg/L of kanamycin. Primers used in the vector construction are listed in Supplemental Table S3. Plants from independent transgenic Arabidopsis lines were used for histochemical staining of GUS, which was detected according to a previously described method (Jefferson et al., 1987). Whole chemically treated seedlings were soaked in the GUS staining buffer (1 mM X-glucuronide in 0.2 M dibasic sodium phosphate, pH 7.0, 0.2 M sodium dihydrogen phosphate, pH 7.0 and 0.1% Triton X-100), and incubated at 37 °C in the darkness overnight. After being washed with 70% ethanol several times, plants were photographed using a ZEISS SteREO discovery. V20 stereomicroscope.

3.6 Identification of interaction between miR828 and MYB75

To analyze the interaction between miR828 and MYB75, RNA ligase-mediated (RLM)-5’ RACE analysis and transient expression in *Nicotiana benthamiana* were performed.

RNA ligase-mediated 5’ amplification of cDNA ends (RLM-RACE)

To validate the predicted targets of miR828, 5’ RACE was carried out with 2 μg of total RNA. To this RNA a synthetic RNA adapter was ligated using T4-RNA ligase 1 (NEB). After phenol/chloroform cleaning RNA was precipitated, resuspended in Diethyldicarbonat (DEPC) treated water and the integrity was checked on the agarose gel. Reverse transcription of mRNAs

was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). For amplification of cDNA ends, a pair of gene-specific and adapter specific primers were designed (Supplemental Table S4). RACE touchdown PCR was performed, and 1 µl of this PCR product was used as template for the following nested PCR. The reaction products were separated on a 2% agarose gel. The bands with the expected size were excised, purified, and cloned into the pGEM®-T Easy Vector (Promega) for sequencing. Primer used for 5'-RACE analysis is listed in Supplemental Table S4.

Transient expression in *Nicotiana benthamiana*

Further, to demonstrate the interaction of miR828 and MYB75, infiltration of transient expression via *Agrobacterium* in *Nicotiana benthamiana* was performed. 3-week-old *Nicotiana benthamiana* plant was used for transient transfection with the *A. tumefaciens* harboring the overexpressing constructs of miR828. The predicted target site, RACE target site, mutated predicted target site, mutated RACE target site were fused to the report gene GUS, namely, TS1, TS2, MT1 and MT2, respectively. Primer used for transient expression analysis is listed in Supplemental Table S4.

3.7 Identification of *Pseudomonas syringae* pv. *tomato* DC3000 and bacteria mutants

Three strains of *Pseudomonas syringae* were applied in this study, including bacteria wild-type strain *Pst* DC3000, mutant *hrpL*, which lacks on its transcriptional factor for T3SS genes (Lam et al., 2014), as well as mutant *hrcQ*, which T3SS is deficient so that not able to translocate its effectors (Wei et al., 2007). Bacteria were cultivated at 28 °C on NYGA medium (5 g/l bactopeptone, 3 g/l yeast extract and 20 m/l glycerol). When appropriate, antibiotics were added to the medium at the following final concentrations: for DC3000, 50 µg/ml kanamycin and 50 µg/ml rifampin; for *hrpL*, 100 µg/ml kanamycin; for *hrcQ*, 50 µg/ml spectinomycin. Bacteria were grown on NYGA agar plates for 48 h at 28 °C and resuspended in 10 mM $MgCl_2$ at an OD_{600} of 0.2, corresponding to about 1×10^8 colony forming units (cfu) per ml. Further serial dilutions were carried out to obtain suspensions for inoculations with different doses. Eight-week-old plants were inoculated by infiltrating bacterial suspensions into the intracellular spaces.

3.8 Statistical analysis

The experiments were arranged in a completely randomized design. Each treatment was composed of three independent biological replicates. Data are plotted as means ± SE in the figures. Phenotypic observations were monitored for two to three generations for consistency. Multiple comparisons of statistical significance were carried out using a two-way ANOVA or Student's *T*-test according to Minitab software (MINITAB, 2000).

4 Results

4.1 miR858 and miR828 co-regulated the CHS expression by targeting distinct TF MYBs

In previous study, it is reported that miR858 and miR828, which target different MYB transcription factor genes, and differentially expressed in response to flg22 and UV-B (Stracke et al., 2007; Zhou et al., 2017). We detected that expression levels of both miR858 and miR828 are drastically downregulated by UV-B treatment, while elevated by flg22 as well as co-treatment F/U (Figure 1C and 1D). While miR828 only targets MYB75, miR858 is able to targets MYB11, MYB12 and MYB111 (Figure 1B). As revealed by a simultaneous transcripts analysis by RT-qPCR, miR828 and miR858 both showed an expression pattern opposite to those observed by MYB75, MYB11, MYB12 and MYB111 in response to flg22, UV-B and F/U, respectively, thus suggesting their involvement in the crosstalk. As expected, MYB4 was vice versa upregulated by flg22 and downregulated by UV-B, also induced significantly by co-treatment (Figure 1B). In Arabidopsis, miR858 is encoded by one functional genomic locus (miR858a, At1g71002) that produces 21-nucleotide mature molecules and targets ten TF MYB mRNAs that contain the miR858 complementary sequences. While, miR828 (At4g27765) produces 22-nucleotide mature sequence and also target TF MYB75. Based on our previous study, a working model has been established in wild-type plant Col-0. This crosstalk was functional in Arabidopsis plant leading to flg22-induced suppression of UV-B-induced FPGs exemplified by CHS expression analysis (Figure 1A). As shown in Figure 1, the crosstalk was functional in Arabidopsis plant leading to flg22-induced suppression of UV-B-induced FPGs exemplified by CHS expression analysis.

Considering that MYB111 and MYB75 might play an important role in the regulation of FPGs the flavonoid accumulation (Pandey et al., 2014). We focus our investigation on the miR858-MYB111 and miR828-MYB75 modules in respect of their role in the crosstalk. MYB11, MYB12 and MYB111 were all directly targeted by miR858 (Shamra et al., 2016; Fahlgren et al., 2007), while miR828 has only been predicted to directly target MYB75 in sweet potato (Lin et al., 2012).

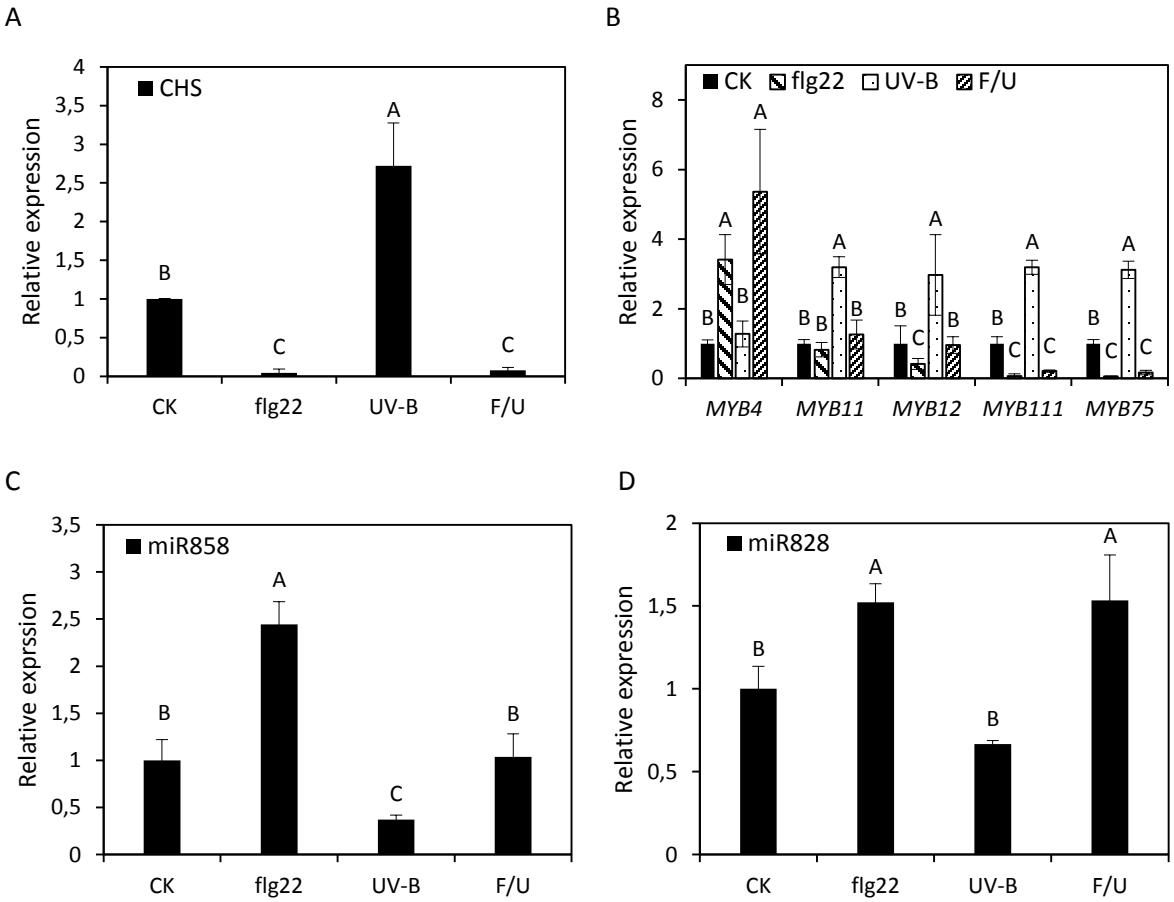


Figure 1 miR858 and miR828 post-transcriptionally downregulates MYB11, MYB12, MYB111 and MYB75 during the crosstalk between flg22 and UV-B induced signaling cascades in Arabidopsis. The abundance of CHS (A), MYBs (B), mature miR858 (C) as well as mature miR828 (D) were measured by RT-qPCR in wild-type plant Col-0, relative to non-treated control plant. The transcript levels of MYB11, MYB12, MYB111 and MYB75 were inversely correlated with the expression levels of miR858 and miR828, respectively. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of genes and miRNAs was carried out using Actin2 and U6 as internal reference genes, respectively. Statistically significant differences between the levels of CK and other treatments transcripts were determined using ANOVA ($P < 0.05$).

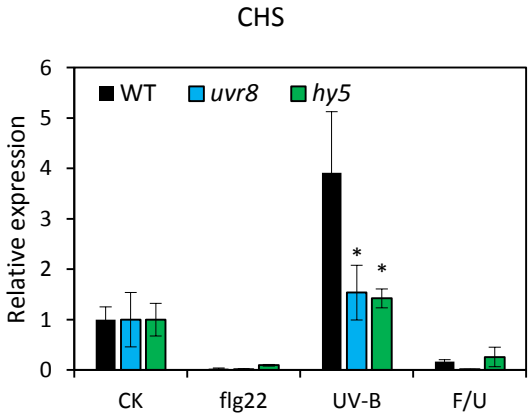
4.2 UVR8 and FLS2 receptors are involved in the crosstalk between the flg22 and UV-B induced signal cascades

To address whether the crosstalk between flg22 and UV-B induced signal cascades in Arabidopsis is mediated by the UV-B and FLS2 receptors, we employ the Arabidopsis UV-B mutant *uvr8*, FLS2 mutant *fls2* and double mutant *uvr8×fls2* in this study. There is no obvious phenotypic difference between the mutants and the wild-type control plants as evaluated by visual inspection (Supplemental Figure S2).

The homozygous mutant plants were treated by flg22, UV-B and F/U. Firstly, we checked the CHS expression levels in the mutants and compared them with the wild-type control. While the induction of CHS expression by UV-B occurred in the wild-type, it was drastically impeded in *uvr8* mutant, in which the suppression of CHS expression by flg22 treatment was obviously not remarkably changed (Figure 2A). Since UVR8 specifically regulates expression of the downstream TF HY5 when the plant is exposed to UV-B (Brown et al., 2005). We extended the analysis to include the Arabidopsis *hy5* mutant. As expected, the *hy5* mutant showed a similar CHS expression pattern as those observed with *uvr8* mutant (Figure 2A), thus demonstrating that the UV-B induced the CHS expression is mediated by a UVR8-dependent signaling cascade. In a similar vein, we analyzed the Arabidopsis *fls2* mutant plants, which lack the leucine-rich repeat serine/threonine protein kinase required for flg22 perception and signal transduction initiation. As shown in Figure 2B, the flg22 triggered suppression of CHS expression was abolished in the mutant *fls2*, in which no significant change in the UV-B-induced CHS expression occurred, supporting for a FLS2-dependent suppression mechanism of the CHS expression by flg22. BAK1, which has been identified as a co-receptor of flg22, also participates in innate immunity responses. FLS2-BAK1 signaling also activates the downstream components such as MPK3, MPK4 and MPK6 (Gao et al., 2009; Li, 2010). Moreover, Li et al. (2014) demonstrated that the FLS2-associated kinase BIK1 directly phosphorylates the NADPH oxidase RbohD to control plant immunity. Moreover, previous studies suggested a role for PLC2 in FLS2-triggered immunity, as it is rapidly phosphorylated in vivo upon treatment with the bacterial MAMP flg22 (Nühse et al., 2007). Considering these, we analyzed mutants *bak1*, *bik1*, *plc2*, *rbohD*, *mpk3* and *mpk6* in respect of their CHS expression in response to distinct treatments in this study. As shown in Figure 2B, all mutants showed a similar regulation upon flg22 and UV-B treatment as those in the wild-type plants: the CHS expression was induced by UV-B while suppressed by flg22 as well as co-treatment F/U, respectively. Following this, we excluded these genes as well related signaling pathways from the crosstalk between flg22 and UV-B induced signaling cascades. In support for this, the CHS gene expression was not significantly affected either by flg22 or by UV-B and by F/U in the double mutant *uvr8×fls2* (Figure 2C), which were identified for genotyping by PCR analysis (Supplemental Figure S3).

In the next step, we analyzed the expression levels of miR858 and miR828 in the treated mutants and compared with the wild-type control. The drastic suppression of miR858 and miR828 (Figure 2D and 2F), along with the induction of MYB111 and MYB75 as observed in the wild-type did not occur in the mutant *uvr8* and *hy5* (Figure 2E and 2G), in which also no significant difference upon the flg22 treatment was given. Somehow, flg22 showed the effect on miR858 and miR828 expression even in *uvr8* and *hy5* plants. In similar, in the *fls2* mutant, the flg22-induction of miR858 and miR828 (Figure 2D and 2F) as well as the corresponding downregulation of MYB111 and MYB75 were obviously also impeded (Figure 2E and 2G). Nevertheless, the UV-B irradiation showed effects on miR828-suppresion and MYB75-induction as well as miR858-suppresion and MYB111-induction in the *fls2* mutant. In the double mutant *uvr8×fls2*, the both miRNAs neither upregulated by flg22 and F/U nor downregulated by UV-B could be observed when compared with the wild-type control (Figure 2D and 2F). The reduction of the two MYBs by flg22 and F/U as well as their induction by UV-B are all significantly impaired in *uvr8×fls2* (Figure 2E and 2G). Taken together, we conclude that flg22 and UV-B differentially regulate the expression of miR858 and miR828 in a UVR8 and FLS2 receptor dependent manner, which in turn interfere with targets leading to fine-tuning of the crosstalk between flg22 and UV-B signaling pathways.

A



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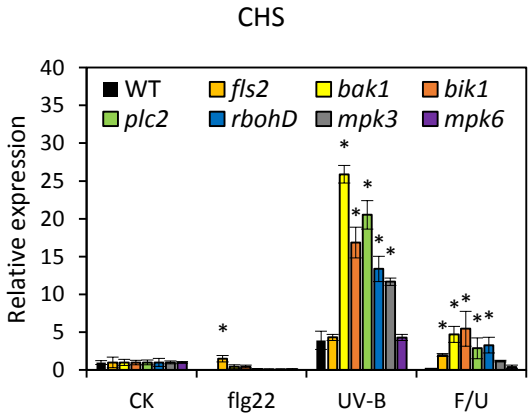

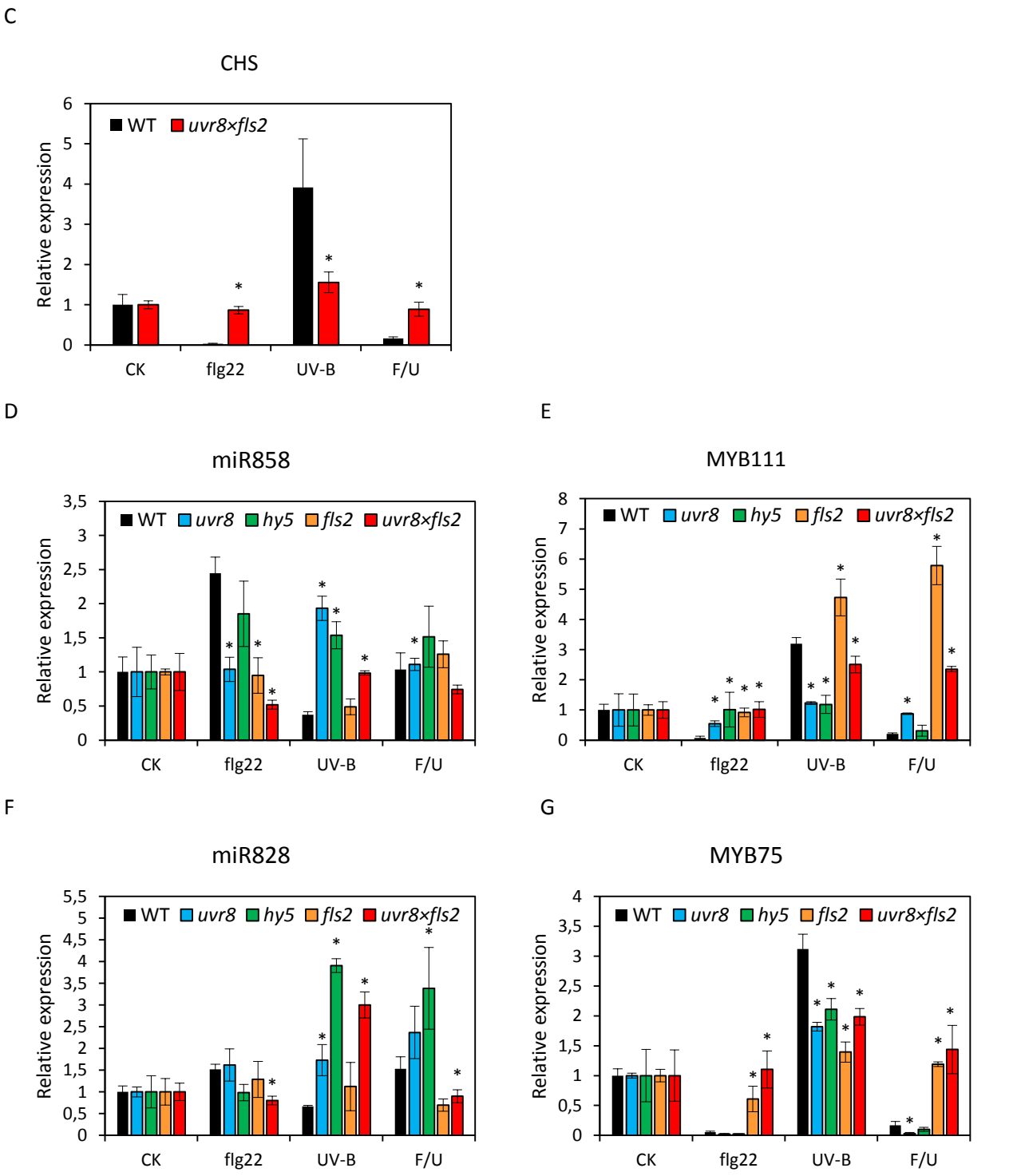

Figure 2 The relative expression of marker gene CHS in UV-B signal pathway mutants (A: *uvr8, hy5*), flg22 signal pathway mutants (B: *fls2, bak1, bik1, plc2, rbohD, mpk3, mpk6*) and the double mutant *uvr8×fls2* (C), as compared with the wild-type control, as well as the relative expression of miR858 (D), MYB111 (E), miR828 (F) and MYB75 (G) in mutants *uvr8, hy5, fls2* and *uvr8×fls2* compared with the wild-type. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of genes and miRNAs was carried out using Actin2 and U6 as internal reference genes, respectively. Statistically significant differences between wild-type plant and mutant transcripts were determined by using Student's T-test (*, $P < 0.05$).

4.3 Overexpression of a mimic sequence for miRNA augments UV-B-induction

The implication of miRNAs in modulating plant response to UV-B-induction was further examined by analysis of transgenic Arabidopsis plants with a reduced miRNA expression. This was accomplished by expressing a mimic sequence for miR858 and miR828 in their mature form (*STTM858* and *STTM828*). The artificial non-cleavable binding site for the mature miRNA contained a three nucleotide bulge (CTA) that does not interfere with miRNA binding but would prevent transcript cleavage and hence sequester miRNA activity. *STTM858* and *STTM828* transgenic lines that showed a 7.7 and 2.7-fold reduction in the mature miR858 and miR828 expression level, respectively (Supplemental Figure S4), were used for further analysis. No noticeable morphological differences between *STTM858*/*STTM828* lines and the wild-type were observed (Supplemental Figure S2). In the both mutants, the CHS expression was significantly increased by UV-B, while as expected the flg22-repression of CHS expression could not be observed, compared with the wild-type (Figure 3). The data provide genetic evidence that miR858 and miR828 were crucially involved in the crosstalk between flg22 and UV-B induced signaling cascades.

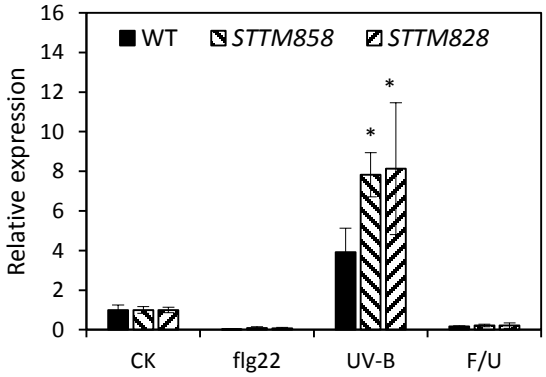

Figure 3 Regulation of the CHS expression in *STTM858* and *STTM828* plants, as compared with the wild-type. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of genes was carried out using Actin2 as internal reference genes. Statistically significant differences between wild-type plant and mutant transcripts were determined by using Student's T-test (*, $P < 0.05$).

4.4 Regulation of CHS is mediated by TF MYBs

To confirm whether the both miRNAs regulate the CHS expression via targeting TF MYBs, T-DNA insertion mutants of MYB4, MYB11, MYB12, MYB111, MYB75 as well as a triple mutant

myb11/12/111 were analyzed. Obvious differences in plant growth and development between the mutants was not observed (Supplemental Figure S2). MYB4, which differs from other TF MYBs, was induced by flg22 but downregulated by UV-B in the wild-type (Figure 1B). Mutant analysis indicated that *myb4* showed a similar expression pattern as those observed in the wild-type (Figure 1A). Hence, its involvement in the crosstalk could not be confirmed. We found that flg22-reduction and UV-B-induction of CHS expression were strongly impaired in mutants *myb11* and *myb12*, respectively. In the mutant *myb111*, the UV-B-upregulation of CHS expression was abolished as compared with the wild-type, in consistent with the *STTM858* data. Unexpectedly, no notable differences in flg22-suppression and UV-B-induction of CHS expression could be observed between the wild-type and the triple mutant *myb11/12/111* (Supplemental Figure S2). But in the mutant *myb75*, the suppression of CHS expression by flg22 is to some content attenuated (Figure 4). These results are in consistence with many reports that the TF MYBs act as crucial player in modulating plant responses and their crosstalk. The data indicates that the UV-B-induction of CHS expression is mostly mediated by MYB12 and MYB111, while flg22-suppression is more dependent on MYB11 and MYB75, respectively.

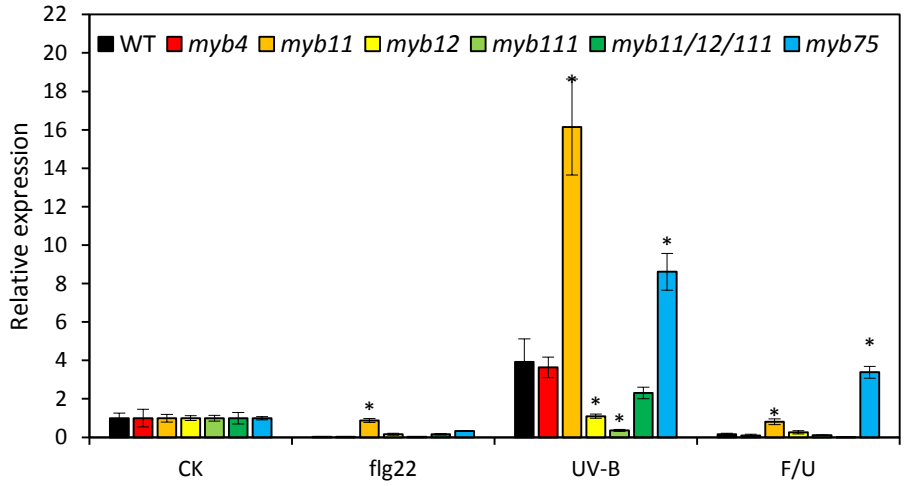

Figure 4 Expression analysis of the CHS expression in *myb* mutants *myb4, myb11, myb12, myb111, myb11/12/111* and *myb75,* respectively as compared with those in the wild-type. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of gene was carried out using Actin2 as internal reference gene. Statistically significant differences between wild-type plant and mutant transcripts were determined by using Student's T-test (*, $P < 0.05$).

4.5 Promoter analysis of miRNAs and MYBs subjects a complex regulation in signal crosstalk

To define the role of transcriptional and post-transcriptional regulation of miR858/miR828 and MYB111/MYB75 in the crosstalk between flg22 and UV-B induced signal cascades, the promoter-GUS fusion constructs of miR858, miR828, MYB111 and MYB75 were generated in which a β-glucuronidase was placed behind the promoter, resulting in the constructs of *pmiR858:GUS*, *pmiR828:GUS*, *pMYB111:GUS* and *pMYB75:GUS*, respectively**.** The gene constructs were transferred into wild-type Col-0 Arabidopsis plants through *Agrobacterium tumefaciens*-mediated transformation (Figure 5A and 5B, Supplemental Figure S5A and S5B). In total, 13, 8, 11 and 2 positive transgenic plants for *pMYB111:GUS, pmiR858:GUS*, *pMYB75:GUS* and *pmiR828:GUS* were selected, respectively and further confirmed by PCR with construct-specific primer (Supplemental Figure S6). Because of a weak GUS activity detectable in both *pMYB75:GUS* and *pmiR828:GUS* transgenic plants (Supplemental Figure S5), these two constructs were excluded for further GUS staining and expression analysis.

In *pMYB111:GUS* transgenic Arabidopsis plants, GUS gene was activated in the whole seedling, including leaves, cotyledons, apical meristems and roots with varied levels (Figure 5C), which is consistence with the observation made by Stracker et al. (2007). Noticeably, in response to the treatment, the GUS expression patterns in *pMYB111:GUS* transgenic plants (Figure 5E) totally differed from those observed by MYB111 transcripts analysis in the wild-type (Figure 1B). The flg22 induced GUS expression was not suppressed by flg22 and co-treatment F/U as well. But, *pmiR858:GUS* transgenic plants showed an intense GUS staining in the whole seedling (Figure 5D), and the GUS expression patterns in response to distinct treatments (Figure 5F) were consistent with the changes in transcript abundance of miR858 observed in the wild-type (Figure 1C). These data strongly support that MYB111 is regulated not only at the transcriptional, but also at post-transcriptional levels in response to flg22 and UV-B challenges, and that miRNA-target interactions act as a determinant in the crosstalk between flg22 and UV-B signal cascades.

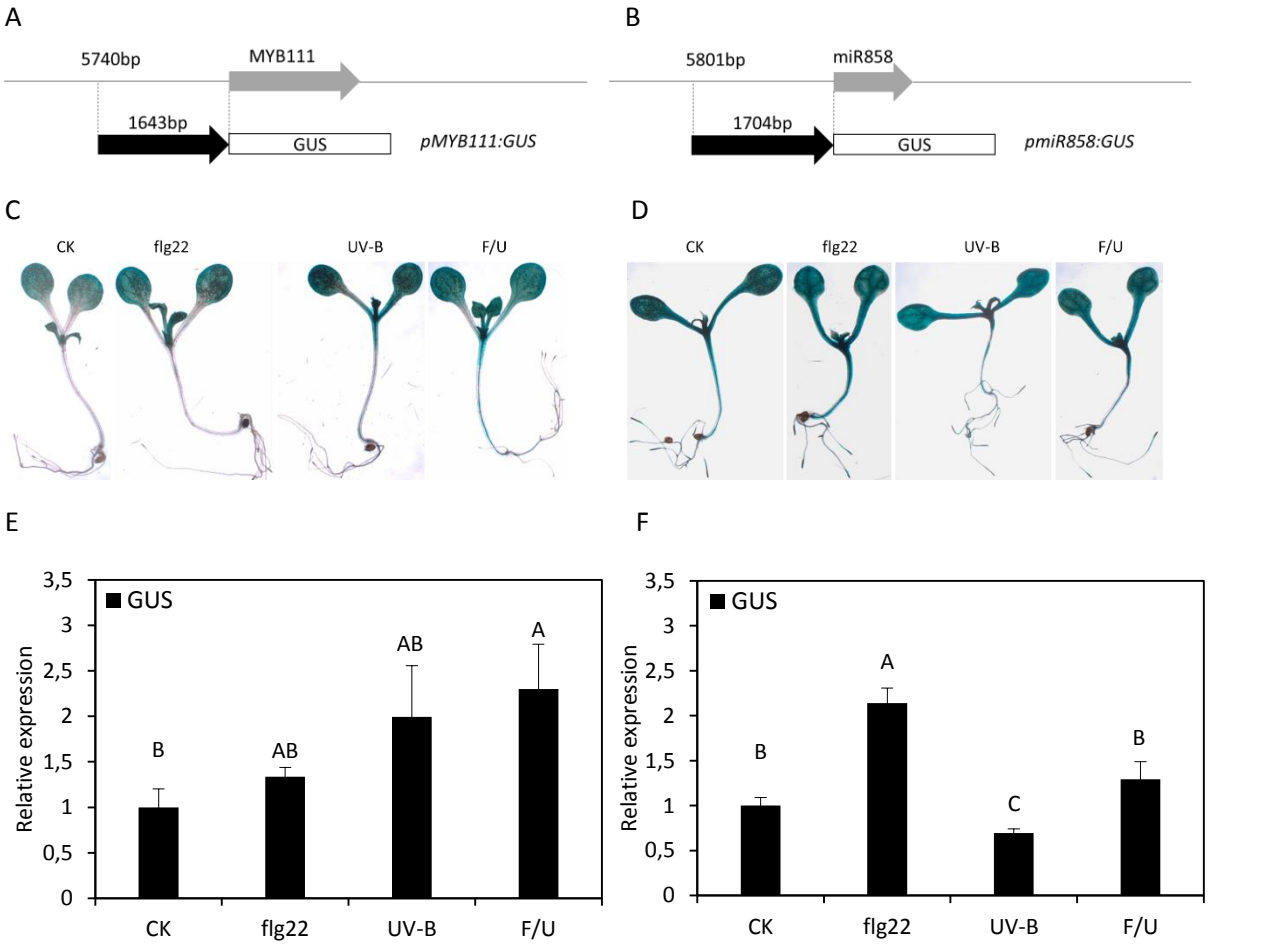

Figure 5 Promoter analysis of miR858 and its target MYB111. (A, B) Schematic presentation of *pMYB111:GUS* and *pmiR858:GUS* constructs. (C, D) Histochemical staining of GUS expression patterns in T_2 generation of two-week-old transgenic Arabidopsis seedlings expressing *pMYB111:GUS* and *pmiR858:GUS*. (E, F) GUS transcript levels in *pMYB111:GUS* and *pmiR858:GUS* transgenic plants treated by flg22, UV-B and co-treatment F/U as compared with the control. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of gene was carried out using Actin2 as internal reference gene. Statistically significant differences between the levels of CK and other treatments transcripts were determined using ANOVA ($P < 0.05$).

4.6 Determination of interaction and cleavage site between miR828 and MYB75

Transcription factor MYB75 is predicted to be targeted by miR828 through psRNATarget (V2, 2017 release, http://plantgrn.noble.org/psRNATarget/analysis). But the interaction and cleavage site between miR828 and MYB75 is still not demonstrated in Arabidopsis. To validate that MYB75 is targeted by miR828, a RACE experiment was conducted. Although all clones showed a possible cleavage, but it occurred before the expected cleavage site (Supplemental Figure S7). Hence, we decided to analyze the interaction and cleavage site between miR828 and MYB75 by infiltration of transient expression via *Agrobacterium* in *Nicotiana benthamiana*. The predicted target site

(between the 10th and 11th nucleotide of miR828) named TS1, while RACE target site by RACE named TS2 (Figure 6A). As control, we mutated predicted target site with 4 nucleotide changes named MTS1 and MTS2, respectively (Red in Figure 6A). Two constructs, pGWB402-pri-miR828 (OE-miR828) and pGWB433-P35S-miR828 (TS), overexpressing miR828 and MYB75 target site or mutated target site were generated, respectively (Figure 6B and 6C).

In *Nicotiana benthamiana*, there was no GUS activity detectable in the OE-miR828. A strong staining of GUS activity could be observed with both TS1 and TS2 alone (Figure 6D). But, TS1 and TS2 both gave a significant reduction in the GUS staining intensity if they co-transformed with OE-miR828, indicating that miR828 is able to target MYB75 at predicted TS1 and/or TS2. Furthermore, MTS1 together with OE-miR828 showed however a lower GUS activity as compared with MTS1 alone, confirming that TS1 is not an only cleavage site for miR828. To contrast, MTS2, together with OE-miR828 did not result in change in GUS staining intensity, suggesting that MTS2 cannot be targeted by miR828 (Figure 6D). Moreover, RT-qPCR analysis of the GUS expression was applied to validate the targeting effect of miR828 on MYB75 (Figure 6E). Co-expression of the TS2, together with OE-miR828 resulted in a significant reduction in the GUS transcript level, supporting a specific targeting at the TS2, but this reduction was clearly impeded by MTS2. Thus, we conclude that miR828 is able to interfere with MYB75 in Arabidopsis, at targeting sites of TS2 and TS1, the latter differs from the predicted one.

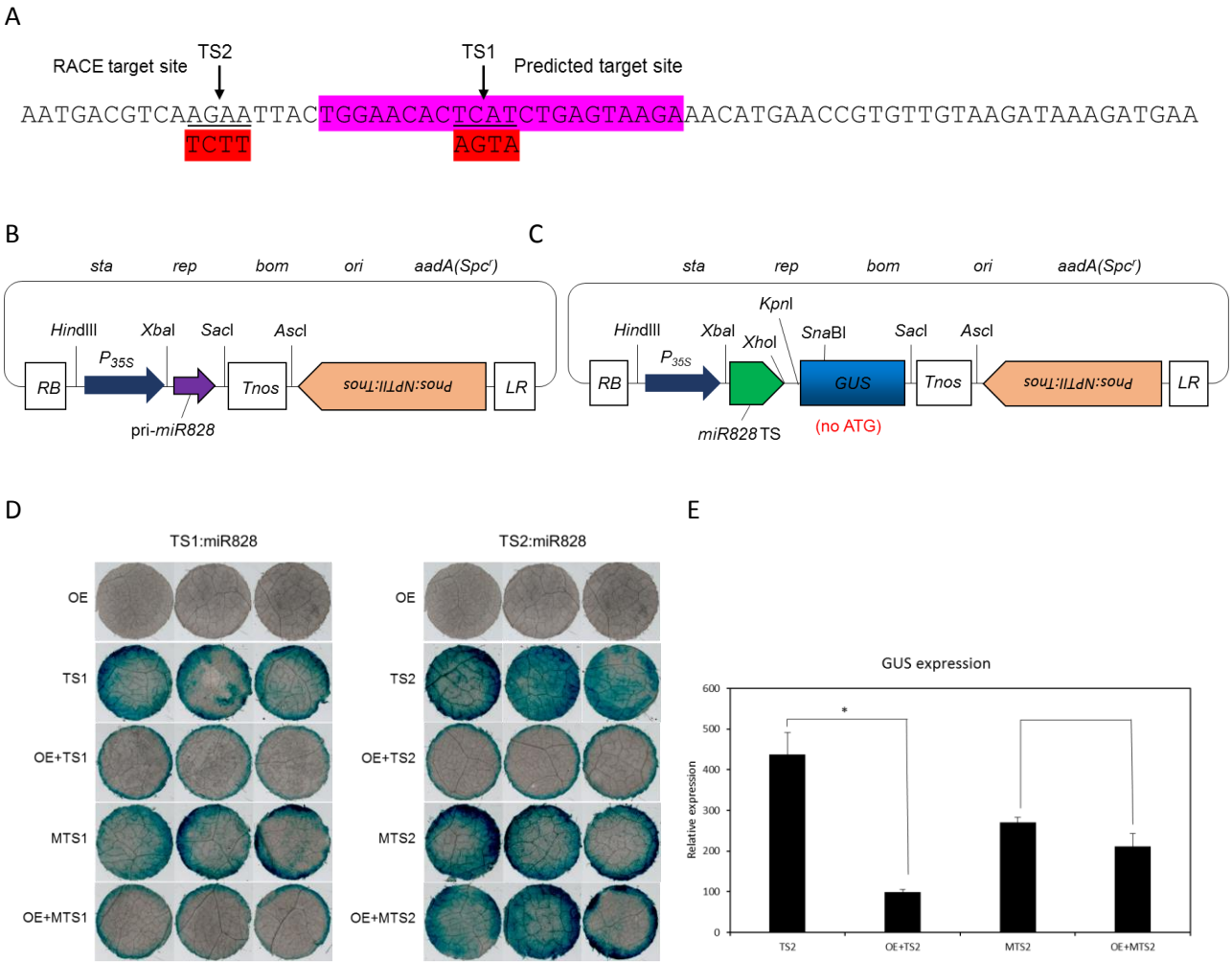

Figure 6 Determination of the interaction and cleavage site between miR828 and MYB75 in transient expression in *Nicotiana benthamiana*. (A) MYB75 sequence targeted by miR828. TS1, predicted target site. TS2, RACE target site by RACE. Highlighted in pink and red is miR828 mature sequence and mutated target site, respectively. (B) pGWB402-pri-miR828 (OE-miR828) construct for overexpression of miR828. (C) pGWB433-P35S-mi828 (TS) construct for overexpression of target site or mutated target site. (D) Histochemical staining of GUS expression of TS1:miR828 and TS2:miR828 in Nicotiana benthamiana. (E) GUS transcript level in TS2:miR828 in Nicotiana benthamiana. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of gene was carried out using NbPP2A as internal reference gene. Statistically significant differences between TS/MTS and TS/MTS together with OE-miR828 transcripts were determined by using Student's T-test (*, $P < 0.05$).

4.7 Functional characterization of effect on *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 growth in the crosstalk

Pst DC3000 infection of *Arabidopsis thaliana* has been widely used to elucidate many of the general principles underlying the plant immune response and bacterial pathogenesis. To study the effect on the pathogenicity of bacterial pathogen *Pst* DC3000 growth in response to the UV-

B/flg22 crosstalk in Arabidopsis, the wild-type Col-0 plants and *STTM858* plants treated by mock (CK), flg22, UV-B and co-treatment (F/U) were infected by *Pst* DC3000, *hrpL* and *hrcQ*. Samples were collected from three plants (3 leaf discs for each) for each of the 3 independent biological replicates, thus the final values represented 9 plants and 27 leaf discs in total. Bacterial populations in infected leaves at 4 dpi were calculated by colony counting. We also tested whether the flg22-induced PTI toward the virulent *Pst* DC3000 is altered in *STTM858* as compared with the wild-type.

Based on the symptom appearance, the wild-type virulent *Pst* DC3000 resulted in a higher infection rate, followed by the mutants *hrpL* and *hrcQ*, respectively, which both have defects in their virulence (Figure 7A). In addition, quantification of bacterial proliferation/growth was performed by colony counting. In the wild-type plant, flg22-pretreated plants exhibited a significant reduction in bacterial populations of *Pst* DC3000 as compared with the control, which is consistent with previous work (Nomura et al., 2011). In contrast, plants pretreated by UV-B showed much more susceptible with a higher bacterial growth rate. As expected, Col-0 showed less susceptible to bacteria mutants *hrpL* and *hrcQ* as compared with the wild-type *Pst* DC3000 (Figure 7B). Significantly, Arabidopsis *STTM858* plants exhibited enhanced resistance to *Pst* DC3000 infection as compared with the wild-type Col-0 plants (Figure 7C). Notably, plants irradiated by UV-B showed much less susceptibility to *Pst* DC3000 in *STTM858* transgenic plant as in the control treatment. The flg22 treatment resulted in reduced colony forming units in *STTM858* transgenic plant as in the wild-type control (Figure 7C). Altogether, these data support for the hypothesis that flg22 induced suppression of FPGs in Arabidopsis benefits plants to effectively active the PTI against pathogen infection, in which miR858 plays a crucial role in the crosstalk between flg22 and UV-B signaling pathways.

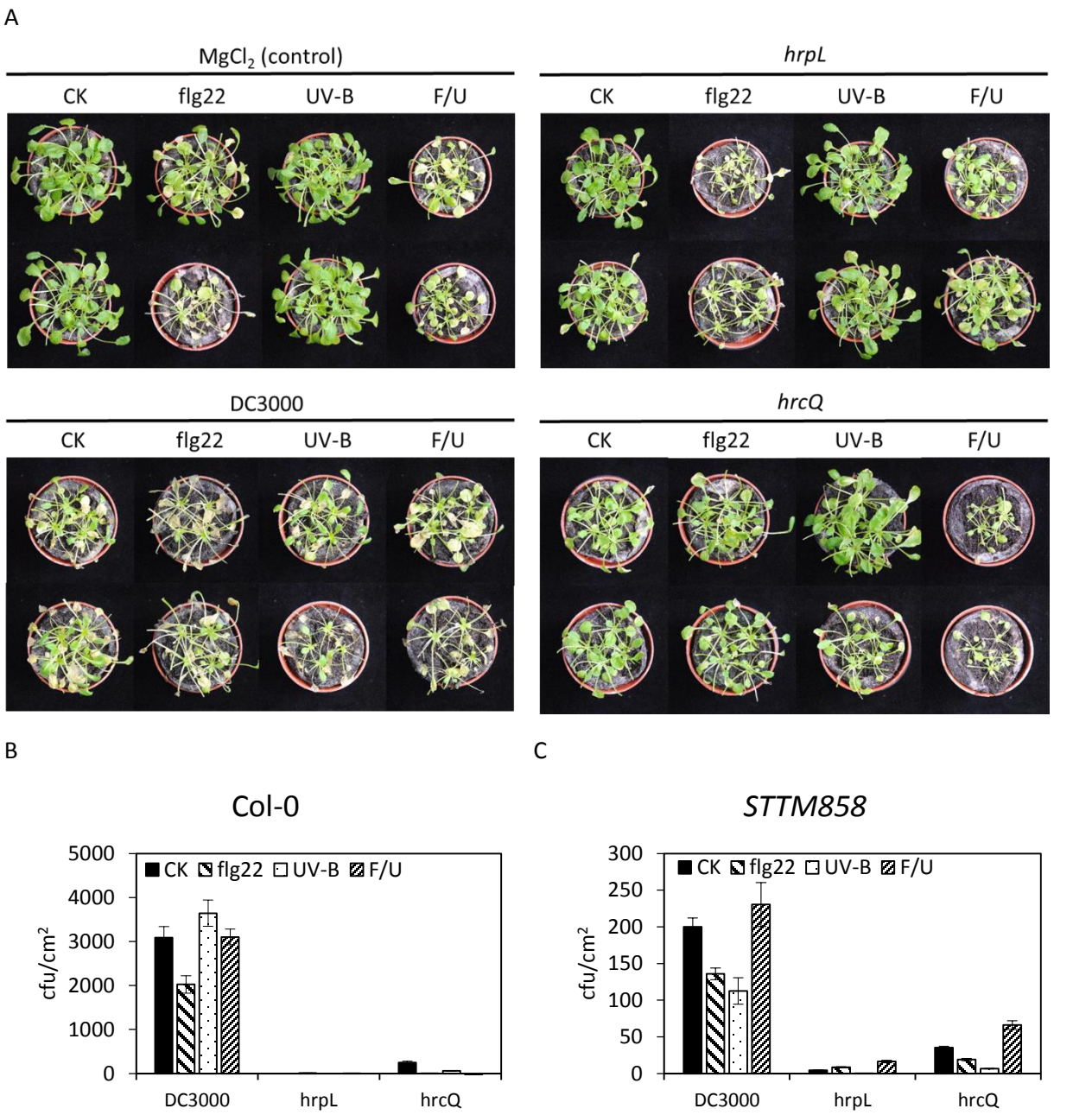

Figure 7 Scoring symptom development and bacterial proliferation in Arabidopsis infected leaves at 4 dpi. (A) Symptoms development 4dpi in Arabidopsis wild-type Col-0. Plants infected Pst DC3000 showed a much severe symptom by comparing with strains, hrpL and hrcQ in which, plants sprayed with $MgCl_2$ were used as control (CK). (B, C) Arabidopsis Col-0 plants (B) and *STTM858* (C) were sprayed with $MgCl_2$ (Mock), Pst DC3000, hrpL and hrcQ (1×10^8 cfu/ml). Comparison of bacterial growth rates upon spray inoculation of Arabidopsis thaliana leaves in response to flg22, UV-B and co-treatment at 4 dpi. Data was normalized to Mock $MgCl_2$-treated plants.

4.8 A proposed possible working function model

Finally, a functional model for miR858-MYB111 and miR828-MYB75 interactions is proposed to elucidate how the miRNAs are involved in the crosstalk between flg22 and UV-B in Arabidopsis (Figure 8). It is known that many MYBs are involved in the crosstalk (Figure 1B), in which we believe that MYB111 and MYB75 represent two key regulatory TFs. We demonstrate that both miR858 and miR828 are regulated in a UVR8 and FLS2 receptors-dependent manner and relying on the respective signaling pathways. The subsequent miR858-MYB111 and miR828-MYB75 interactions consequently modulate the crosstalk between flg22 and UV-B induced signaling pathways, resulting in regulation of FPGs expression and ensuring plants to effectively activate the PTI against pathogen infection. Following these, we conclude that miRNAs constitute a crucial regulatory layer in the crosstalk of plant responses to flg22 (biotic) and UV-B (abiotic) stress.

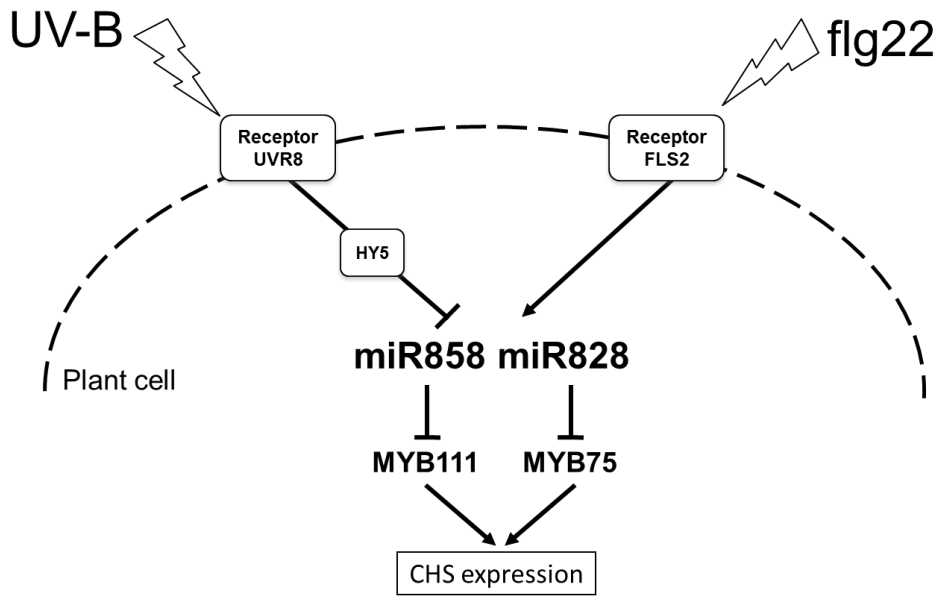

Figure 8 Model for miRNA-MYB interaction in the crosstalk of plant response to flg22 (biotic) and UV-B (abiotic) stress. miR858 and miR828 post-transcriptionally regulates the expression of MYB111 and MYB75, respectively, leading modulate the crosstalk between flg22 and UV-B induced signaling pathways, resulting in re-regulation of FPGs expression and ensuring plants to effectively activate the PTI against pathogen infection. Positive interactions are represented with arrows, and negative interactions with bars.

5 Discussion

The discovery of miRNAs in many plant and animal species, and the growing evidence that these molecules regulate the activity of genes involved in a wide range of developmental and physiological processes represent a significant advance in our understanding of the regulatory mechanisms that control gene expression (Carrington and Ambros, 2003; Kidner and Martienssen, 2005). Here, we show that miR858 and miR828 regulate the expression of their targets MYB111 and MYB75 at post-transcriptionally level, respectively, thus constituting the crosstalk between the flg22 and UV-B induced signal cascades in Arabidopsis.

It is known that plant responses to environmental stimuli involve a network of molecular mechanisms that vary depending on the nature of environmental signal. In the signal transduction network that leads from the perception of stress signals to the expression of stress-responsive genes, transcription factors (TFs) play an essential role. TFs are the central core of the gene regulatory networks and precisely modulate the transcription rate through either repression or activation of gene expression. Often, they are multi-functional and mediate diverse aspects of developmental processes and responses to various biotic and abiotic stimuli in plants (Tsuda and Somssich, 2015). Most TFs impact multiple physiologic processes such as metabolism, cell cycle progression, growth, development and reproduction (Fujita et al., 2009; Zhou et al., 2010; Hussain et al., 2011). MYB proteins are classified into four classes, 1R, 2R (R2R3), 3R (R1R2R3), and 4R (Dubos et al., 2010), depending on the number of adjacent repeats homologous to animal c-MYB (Klempnauer et al., 1982). In plants, the MYB family has selectively expanded through the R2R3-MYBs. R2R3-MYB transcription factors regulate secondary metabolism, while other MYBs regulate stress responses and development. The R2R3-MYBs, along with two other proteins, bHLH and WD40, can act as both activators and repressors of the phenylpropanoid pathway (Ramsay and Glover, 2005).

The phenylpropanoid pathway is an essential pathway in higher plants required for the production of metabolites such as lignin, flavonols, proanthocyanins, and anthocyanins (Fraser and Chapple, 2011). Our results identified several TFs of MYB family (MYB11, MYB12, MYB111 and MYB75), which are functionally involved in the crosstalk (Figure 1B) and might play a determinant role of MYBs in forming a complex and highly interconnected regulatory network in

the flg22/UV-B signal cascades to regulate the FPGs expression. Recently, of the TFs MYB, MYB108, which regulates wound-induced cell death in an abscisic acid-dependent manner (Cui et al., 2013), and MYB51, a key regulator of indole glucosinolate biosynthesis (Frerigmann and Gigolashvili, 2014) were found. Additional TFs MYBs, included MYB12 and MYB59 that are involved in phenylpropanoid biosynthesis and cell cycle progression, respectively (Mehrtens et al., 2005; Mu et al., 2009), suggesting a cross-regulation among certain TFs MYB in the crosstalk can constitute a sub-regulatory network that contributes to the establishment of a transcriptional program. In support for our finding, a large number of experiments have shown that a subgroup of R2R3-MYBs, such as MYB11, MYB12 and MYB111, apparently influence the accumulation of flavonols by activating a branch of the phenylpropanoid pathway (Mehrtens et al., 2005; Czemmel et al., 2009; Misra et al., 2010; Stracke et al., 2010; Liu et al., 2015). These MYBs participate in fine-tuning the transcriptional activation of a set of genes engaged in flavonol biosynthesis and maintain a subtle balance with other branches of the phenylpropanoid pathway (Xu et al., 2015). Moreover, the Arabidopsis R2R3-MYB transcription factor MYB75 plays important roles in the regulation of anthocyanin biosynthesis and therefore functions as a convergence point for various internal and external signals including light, sugar, hormones and nutrient status (Koes et al., 2005; Das et al., 2012).

So far, very few TFs have been reported to take part in the crosstalk between abiotic and biotic stress signaling networks. The mode of action underlying remains so far unsolved. For instance, the basic helix-loop-helix (bHLH) domain-containing transcription factor *AtMYC2* is a positive regulator of ABA signaling. The genetic lession of AtMYC2 however results in elevated levels of basal and activated transcription from JA-ethylene responsive defense genes (Abe et al., 2003; Anderson et al., 2004). Also, MYC2 differentially regulates two branches of JA-mediated responses; it positively regulates wound-responsive genes, including *VSP2*, *LOX3*, and *TAT*, but represses the expression of pathogen-responsive genes such as *PR4*, *PR1*, and *PDF1.2*. These complex interactions are co-mediated by the ethylene-responsive transcription factor ERF1 (Lorenzo et al., 2003; Lorenzo et al., 2004). In addition, the botrytis susceptible 1 (*BOS1*) gene of *Arabidopsis* encodes an R2R3-MYB transcription factor that mediates responses to certain signals, probably through ROS intermediates from both biotic and abiotic stress agents (Mengiste et al., 2003). There are also four members of the *NAC* family of genes that encode plant-specific

transcription factors involved in diverse biological processes. *OsNAC6*, *Arabidopsis transcription activation factor 1* (*ATAF1*), *ATAF2* and *dehydration 26* (*RD26*) are potentially involved in regulation of responses to abiotic and biotic stresses (Wu et al., 2009).

Genes of the member in UV-B signaling cascade have been characterized via forward genetics approaches following mutant screens relying on sensitivity to UV-B irradiation. The principal genes identified as mediators of UV-B photomorphogenic response were COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) (Oravecz et al., 2006) and HY5 (ELONGATED HYPOCOTYL 5) (Ulm et al., 2004), while UVR8 has been identified as a UV-B receptor (Rizzini et al., 2011). In the UV-B signaling transduction cascade, the bZIP transcription factor, HY5, a mediator of several photomorphogenic pathways, is required for UV-B-mediated gene expression (Oravecz et al., 2006). MYB75 and MYB12 are regulators of flavonol/anthocyanin pathways (Stracke et al., 2010; Tohge et al., 2005). MYB4 is known as negative regulator of phenylpropanoids, because MYB4 downregulates C4H expression (Hemm et al., 2001). These regulators are all under the control of HY5 suggesting that the complex structure of light signaling cascades can adapt to changes of light intensity to survive under more severe environments. In addition, obvious evidence for the importance of flavonoid in protection against UV-B has been obtained in experiments to confirm the regulation of CHS and MYB12 by HY5 (Oravecz et al., 2006; Stracke et al., 2010). Phenylpropanoids which are derived from phenylalanine are regarded as major UV-B protectants, because knockout mutant of MYB4 gene which negatively regulates cinnamate 4-Hydroxylase gene (AtC4H) expression has a reduced tolerance to UV-B light (Jin et al., 2000). Moreover, MYB4 and its homolog MYB7 have also been suggested to repress flavanoid biosynthesis (Jin et al., 2000; Fornalé et al., 2014). MYB4, along with the closely related homologs MYB7 and MYB32, therefore are believed to play important roles in regulating flavonoid biosynthesis (Wang et al., 2019).

FLS2 is currently the most intensively studied *Arabidopsis thaliana* PRR and is activated upon binding of bacterial flagellin or flg22, which is a conserved epitope present in the flagellin N terminus (Zipfel et al., 2004; Sun et al., 2013; Kadota et al., 2014). Upon flg22 perception, several immediate host responses can be observed, including the influx of H^+ and Ca^{2+}, the generation of reactive oxygen species, and the activation of calcium-dependent protein kinases and MAP kinase cascades (Vidhyasekaran, 2014). Subsequently, as with other ligand-PRR interactions, binding of

flg22 to FLS2 results in rapid and massive transcriptional reprogramming within the host cell (Zipfel et al., 2004; Wan et al., 2008). Prominent among the TF genes that were induced by flg22 early on during MTI are members of the WRKY TF family. WRKY18, WRKY33 and WRKY40 were identified to be important functional HUBs within a proposed WRKY regulatory network (Choura et al., 2015). The potential importance of WRKY factors in modulating early MTI responses was further supported by the analysis of promoter sequences of flg22-induced genes, which revealed an overrepresentation of the W-box *cis*-acting DNA element, the consensus binding site of WRKY TFs (Navarro et al., 2004). Similarly, the W-box was overrepresented within promoters of the large group of early flg22-induced receptor-like kinase (RLK) genes (Zipfel et al., 2004). MAMP recognition triggers protein kinase cascades that activate downstream defense responses (Meng and Zhang, 2013; Buscaill and Rivas, 2014). For instance, the camalexin pathway is activated by upstream mitogen-activated protein kinase (MAPK) kinases, which in turn phosphorylate partially redundant MPK3 and MPK6 (Ren et al., 2008). Mao et al. (2011) showed that TF WRKY33 is one of the molecular targets of the MPK3/6 cascade.

Proper gene expression is fundamental to the integrity and function of biological processes and developmental programs. Also, gene expression is deliberately controlled through sophisticated regulatory networks. In addition to transcription factors, miRNAs play crucial roles in the regulatory networks by modulating gene expression at the posttranscriptional level (Voinnet, 2009). In the context of regulatory networks, identification of regulatory gene circuits incorporating transcriptional control mechanisms will provide much needed insights into gene activity that is critical for plant responses to a/biotic stresses.

In Arabidopsis, miR828 is encoded by a single locus, MIR828a (AT4G27765), located on chromosome 4, and is predicted to form a stem-loop hairpin (Yang et al., 2013). MiR828 was initially identified in Arabidopsis (Rajagopalan et al., 2006; Hsieh et al., 2009), and constitutively expressed in different tissues of Arabidopsis. Low GUS activity of miR828 can be observed in meristem and leaf (Supplemental Figure S5D). miR858 is transcribed from two genomic loci (miR858a and miR858b) in Arabidopsis and targets similar genes. The additional targets of miR858a identified in this study might be involved in regulating diverse developmental aspects in the plant, which would confirm the significance of miR858a in specific developmental programs

in Arabidopsis. The earlier function of miR858 has been recognized in trichome fiber development by regulating MYB2 in Arabidopsis (Guan et al., 2014) and anthocyanin biosynthesis in tomato (Jia et al., 2015). These reports are in agreement with the idea that miR858 might target a number of R2R3-MYB genes and may be involved in the regulatory responses leading to secondary metabolite production and specific developmental programs. The expression of miR858a was found to be widespread in Arabidopsis, as is evident from RT-qPCR results and promoter activity (Figure 5D). The GUS staining observed in the different treatments of Arabidopsis supports for an active participation of miR858-mediated regulation in the crosstalk of plant response to a/biotic stresses.

Arabidopsis miR858/miR828 has been shown to regulate various growth and plant developmental processes (Guan et al., 2014; Jia et al., 2015; Sharma et al., 2016; Tirumalai et al., 2019). However, a regulatory function of miR858 in the crosstalk between UV-B and flg22 interactions and plant-pathogen interactions are ne2. Here, we report a crucial regulatory role of miR858 during the crosstalk between UV-B and flg22-induced MTI in Arabidopsis (Figure 1). In response to the flg22/UV-B signal cascades, expression of miR858 and miR828 is upregulated by flg22, but downregulated by UV-B, whereas TF MYB111 and MYB75 exhibit opposite patterns to miRNA expression, thus supporting that MYB111 and MYB75 are targeted by miR858 and miR828, respectively (Figure 1B, 1C and 1D). Mutant analysis provided genetic evidence as the induction of CHS expression by UV-B was impeded in both *uvr8* and *hy5* mutants. While, expression of CHS treated by flg22 presented no dramatic change as compared with the wild-type. Additionally, flg22-induction of miR858/miR828 and concurrent downregulation of MYB111/MYB75 were impaired in *fls2* mutant. Of most interest, the double mutant *uvr8×fls2* showed the induction of CHS and MYBs by UV-B as well as reduction by flg22 and F/U were all impeded. And expression pattern of miR858/miR828 in *uvr8×fls2* totally differed from those in the wild-type. Therefore, it could be concluded that UVR8 and FLS2 receptors were required for miRNA-mediated the crosstalk between flg22 and UV-B induced signal cascades (Figure 2). In support, inactivation of miR858 and miR828 by overexpressing an artificial target mimic sequence let to the UV-B-induction of CHS expression significantly (Figure 3).

It is worth mentioning that miR858a cleaves flavonol-specific MYBs such as MYB11, MYB12, and MYB111, which are predicted to be potential targets using online target prediction tools (Supplemental Table S5). All the MYBs have been verified as targets through RLM-RACE analysis (Fahlgren et al., 2007; Sharma et al., 2016). Thus, the influence of miR858 on the crosstalk between flg22 and UV-B induced signal cascades seems to be mediated through posttranscriptional regulation of its targets of TF MYB genes, e.g. via MYB111. The fact that as revealed by promoter-GUS assays both of miR858 and MYB111 are predominantly active in the whole seedling in control and all treatments as well (Figure 5C and 5D), the suppression of GUS expression by UV-B while activation by flg22 occurred only in *pmiR858:GUS*, but not in *pMYB111:GUS* evidences that MYB111 must undergo a post-transcriptional regulation to constitute its role in the crosstalk of plant response towards concurrent flg22 and UV-B challenges, in which miRNAs act as a determinant factor.

Arabidopsis plants pretreated by UV-B showed an enhancement of plant susceptibility to *Pst* DC3000, whereas flg22-pretreated plants rendered the plants less susceptible (Figure 7). This provides for the first time evidence supporting the hypothesis that flg22 induced suppression of FPGs in Arabidopsis benefits plants to effectively active the PTI against pathogen infection, in which miR858 plays a crucial role in the crosstalk between flg22 and UV-B induced signaling pathways.

6 Conclusion

On the basis of our data, a functional model for miR858-MYB111 and miR828-MYB75 interactions is proposed to elucidate how the miRNAs are involved in the crosstalk between flg22 and UV-B in Arabidopsis (Figure 8). It is known that many MYBs are involved in the crosstalk (Figure 1B), in which we believe that MYB111 and MYB75 represent two key regulatory TFs. We demonstrate that both miR828 and miR858 are regulated in a UVR8 and FLS2 receptor-dependent manner and relying on the respective signaling pathways. The subsequent miR858-MYB111 and miR828-MYB75 interactions consequently modulate the crosstalk between the flg22 and UV-B induced signaling pathways, resulting in regulation of the FPGs expression and ensuring plants to effectively activate the PTI against pathogen infection. Following these, we conclude that miRNAs constitute a crucial regulatory layer in the crosstalk of plant responses to flg22 (biotic) and UV-B (abiotic) stress.

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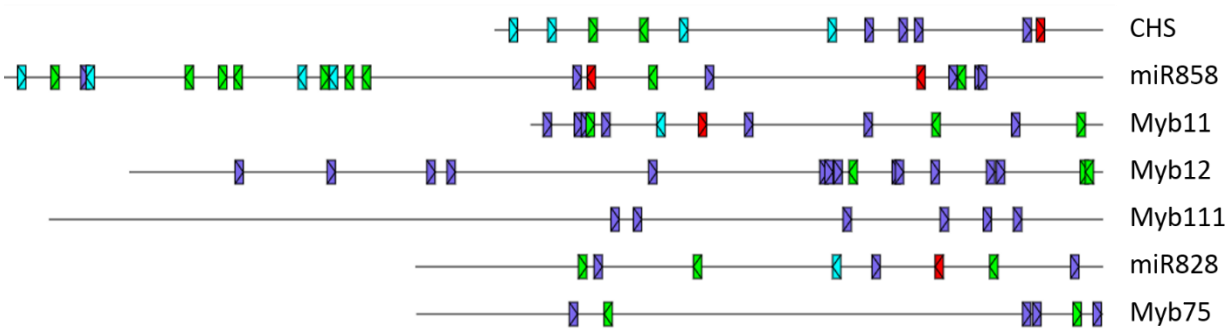
8 Supplementary data

A



B

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miR858  -------------------------AAGGUCGAACAGACAACGAAA--------------------
MYB11   ...GTCAACAATTGCGAGCAATCTACCGGGAAGAACAGACAACGAAATAAAAAACTATTGGAAT...
MYB12   ...GTCACTAATCGCGGGTCATCTACCAGGGAGAACAGACAACGAAATAAAAAATTATTGGAAC...
MYB111  ...GTCACTTATTGCAACACATCTACCAGGAAGAACAGACAACGAAATTAAAAACTATTGGAAC...

miR828  ----------------------------TGGAATACTCATTTAAGCAAGA----------------
MYB75   ...GGACCGCAAATGACGTCAAGAATTACTGGAACACTCATCTGAGTAAGAAACATGAACCGTG...
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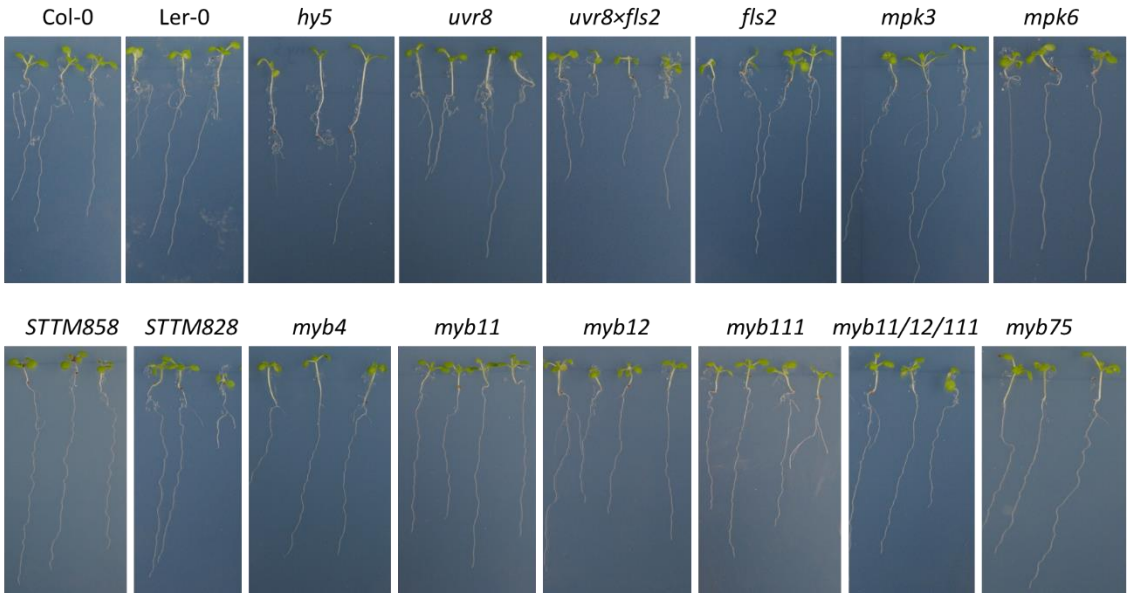
Figure S1 Potential involvement of a miRNA in regulation of the positive acting MYBs. (A) CHS, miRNAs and MYBs promoter comparison focusing on these *cis*-elements: ACE element: ACCTACC (binding of bZIP TFs, such as HY5), W-box: TTGAC(C|T) (binding site of WRKY TFs), G-box: CACGT (binding site of bZIP, bHLH and NAC TFs) and MRE elements: TTGGGC, which are potential MYB TF binding. (B) miR858 has the potential to target MYB 11, MYB 12 and MYB 111, while miR828 has the potential to target MYB75.

A



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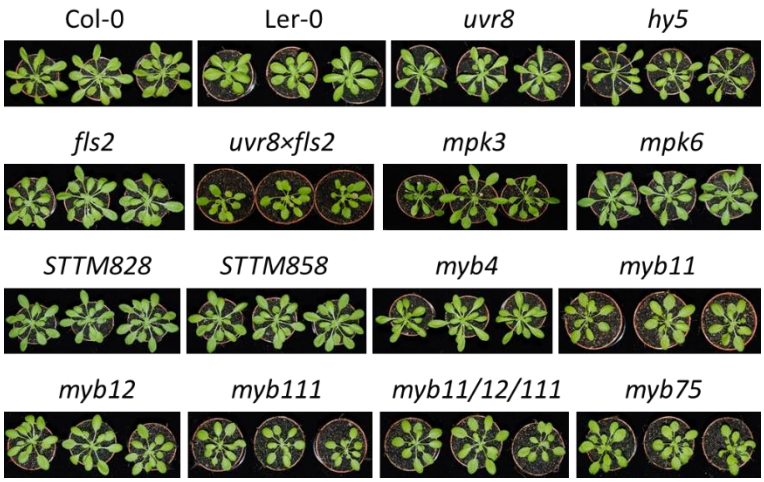

Figure S2 Phenotypic analysis of all materials involved in this study. (A) 2-week-old of seedlings were grown on one-half-strength ½ MS medium under short-day condition. (B) 5-week-old of seedlings were grown in the soil under short-day condition.

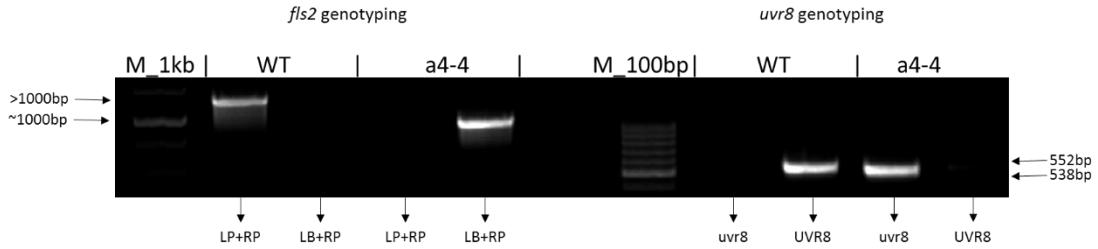

Figure S3 Identification of F_3 generation plants homozygous for the *uvr8* and *fls2* mutation for genotyping by PCR analysis.

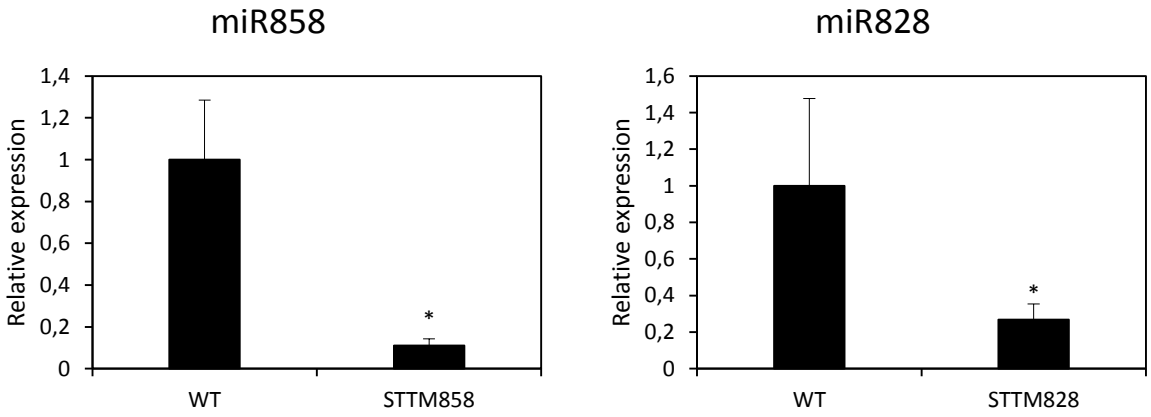

Figure S4 Mature miR858 and miR828 downregulation in the *STTM858* and *STTM828* transgenic line, respectively. Data were obtained from three biological samples and represented as mean ± SE. Normalization of the expression levels of miRNAs was carried out using U6 as internal reference genes. Statistically significant differences between wild-type plant and STTM mutant transcripts were determined by using Student's T-test (*, $P < 0.05$).

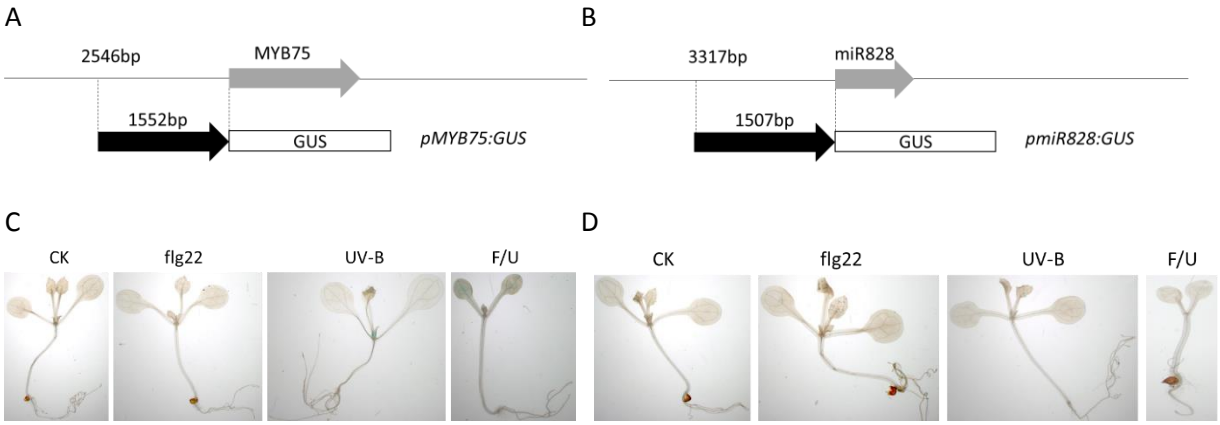

Figure S5 Promoter analysis of miR828 and its target MYB75. (A, B) Schematic representation of *pMYB75:GUS* and *pmiR828:GUS* constructs. (C, D) Histochemical staining of GUS expression pattern in T_2 generation of two-week-old transgenic Arabidopsis seedlings expressing *pMYB75:GUS* and *pmiR828:GUS.*

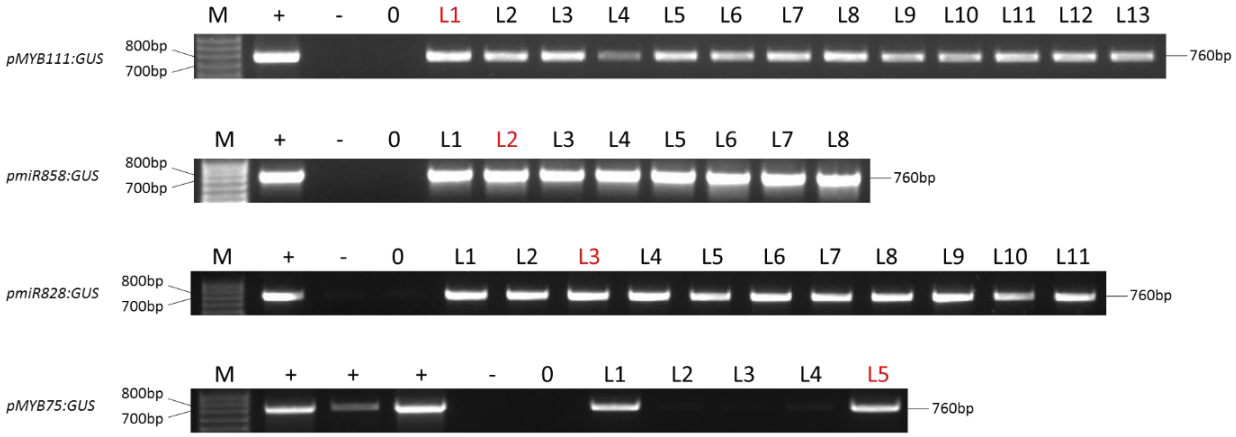

Figure S6 Identification of T_1 generation *pMYB111:GUS*, *pmiR858:GUS*, *pMYB75:GUS* and *pmiR828:GUS* transgenic plants by PCR analysis. M, 100bp DNA ladder marker; +, plasmid construct as positive control; -, wild-type Col-0 as negative control; 0, no template. Highlighted in red is the transgenic lines used for further analysis.

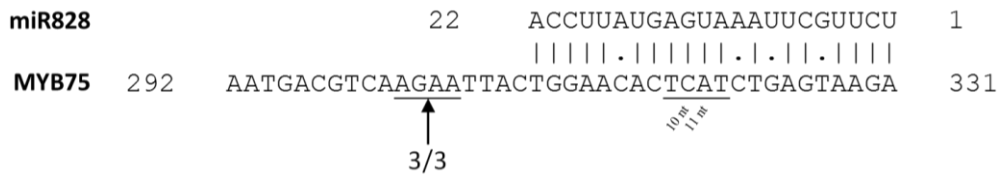

Figure S7 Validation of miR828 predicted target MYB75 by RLM-5' RACE. Arrows and numbers indicate free RNA ends with the frequency of cut points identified; the cleavage site occurs between 10/11 nucleotides of the small RNAs.

Table S1 Primer used for mutant genotyping

Gene		Sequence (5'-3')	Purpose
uvr8	forward	GTGACCTCTATGGATACGGA	Genotyping of mutant *uvr8*
UVR8	forward	TGACCTCTATGGATGGGGCT	Genotyping of mutant *uvr8*
	reverse	AACTGATATTGTGTGCCGCC	Genotyping of mutant *uvr8*
fls2	LP	TGTCCGGTGATGTTCCTGAG	Genotyping of mutant *fls2*
	RP	CAGCTCTCCAGGGATGGTTC	Genotyping of mutant *fls2*
	LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping of mutant *fls2*

Table S2 Primer used for RT-qPCR

Gene		Sequence (5'-3')	Purpose
Actin2	forward	ACCTTGCTGGACGTGACCTTACTGAT	Reference gene for *Arabidopsis thaliana*
	reverse	GTTGTCTCGTGGATTCCAGCAGCTT	Reference gene for *Arabidopsis thaliana*
CHS	forward	GTTCAAGCGCATGTGCGACAAG	Gene expression of CHS
	reverse	GCCGCTTCTTTGCCTAGCTTA	Gene expression of CHS
MYB4	forward	GCCACGTTGTTTCAAGTGCA	Gene expression for MYB4
	reverse	TCCATTGCTCATGTCACTCC	Gene expression for MYB4
MYB11	forward	TTTGGGAACCAGGTGGTCAAC	Gene expression for MYB11
	reverse	TCTTCCAGGTCTACGCTTAGG	Gene expression for MYB11
MYB12	forward	TCAGACCTCAAGCGTGGAAAC	Gene expression for MYB12
	reverse	TGGTAGATGACCCGCGATTAG	Gene expression for MYB12
MYB111	forward	GGCAACAGATGGTCACTTATTG	Gene expression for MYB111
	reverse	GCTCCAGAAGACGATGAACAAG	Gene expression for MYB111
MYB75	forward	CGAAAAGGTGCTTGGACTAC	Gene expression for MYB75
	reverse	CGGTTTAGCCCAGCTCTTACA	Gene expression for MYB75
U6-RT	stem-loop RT	TTGGACCATTTCTCGATTTATG	Reference gene for miRNA expression
miR858-RT	stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACAAGGTC	cDNA synthesis for miR858
miR828-RT	stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTGGAAT	cDNA synthesis for miR828
U6	forward	TTGGAACGATACAGAGAAGATTAGCA	Reference gene for miRNA expression
	reverse	TTGGACCATTTCTCGATTTATG	Reference gene for miRNA expression
miR858	forward	GCGACTTTCGTTGTCTGTTCGA	miR858 expression
miR828	forward	GCGACTCTTGCTTAAATGAGTATTCCA	miR828 expression
miRNA	reverse	CCAGTGCAGGGTCCGAGGT	Universal miRNA reverse primer
NbPP2A	forward	GACCCTGATGTTGATGTTCGCT	Reference gene for *Nicotiana benthamiana*
	reverse	GAGGGATTTGAAGAGAGATTTC	Reference gene for *Nicotiana benthamiana*
GUS	forward	GTTGACTGGCAGGTGGTGG	Gene expression for GUS
	reverse	GGTAGATATCACACTCTGTCTGG	Gene expression for GUS

Table S3 Primer used for gateway clone

Gene		Sequence (5'-3')	Purpose
MYB111-GUS	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCG AGGATTTAATTTACACTGGC	Gateway clone for MYB111 promoter-GUS construct
	reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTTG CTTCTCGGTCTCTTCTG	Gateway clone for MYB111 promoter-GUS construct
miR858-GUS	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTGT TGGTTTTGACCCATTAGC	Gateway clone for miR858 promoter-GUS construct
	reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGA TTGCTCACTTTGTCAAGC	Gateway clone for miR858 promoter-GUS construct
MYB75-GUS	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTGA CTAGAGTTCACAGGTTC	Gateway clone for MYB75 promoter-GUS construct
	reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGG AACAAAGATAGATACGTAAA	Gateway clone for MYB75 promoter-GUS construct
miR828-GUS	forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCT GGTCTTGTCAATTGGTTG	Gateway clone for miR828 promoter-GUS construct
	reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGA GAGTAAGAGAGATGATGACTC	Gateway clone for miR828 promoter-GUS construct

Table S4 Primer used for identification of interaction between miR828 and MYB75

Gene		Sequence (5'-3')	Purpose
5'RACE	forward	CGACTGGAGCACGAGGACACTGA	Universal forward primer for first round RACE-PCR
5'RACE-nested	forward	GGACACTGACATGGACTGAAGGAGTA	Universal forward primer for second round RACE-PCR
MYB75-RACE	reverse	CGTCAAAAGCCAAGGTGTCCCCC	Reverse primer for first round RACE-PCR
MYB75-nested	reverse	GGCATTGAGATGGTTGCAGTC	Reverse primer for second round RACE-PCR
OE-miR828	forward	GACTCTAGAACTCTCATGGTGACAAAGTCAC	Gateway clone for overexpression of miR828
	reverse	TTCGAGCTCGATTCACGTGAAGTGGGCACGA	Gateway clone for overexpression of miR828
TS1	forward	CTAGAATGTGGAACACTCATCTGAGTAAGAC	Gateway clone for TS1-GUS construct
	reverse	TCGAGTCTTACTCAGATGAGTGTTCCACATT	Gateway clone for TS1-GUS construct
MT1	forward	CTAGAATGTGGAACACAGTACTGAGTAAGAC	Gateway clone for MT1-GUS construct
	reverse	TCGAGTCTTACTCAGTACTGTGTTCCACATT	Gateway clone for MT1-GUS construct
TS2	forward	CTAGAATGAATGACGTCAAGAATTACTGGAACAC	Gateway clone for TS2-GUS construct
	reverse	TCGAGTGTTCCAGTAATTCTTGACGTCATTCATT	Gateway clone for TS2-GUS construct
MT2	forward	CTAGAATGAATGACGTCATCTTTTACTGGAACAC	Gateway clone for MT2-GUS construct
	reverse	TCGAGTGTTCCAGTAAAAGATGACGTCATTCATT	Gateway clone for MT2-GUS construct

Table S5 Putative targets of Arabidopsis miR858a

Target accession	Target description	Function
AT1G17760	CSTF77	Involve in mRNA polyadenylation
AT3G59470	FRF1	Proximal promoter sequence-specific DNA binding
AT2G26950	MYB104	Cell differentiation, regulation of transcription
AT3G02940	MYB107	DNA-dependent, response to SA stimulus
AT3G62610	MYB11	Phenylpropanoid pathway/flavonol biosynthesis
AT5G49330	MYB111	Phenylpropanoid pathway/flavonol biosynthesis
AT2G47460	MYB12	Phenylpropanoid pathway/flavonol biosynthesis
AT5G55020	MYB120	Participate in pollen tube reception
AT3G30210	MYB121	Response to abscisic acid, sequence-specific DNA binding
AT5G35550	MYB123	Phenylpropanoid pathway/proanthocyanindins biosynthesis
AT1G06180	MYB13	Abiotic stress response/drought, light and wounding
AT2G31180	MYB14	Regulates seed mucilage biosynthesis and trichome branching
AT3G61250	MYB17	Flower development, response to JA and SA stimulus
AT5G52260	MYB19	Cell differentiation
AT1G66230	MYB20	Secondary cell wall biosynthesis
AT3G27810	MYB21	Stamen development, GA- and JA-mediated
AT1G22640	MYB3	Phenylpropanoid pathway
AT3G24310	MYB305	Sequence-specific DNA binding
AT4G34990	MYB32	Phenylpropanoid pathway
AT5G60890	MYB34	Glucosinolate biosynthesis / indolic pool
AT5G23000	MYB37	Axillary meristem regulation / lateral organ formation
AT4G17785	MYB39	Sequence-specific DNA binding transcription factor
AT5G14340	MYB40	Cell differentiation
AT4G12350	MYB42	Secondary cell wall biosynthesis
AT3G46130	MYB48	Response to salicylic acid stimulus
AT5G54230	MYB49	Cellular cadmium ion homeostasis
AT4G09460	MYB6	Response to gibberellin, jasmonic acid and salicylic acid
AT1G09540	MYB61	Mucilage deposition and extrusion
AT1G79180	MYB63	Phenylpropanoid pathway/lignin biosynthesis
AT3G11440	MYB65	Stamen development/anther development (tapetum)
AT4G13480	MYB79	Regulation of transcription
AT2G26960	MYB81	Regulation of transcription
AT5G52600	MYB82	Regulation of trichome development
AT3G08500	MYB83	Secondary cell wall biosynthesis
AT3G49690	MYB84	Axillary meristem regulation/lateral organ formation
AT5G26660	MYB86	Negative regulation, flavonol biosynthesis
AT5G10280	MYB92	Branching, GA-mediated
AT1G34670	MYB93	Proanthocyanidin biosynthetic process
AT4G26930	MYB97	Participate in pollen tube reception
AT1G71030	MYBL2	Proanthocyanidin biosynthetic process

Chapter V: General discussion

In nature, plants are often simultaneously challenged with different stresses factors. The abiotic stress UV-B-light induces the production of UV protective flavonols in plants, but their accumulation is attenuated by concurrent treatment with pathogen or by application of elicitors (simulating biotic stress), e.g. the bacterial peptide flg22, a defined Microbe Associated Molecular Patters (MAMP) inducing Pattern Triggered Immunity (PTI) in plants. This crosstalk is partially reciprocal, but the response to biotic stress is largely dominant and thought to enable the plant to direct its secondary metabolism to production of phytoalexins and lignin instead of flavonols to guarantee a more efficient pathogen defense. We employed Arabidopsis plants to unravel the molecular mechanism underlying this signal crosstalk. We found that this crosstalk mainly relies on an antagonistic regulation of several MYB transcription factors (TFs) including MYB11, MYB12, MYB111 and MYB75 by UV-B and flg22, respectively. This is consistent with the previous results from the cell culture system with exception for MYB4, which role in the crosstalk could not be clearly defined (Schenke and Cai, 2014). As the post-transcriptional regulation relying on miRNAs is mostly associated with the regulation of mRNA levels of TFs, we thus investigated the role of miRNA858 (targeting MYB12 and MYB111 mRNA) and miRNA828 (targeting MYB75 mRNA) in the crosstalk. Strikingly, the expression of miR828 and miR858 was found to be reciprocally regulated by UV-B und flg22, respectively in a UVR8 and FLS2 receptor- dependent manner. We demonstrate that miRNAs constitute an additional regulatory layer in the crosstalk of plant responses to biotic (flg22) and abiotic (UV-B) stresses via post-transcriptional regulation of their targets (TFs) expression. A functional model is postulated.

1 *Arabidopsis thaliana* seedlings serve as a model system

We have firstly established *Arabidopsis thaliana* seedlings as an experimental system to study the crosstalk between flg22 and UV-B induced MTI in mutant plants. We could show that this crosstalk is fully functional in planta leading to flg22-induced suppression of UV-B-induced flavonol pathway genes (FPGs) and that this reaction is attenuated in mutant plants exemplified by the flg22-insensitive *fls2* KO plants. Arabidopsis in planta offers a valuable experimental system to

dissect the mechanism underlying the crosstalk, e.g. by analysis of loss-of-function mutants or gain-of-function transgenic plants.

In consistence with reports made from cell cultures, MYB TFs have been implicated in the regulation of FPGs and flavonol content in *Arabidopsis thaliana*, in which one negative regulator MYB4 (Jin et al., 2000) and three MYB TFs (Stracke et al., 2007) appear to play a major role. Differing from the cell culture results, reported previously, we observed a much stronger regulation of MYB111 compared to MYB12 or MYB11. We believe that the cell culture used in our former studies were derived from root material and that these cells could memorize their origin stably over several years and show thus a dominant regulation of MYB12. In accordance, the expression of MYB12 in seedlings was not as strong as expected. So far, regulation of MYB111 is not as well investigated as MYB12. Furthermore, the previously proposed direct regulation by WRKYs can't be uphold, since the MYB111 promoter does not contain any W-Boxes required for WRKY binding (Schenke et al., 2011). Thus, there must be an additional layer of regulation. Recently, flavonoids have been reported to be regulated by miR858 (Sharma et al., 2016). However, until now miR858 has not been reported among e.g. flg22-upregulated miRNAs. Nevertheless, miRNAs have been often associated with post-transcriptional regulation of various TFs (Megraw et al., 2016), and thus it is reasonable to believe that miRNAs can serve as an explanation how the TFs in flavonol production could be downregulated during MTI. With the description of this Arabidopsis in planta system we are closer to natural conditions compared to the cell culture based system we used before and now it should be possible to test various factors which could contribute to the molecular mechanisms underlying this crosstalk. With an update of our model we summarize these regulatory possibilities, but not excluding that there might be also other TFs involved in this crosstalk.

2 A set of miRNAs is responsive to the crosstalk

miRNAs proved to be important regulators of gene expression at the post-transcriptional level and play vital roles in plant development and response to a/biotic stresses (Gleeson et al., 2014; Bertolini et al., 2013). Since so far, an integrated view of the transcriptional regulation in the crosstalk between flg22 and UV-B signal cascades is still lacking. Identification of miRNAs that are

involved in this crosstalk is an essential step to assert the process. Thus, we focused on the identification and characterization of miRNAs from Arabidopsis whole seedlings treated by flg22, UV-B and F/U as well as by mock treatment through high-throughput sequencing. Genome-wide parallel sequencing of miRNAs can greatly facilitate the identification and understanding of the function and regulatory mechanism of miRNAs in the crosstalk between flg22 and UV-B. Analysis of small RNAseq data identified 128, 129 and 121 miRNAs with altered abundance in Arabidopsis seedlings treated by flg22, UV-B and F/U, respectively, from which 75, 51 and 52 miRNAs were upregulated while 53, 78 and 69 were downregulated by flg22, UV-B and F/U, respectively. Identification of a large number of miRNAs responsive to flg22, UV-B and F/U treatments supports the idea that a multiple complex network of miRNAs constitutes a regulatory layer in the crosstalk of between flg22 and UV-B signal cascades. it is of great interest and importance to further characterize miRNAs and their targets, which were newly identified to be regulated by flg22 or UV-B irradiation in this study. It is reasonable to believe that the understanding such miRNA-target interactions may shed more light on the multiple complex mechanism plants responses to a/biotic stresses.

2.1 miRNAs upregulated by flg22 but downregulated by UV-B

The identification of 22 candidate miRNAs, which are clearly distinguished between flg22 and UV-B as well as F/U treatments in their expression levels provoked us to seek their possible involvement/role in the crosstalk. Since the function of miRNAs is merely relying on the function of their targets, we firstly predict targets of candidate miRNAs, and then characterized them *in silico* and finally determine the involvement of miRNA-target interactions in the crosstalk by simultaneously determining the changes in their expression levels in response to distinct treatments. In agreement with the literatures (Samad et al., 2017; Jangra et al., 2018), the majority of potential targets predicted are transcription factors, including several well studied transcription factors, like miR858-MYB11, -MYB12, -MYB111, as well as miR828-MYB75 (Sharmar et al., 2016; Yang et al., 2013), which proved to be involved in anthocyanin accumulation and biosynthesis, imply their role in the crosstalk. Simultaneous monitoring the expression levels of miRNAs and their targets in response to distinct treatments reveals two modulations of miRNA-target interactions in the crosstalk between the flg22 and UV-B signal cascades. The first on

consisting of miR158, miR165, miR166, miR167, miR168, miR172, miR391, miR393, miR447, miR824, miR828, miR846 and miR858, which were all repressed by UV-B irradiation, resulting in an increased transcript accumulation of the targets and the activation of CHS gene expression consequently, while they were all drastically upregulated by flg22, going along with the decrease in the transcript levels of the targets as well as the CHS gene expression. In accordance with many reports that the UV-B-induced flavonol pathway gene expression was abolished when a concomitant elicitation by flg22 occurs (Serrano et al., 2012; Schenke et al., 2019), the flg22 effect on miRNA expression occurs also by co-treatment flg22/UV-B in our study. Thus, it is reasonable to believe that the flg22 triggered PTI plays a dominant role in reprogramming the secondary metabolism in the crosstalk between flg22 and UV-B induced signal cascades, which is partially relying on the modulation of miRNA-target interactions. There are already indications that this suppression is mediated by transcription factors (TFs) regulation. Two MYB TFs, the positive regulator MYB12 (UV-B upregulated and flg22 suppressed) and the negative regulator MYB4 (activated by UV-B, but much faster by flg22) have been implicated in the regulation of the FPGs (Schenke and Cai, 2014). The modulation of miRNA on CHS gene expression could be evidently demonstrated by investigation on Arabidopsis *STTM858* and *miR858_OE* as well as *miR164b_KO* and *miR164b_OE* mutant plants. Thus, we could provide the first genetic evidence that miRNAs identified in this study constitute an additional layer in regulating the crosstalk between flg22 and UV-B induced signaling cascades.

miR165/miR166: In Arabidopsis, the CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) transcription factors family consists of five members: PHABULOSA (PHB), PHAVOLUTA (PHV), REVOLUTA (REV), THB-8 and ATHB-15, which are known targets of the miR165/166 family (Teotia et al., 2016). Accumulating evidences have demonstrated that miR165/166 and their targets, HD-ZIP III genes, regulate important processes in plant development, such as shoot apical meristem (SAM) maintenance, xylem patterning and embryo formation (Armenta-Medina et al., 2017; Yu et al., 2017). Additionally, miR165/166 are also involved in the establishment of leaf polarity by repressing the expression of targets on the abaxial side of the leaf primordia (Ramachandran et al., 2018; Merelo et al., 2016). Intriguingly, recent studies have also proven the role of miR165/166 in auxin and ABA signaling, suggesting that auxin is a regulator in miR165/166 controlled leaf development and ABA is a player in stress responses directed by miR165/166 (Yan

et al., 2016; Jia et al., 2015). In this study, we focus on PHB only because of RNAseq data analysis. Our finding indicates the predominant regulatory function of miR166c on PHB. This is supported by findings of Jung and Park (2007), who showed that overexpression of miR166 suppresses especially PHB. Overexpression of miR165a-3p/165b in contrast, suppresses all the above mentioned HD Zip III TFs, but in a lesser extent (Zhou et al., 2007a). Interestingly, Zhou et al. (2007b) reported a relationship between miR165a and flavonoid biosynthesis. They executed a GO term enrichment analysis, yielding terms such as 'transcription factor activity', 'response to abiotic stimulus', and 'flavonoid 3'-monooxygenase activity/flavonoid 3'-hydroxylase (F3'H)'. Furthermore, they found defense related GO terms, such as 'response to pathogen', 'response to biotic stimulus', and 'response to stress', for miR166f (identical to 166c). Conclusively, these findings support the potential involvement of miR165a-3p/165b and miR166c in the crosstalk signaling.

miR168: ARGONAUTE (AGO) proteins are considered to be integral players in all known small RNA-targeted regulatory pathways (Vaucheret, 2008). Among the AGO proteins in Arabidopsis, AGO1 is a core component of the RNA-induced silencing complex, which associates with miRNAs and inhibits target genes by mRNA cleavage and/or translational repression (Voinnet, 2009). In the literature there are several reports on the involvement of AGO1 in plant antiviral defense (Várallyay et al., 2014). AGO1 also contribute to flg22-induced disease resistance in Arabidopsis plants (Li et al., 2010). Shen et al. (2014) described a decrease in miR168 accumulation with a simultaneous increase in AGO1 transcript abundance in roots of oilseed rape (*Brassica napus*) infected with the soil-borne pathogenic fungus *Verticillium longisporum* (at 6 dpi). In other studies, miR168 was reported to be induced by abiotic stresses (drought, salinity, cold) (Li et al., 2012). Then, it is noteworthy that miR168 is regulated by both biotic and abiotic stress conditions, these findings highlighting the importance of miRNA functioning in plant adaptive processes to environmental stress. Clearly, a fine-tuned adjustment of miR168 and AGO1 levels would provide a flexible system for the control of processes that are critical to ensure plant survival under adverse environmental conditions. In this study, the expression level of miR168a were strongly induced by flg22 and suppressed by UV-B, and its target AGO1 exhibited the opposite expression pattern, indicating a potential role of miR168-AGO1 modulation was involved in the crosstalk as well.

miR172: In Arabidopsis, three mature miR172s and six target genes, including APETALA2 (AP2), TARGET OF EAT 1 (TOE1, TOE2, and TOE3), SCHLAFMUTZE (SMZ) and SCHNARCHZAPFEN (SNZ)., were identified (Fornara and Coupland, 2009; Mathieu et al., 2009). However, only TOE family genes were involved in the crosstalk between flg22 and UV-B in Arabidopsis. Previous study demonstrated that TOE1 and TOE2 positively regulate miR172 by a negative feedback loop (Wu et al., 2009). Recent research found that miRNA172 is modulated by auxins (Diaz-Manzano et al., 2018). It has been shown that miR172 is involved in various developmental processes in plants, including stem cell fate (Zhao et al., 2007), developmental timing (Fouracre and Poethig, 2016), floral organ identity and flower pattern (Lee and An, 2012), spike architecture and grain threshability in bread wheat (Debernardi et al., 2017; Liu et al., 2018), tuberization in potato (D'Ario et al., 2017), and nodulation in soybean (Yan et al., 2013). Also miR172 was found to have a role in the abiotic response of Arabidopsis (Han et al., 2013) and biotic stress resistance in tomato (Luan et al., 2018). In our study, TOE family, including TOE1, TOE2 and TOE3, was the target of miR172 with an inverse expression as compared to the miR172 expression. miR172a was significantly suppressed by both flg22 and the co-treatment but induced by UV-B irradiation. Therefore, the suppression of miR172a might be an effect induced by pathogen attack.

miR393: Previous study demonstrated that miR393 targets TIR1 and the three functional paralogous AFB1, AFB2, AFB3 (Navarro et al., 2006). Each of these target genes ultimately promotes the expression of auxin responsive genes in the auxin signaling pathway (Cui et al., 2013). In leaves, enhanced innate immunity in response to bacterial infection involves miR393-guided cleavage of TIR1, AFB2, and AFB3 transcripts and repression of AFB1 transcription (Navarro et al., 2006). In roots, response to nitrate involves miR393-guided cleavage of AFB3 mRNAs, but not mRNAs encoded by the other AFBs genes (Vidal et al., 2010). Downregulation of OsTIR1 and OsAFB2 via OsmiR393 led to reduced tolerance to salt and drought in rice (Xie et al., 2010). In contrast, overexpression of a miR393-resistant form of mTIR1 enhanced salt tolerance in Arabidopsis (Chen et al., 2014). In addition, miR393 can be induced by flg22 treatment, and positively contributes to resistance against a virulent *Pseudomonas syringae* strain pv. *tomato* by suppressing auxin signaling and silencing auxin receptors such as *transporter inhibitor response 1* (TIR1) (Navarro et al., 2006). Similar results have been reported for flg22 treated Arabidopsis

seedlings in our study. Thus, miR393 was involved in defense response upon pathogen attack as mimicked by flg22. However, miR393 was also significantly downregulated by UV-B, whereas TIR1 and AFB2 were upregulated. Moreover, auxin response factors (ARFs) are transcription factors binding to an auxin-responsive element (TGTCTC) in the promoter of auxin-response genes, mediating their transcription (Teale et al., 2006). In our study, miR167 as well as the target gene ARF8 showed the expected expression under flg22 and UV-B treatment. In conclusion, these findings suggest the suppression of TIR1, AFB2, and ARF8 by flg22 which consequently represses auxin responsive gene expression. UV-B irradiation, in contrast, promotes the auxin responsive gene expression by suppression of miR393 and miR167, which indicates a potential role of auxin signaling in the crosstalk.

2.2 miRNAs upregulated by UV-B but downregulated by flg22

An antagonistic regulation of the FPGs and production of flavonoids (Stracke et al., 2007) mediated by the positive regulator MYB12 and the negative regulator MYB4 was postulated by Schenke et al. (2011) and Zhou et al. (2017). In support of this, we could show that in the second group of miRNAs, their expression was vice versa upregulated by UV-B while repressed by flg22, supporting for their involvement in the crosstalk in an antagonistic manner (Zhou et al., 2017). To note the group II contains a few but prominent plant-derived miRNAs like miR159, miR164, miR171 and miR822, which target several well-known TFs involving plant response to a/biotic stresses (MYBs, NACs and HAMs). Thus, further characterization of their role in modulation of the crosstalk between flg22 and UV-B signaling cascades is needed in order to gain more insights into the regulation of plant response to abiotic and biotic stresses.

miR159: In plants, the Arabidopsis miR159 family has been extensively studied as a model for plant miRNA-mediated gene regulation (Palatnik et al., 2007). The family has two major isoforms, miR159a and miR159b, which are strongly expressed throughout Arabidopsis (Palatnik et al., 2007; Li et al., 2016). In Arabidopsis, miR159 is bioinformatically predicted to regulate more than 20 targets, including eight genes encoding conserved R2R3 domain MYB transcription factors (Palatnik et al., 2007). These targets include MYB33 and MYB65, which act in the endosperm and in anthers to promote programmed cell death (Alonso-Peral et al., 2010). Our RT-qPCR results of miR159a and miR159b as well as their target genes, MYB33 and MYB65, represent antagonistic

regulation patterns. Thus, miR159 are suggested as interesting candidate miRNAs regulating these two MYBs in the crosstalk. This finding is supported by Zhang et al. (2011) and Zhou et al. (2007b), that miR159 was downregulated in *Pst* DC3000 *hrcC* infected in Arabidopsis, and that UV-B responsive *cis*-elements, such as the GATA-box, are found in their respective MIR gene promoter. In addition, miR159 plays the key role in resetting ABA responses by directing MYB33 mRNAs degradation during seed germination (Reyes and Chua, 2007) and has also been found to be regulated by gibberellic acid (GA) and ethylene (ET) (Achard et al., 2004; Liu et al., 2009).

miR164: In Arabidopsis, miRNA164 proved to cleave mRNAs of several NAC (NAM, ATAF1/2, and CUC2) domain-containing genes, including CUC1, CUC2, NAC1, NAC2, NAC4, NAC5 and AT3G12977 (Guo et al., 2005). NAC genes belong to a unique class of transcription factors in plants. The common characteristics of the NAC proteins are the presence of a conserved NAC domain, comprising of about 150 amino acids in the N-terminus and a highly variable C-terminal transcriptional regulation region in C-terminals. Extensive studies have revealed that NAC transcription factors not only play important roles in plant growth and development, but also have functions in regulation of plant responses to biotic and abiotic stresses. Several studies have shown that miR164 may also be involved in response to abiotic and biotic stress in plants (Jia et al., 2009; Zhao et al., 2012). Our findings indicate the regulation of the crosstalk by miR164b and the target TFs NAC1, NAC2, NAC4 and AT3G12977. It is reported that downregulation of NAC1 by miR164b in response to abiotic stresses was shown to reset auxin signals (Guo et al., 2005). In addition, repression of NAC2 has already been reported to improve abiotic stress resistance (Patil et al., 2014). NAC4 promotes hypersensitive cell death by suppressing its target genes and this immune process is fine-tuned by the negative action of miR164 (Lee et al., 2017). AT3G12977, which is similar to NAC1, regulates endosperm cell expansion during germination (Sánchez-Montesino et al., 2019). In addition, miR164b has been shown to be UV-B responsive and auxin inducible as well (Guo et al., 2005; Jia et al., 2009). Our study showed that NAC1 could play a role in flg22 signal cascade, although no significant suppression of NAC1 was induced by UV-B treatment. And other NACs, including NAC2, NAC4 and AT3G12977 were identified to be involved in the crosstalk between flg22 and UV-B.

miR171: miR171, a member of the GRAS (GAI-RGA-SCR) gene family of transcription factors, participate in plant growth and development. In Arabidopsis, four HAIRY MERISTEM (HAM) copies exist, three of which (HAM1, HAM2 and HAM3) are cleaved by the miR171 family (Llave et al., 2002; Engstrom et al., 2011). They participate in a wide variety of developmental processes, such as the regulation of reproductive transition, meristem determinacy and trichome patterning in various species (Curaba et al., 2013; Xue et al., 2014). In our study, the regulation of HAM3 indicate a transcriptional reprogramming upon flg22 and UV-B, although the suppression of HAM1 by UV-B and induction of HAM2 by flg22 were not significant in the RT-qPCR results. These results suggested the involvement of miR171-HAMs in the crosstalk.

With improvements in high through-put technology and bioinformatics, several miRNAs are identified that play pivotal role in response to biotic and abiotic stresses in plants. For instance, upon UV-B radiation in Arabidopsis, miR396 has been predicted to target GROWTH REGULATING FACTORs (GRFs) to mediate the inhibition of leaf growth, which is an adaptive strategy of plants to arrest the cell cycle, allowing time to repair UV-B-induced DNA damage (Casadevall et al., 2013; Jiang et al., 2011). While, miR393 is transcriptionally induced by flg22 to downregulate the levels of the F-box auxin receptors TIR1, AFB2 and AFB3 (Navarro et al., 2006). Both miR858 and miR828 are known to target the MYB transcription factors. We observed that by targeting MYB111 and MYB75, miR858 and miR828 were induced by flg22 and suppressed by UV-B, respectively. The regulation of miR858 and miR828 to plant responses against abiotic and biotic stresses are also been reported before. For instance, Camargo-Ramírez (2017) demonstrated that miR858-mediated regulation of flavonoid-specific MYB transcription factor genes negatively controlled resistance to fungal pathogen infection in Arabidopsis. In addition, Piya et al. (2017) described that miR858 post-transcriptionally regulates MYB83 during cyst nematode parasitism, a process in which miR858 and MYB83 expression appear to be connected through a feed-back circuit. Very recently, Sunitha et al. (2019) revealed that miR828 would regulate the AtMYB113-ortologues MYBA5, A6 and A7 via a widely conserved auto-regulatory loop involving miR828 and phasi TAS4abc RNAs in grapevine. In other studies, miR828 and miR858 were reported to regulate the expression of MYB2 gene homologs that function in Arabidopsis trichome formation and cotton fiber development, these particular miRNAs also being regulated during adaptation to high temperature in cotton (Guan et al., 2014). MiR858 was also reported to mediate tolerance to

drought stress in the desert plant *Ammopiptanthus mongolicus* (Gao et al., 2016). Together, these observations point to a functional role for miR858 during adaptation to biotic and abiotic stresses. Whether miR858 or miR828 is a common component of plant adaptive responses to different types of environmental stresses needs to be further investigated.

3 MYBs regulates the crosstalk between flg22 and UV-B signaling in *Arabidopsis thaliana*

In accordance with literatures, we could show a large set of MYB TFs as direct targets of miRNAs identified in this study. MYB TFs represent a family of proteins that control many aspects of plant secondary metabolism, for instance the phenylpropanoid metabolism in Arabidopsis.

In addition to miR858 and miR828, several miRNAs obtained from this study, including miR159, miR396, miR5654, miR838, miR844 and miR866 can also regulate different MYB TFs. For instance, in addition to MYB111 and MYB75, AtMYB66 are required for normal epidermal-cell patterning, and regulated the position-dependent expression of GL2 (GLABROUS 2) (Lee and Schiefelbein, 1999). Additionally, AtMYB91 was found to be a homologue of AmMYBPHAN from *A. majus* and ZmMYBRS2 from *Z. mays*. Moreover, AtMYB4 acts as a negative regulator of cinnamate 4-hydroxylase gene expression as well as negatively regulates several steps of phenylpropanoid metabolism in a dose-dependent way (Jin et al., 2000). The miRNA-MYB interactions are implicated in the regulation of FPGs and flavonol content (Sharma et al., 2016; Wang et al., 2016). For instance, MYB11, MYB12 and MYB111 are prominent targets of miR858 (Wang et al., 2016). Flavonoids have been reported to be regulated by miR858 affecting MYB11, MYB12 and MYB111 expression. In addition, miR858a is obviously able to enhance anthocyanin biosynthesis in Arabidopsis seedlings by repressing translation of MYBL2, the negative regulator of anthocyanin biosynthetic pathway (Sharma et al., 2016; Wang et al., 2016). Recently, Camargo-Ramírez et al. (2017) stated miR858 functioned as a negative regulator of Arabidopsis immunity by controlling accumulation of antifungal phenylpropanoid compounds. Also, miR828 seems to be functionally analog to miR858, which is downregulated by UV-B and upregulated by flg22. Recent studies indicated that miR828 was induced by sugar and attenuated in abscisic acid in Arabidopsis (Luo et al., 2011) and wound inducible in sweet potato leaves (Lin et al., 2012). The sequence of miR828 was complementary to a region of an another MYB factor, MYB75, which caused a

massive activation of phenylpropanoid biosynthetic genes and enhanced accumulation of lignin, hydroxycinnamic acid esters and purple anthocyanins (Hsieh et al., 2009; Yang et al., 2013). miR158 was induced by flg22 but repressed by UV-B. miR158 targets numerous mRNAs coding for pentatricopeptide repeat proteins (PPR) (Allen et al., 2004). The PPR gene family was one of the largest families of putative RNA-binding proteins in plants containing more than 400 genes (Small and Peeters, 2000). It was predicted that the PPR family plays a central and broad role in regulating gene expression in organelles (Lurin et al., 2004; Schmitz-Linneweber et al., 2005). One of the hallmark characteristics of the syncytium was the increasing number of organelles such as mitochondria (Jones, 1981). Nothing is known about gene expression and RNA processing in organelles during feeding site formation and this miRNA may be useful as novel investigative target in this context.

Based on the number of MYB domains, the MYB protein family has been classified into 4 different groups: 4R-MYB (four repeats), R1R2R3-MYB (three repeats), R2R3-MYB (two repeats) and 1R-MYB (one repeat) proteins (Ambawat et al., 2013; Dubos et al., 2010). The R2R3-MYB class is unique to and is the most abundant type in plants. In plants, majority of the MYB proteins belong to the R2R3-MYB subfamily. Several R2R3-MYB genes are involved in regulating responses to biotic and abiotic stresses such as: AtMYB2 was induced by dehydration and salt stress (Abe et al., 2003); AtMYB62 is reported to be involved in phosphate starvation (Devaiah et al., 2009); AtMYB96 acted through the ABA signaling mediate to drought stress (Seo et al., 2009); AtMYB41 and AtMYB102 transcription factor genes were contributed to plant resistance against wounding and osmotic stress (Lippold et al., 2009). OsMYB3R-2 transgenic plants encode a stress-responsive MYB transcription factor having a regulatory role in enhanced tolerance to freezing, dehydration and salt stress and decreased sensitivity to ABA in rice (Yang et al., 2012). AmMYB1 transcription factor enhanced the tolerance to NaCl stress in transgenic tobacco (Ganesan et al., 2012). GmMYB72, GmMYB96 and GmMYB117 were induced by ABA, salt, drought and/or cold stress treatment in soybean (*Glycine max*) (Liao et al., 2008). Totally, more than over 100 R2R3-MYB members have been reported in dicots and monocots (Wilkins et al., 2009).

3.1 Regulation of plant response to abiotic stress by MYB TFs

Light is one of the most vital environmental elements controlling plant growth and development. Light stresses, such as damaging ultraviolet and differential light qualities irradiation, affect the synthesis of sunscreen flavonols in plants. Several members of R2R3-type MYB transcription factors are involved in the regulation of phenylpropanoid pathway which produces various secondary metabolic compounds involved in abiotic stress response in plants. Among the various secondary metabolites produced in plants, the sinapate esters and flavonoids act as key UV-B absorbing sunscreen compounds to protect plants against the harmful effects of UV-radiation. Plants produce higher levels of UV-B absorbing compounds under low doses of UV-B to compromise the initial damage of the major UV-B targets like nucleic acids, proteins and lipids. Molecular and genetic analysis in Arabidopsis mutants impaired in UV-B response have revealed key role of flavonoids and phenolics in UV-B absorption, facilitating enhanced UV-B tolerance (Bieza and Lois, 2001). Recent studies have indicated important role of MYB transcription factors in the regulation of biosynthesis of secondary metabolites involved in UV-B absorption in plants. DcMYB1 is required for UV-B irradiation-induced DcPAL1 by binding to the promoter of DcPAL1 in carrot (Maeda et al., 2005). Production of flavonol glycosides 1 (PFG1)/AtMYB12 and PFG3/AtMYB111 genes are activated under UV-B, and overexpression of PFG1 confers an increased UV-B tolerance in Arabidopsis (Stracke et al., 2001). Arabidopsis MYB4, a member of R2R3 subgroup, represses the transcription of the gene encoding cinnamate 4-hydroxylase, involved in hydroxycinnamate ester biosynthesis. The MYB4 loss-of-function mutant showed UVB tolerance due to increased accumulation level of hydroxycinnamate esters, while MYB4 overexpression caused reduced level of UV-B absorbing compounds, resulting in UV-B hypersensitivity (Jin et al., 2000). Another R2R3 MYB protein in Arabidopsis, AtMYB7 has been shown to be involved in regulating accumulation of UV-B absorbing phenylpropanoid compounds. The *atmyb7* mutants showed induction of several flavonoids biosynthetic genes. Interestingly, under UV-B stress, along with its own transcriptional repression, AtMYB4 was also found to inhibit AtMYB7 expression, which was consistent with the reduced flavonoids contents in *atmyb4* mutant, indicating repression of flavonoids biosynthesis by AtMYB7 and functional involvement of both AtMYB4 and AtMYB7 in maintaining the balance of accumulation of UV-B absorbing compounds in plants (Fornalé et al., 2014). MYB32, which is another TF R2R3-MYB, was previously

shown to affect expression of flavonoid biosynthesis pathway genes and pollen development. Zhong and Ye (2011) suggested that MYB32, MYB7, and MYB4 may be involved in fine tuning the regulation of lignin biosynthesis during secondary wall deposition. Expression of MYB75, MYB4, MYB12 and MYB111 in the turnip *Brassica rapa* is regulated by different light spectra, suggesting their roles in response to light stress (Wang et al., 2012). As a CHS promoter binding protein, the R2R3-type MYB gene pericarp color1 (P1), expressed in leaves, positively controls UV-B tolerance in high-altitude maize (Rius et al., 2012). AtPAP1 and AtPAP2, two major MYB transcription factors in anthocyanin biosynthesis, are induced by high light stress and play important roles in the regulation of high light driven anthocyanin synthesis (Vanderauwera et al., 2005). HbMYB1 is also involved in UV-B stress, evidenced by the fact that HbMYB1 overexpression in tobacco leads to enhanced resistance to UV-B stress (Peng et al., 2011).

3.2 Regulation of plant response to biotic stress by MYB TFs

MYB TFs also play a role in pathogen defense in various plant species (Dubos et al., 2010; Stracke et al., 2001). Overexpression of some R2R3-MYB TFs activates the expression of plant PR genes and triggers systemic acquired resistance (SAR). This response protects the plant against bacterial, fungal and viral pathogens and is modulated by phytohormones, especially JA and SA (Bostock, 2005). For example, the overexpression of AtMYB96 results in enhanced disease resistance in transgenic Arabidopsis. Overexpression also results in a subset of PR genes being upregulated (Seo and Park, 2010). Similarly, the expression of AtMYB44 is upregulated upon pathogen infection and by treatment with defense-related phytohormones. Transgenic plants overexpressing AtMYB44 demonstrate higher levels of PR gene expression and display enhanced resistance when infected with *P. syringae* (Zou et al., 2013). Arabidopsis AtMYB30 also acts as a positive regulator of the hypersensitive response and is dependent on SA accumulation (Raffaele et al., 2006). Transgenic plants that overexpress AtMYB30 also display increased resistance to bacterial and fungal pathogens (Vailleau et al., 2002). In tomato, SpMYB expression is significantly induced after infection with *F. oxysporum* and *B. cinerea*. Transgenic tobacco plants overexpressing SpMYB exhibit enhanced resistance to *F. oxysporum* and *B. cinerea* compared with wild-type plants. This resistance is coupled with enhanced expression of some defense-related genes and genes related to the JA signaling pathway (Liu et al., 2016). The *Thinopyrum*

intermedium TiMYB2R-1 gene is significantly induced following *Gaeumannomyces graminis* infection. TiMYB2R-1 overexpression significantly enhances resistance to take-all disease caused by the fungus *G. graminis* in transgenic wheat lines; the transcript levels of at least six wheat defense-related genes are significantly elevated as a result (Liu et al., 2013). The wheat TaRIM1 has been reported to participate in the resistance response against the pathogen *Rhizoctonia cerealis*. TaRIM1 overexpression significantly increases the resistance of transgenic wheat and TaRIM1 positively regulates the expression of five defense-related genes (Shan et al., 2016).

3.3 MYBs regulate the crosstalk of plant responses to abiotic and biotic stresses

So far, very few TFs have been reported to take part in the crosstalk between abiotic and biotic stress signaling networks. The mode of action underlying remains so far unsolved. For instance, the basic helix-loop-helix (bHLH) domain-containing transcription factor *AtMYC2* is a positive regulator of ABA signaling. The genetic lession of AtMYC2 however results in elevated levels of basal and activated transcription from JA-ethylene responsive defense genes (Abe et al., 2003; Anderson et al., 2004). Also, MYC2 differentially regulates two branches of JA-mediated responses; it positively regulates wound-responsive genes, including *VSP2*, *LOX3*, and *TAT*, but represses the expression of pathogen-responsive genes such as *PR4*, *PR1*, and *PDF1.2*. These complex interactions are co-mediated by the ethylene-responsive transcription factor ERF1 (Lorenzo et al., 2003; Lorenzo et al., 2004). In addition, the botrytis susceptible 1 (*BOS1*) gene of *Arabidopsis* encodes an R2R3-MYB transcription factor that mediates responses to certain signals, probably through ROS intermediates from both biotic and abiotic stress agents (Mengiste et al., 2003). There are also four members of the *NAC* family of genes that encode plant-specific transcription factors involved in diverse biological processes. *OsNAC6*, *Arabidopsis transcription activation factor 1* (*ATAF1*), *ATAF2* and *dehydration 26* (*RD26*) are potentially involved in regulation of responses to abiotic and biotic stresses (Wu et al., 2009).

Several TF MYB genes regulating flavonoid biosynthesis were implicated in response to both abiotic and biotic stress. The rice OsMYB4 gene is strongly induced by cold. Its overexpression in Arabidopsis plants upregulates genes involved in cold, drought, salt, and oxidative stress tolerance, as well as pathogen attack. Transgenic plants have effectively demonstrated improved tolerance/resistance to these stress conditions (Vannini et al., 2006). Tomato plants

overexpressing OsMYB4 acquire a higher tolerance to drought stress and can effectively protect plants against viral infection; however, these transgenic plants do not appear to be more cold tolerant than the controls (Vannini et al., 2007). The authors suggested that OsMYB4 represents a crucial knot in the crosstalk of stress signaling cascades through the activation of multiple components but that the activity depends on the host genomic background. Tomato SpMYB expression is strongly induced by fungal pathogens. Transgenic tobacco plants overexpressing SpMYB have enhanced salt and drought stress tolerance and demonstrate significantly improved resistance to *Alternaria alternata* (Li et al., 2014). Transgenic wheat overexpressing TaPIMP1 demonstrates significantly enhanced resistance to both the fungal pathogen *Bipolaris sorokiniana* and drought stress. Microarray analysis showed that a subset of defense and stress-related genes was upregulated by TaPIMP1 (Zhang et al., 2012). The expression of the same TF gene in transgenic tobacco enhances resistance to *Ralstonia solanacearum*, drought and salt stresses, which endorses its important role in modulating responses to abiotic and biotic stresses (Liu et al., 2011). In Arabidopsis, AtMYB108/BOS1, closely related to AtMYB2, is also one of the first identified MYB genes involved in the crosstalk among abiotic and biotic stresses (Mengiste et al., 2003). Its expression was induced by Botrytis infection, whereas it was blocked in the JA-insensitive *coi1* mutant, suggesting that AtMYB108/BOS1 may play a role in the defense response regulated by JA. Moreover, the expression of the PR-1 gene was induced more strongly in the *bos1* background than in the wild-type plants. These data clearly demonstrate a role for AtMYB108/BOS1 in resistance to *Botrytis*. Since ROS have been implicated in signaling in response to both pathogens and abiotic stresses, these data suggested that AtMYB108/BOS1 could regulate the responses to ROS-mediated signaling from both abiotic and biotic stresses (Mengiste et al., 2003). AtMYB96, which its role in drought response through the regulation of lateral root growth, stomatal opening and cuticle components accumulation (Baldoni et al., 2015), was also characterized for its positive regulation of freezing (Guo et al., 2013) and pathogen responses (Seo and Park, 2010). AtMYB96 expression was upregulated very early (within one hour) in response to the treatment with flg22, which efficiently triggers plant defense response, before the induction of the pathogen related (PR)-1 and the SA biosynthetic SALICYLIC ACID INDUCTION DEFICIENT2 (SID2) genes. These data clearly indicate that AtMYB96-mediated ABA signals enhance plant disease resistance by inducing SA biosynthesis (Seo and Park, 2010). Another well-

characterized gene that is involved in the response to different stresses is AtMYB15. The expression of AtMYB15 was highly induced by wounding and its overexpression in transgenic plants resulted in elevated expression of almost all the genes involved in the shikimate pathway, suggesting that AtMYB15 could be a direct regulator of this pathway in response to wounding (Baldoni et al., 2013). While, AtMYB15 expression was upregulated by treatments with ethylene and with the hairpin protein $HrpN_{Ea}$, an elicitor secreted by *Erwinia amylovora*. A mutant line generated by T-DNA insertion into the exon region of AtMYB15 showed a greater susceptibility to the green peach aphid, indicating that AtMYB15 can be important to activate the ethylene defensive pathway (Liu et al., 2010).

Based on our previous study, MYB12 is also involved in a precise mechanism of the crosstalk between abiotic and biotic stresses (Schenke et al., 2011; Zhou et al., 2017). Particularly, in Arabidopsis cell suspension culture and seedlings, ultraviolet-B (UV-B) light induced the flavonol gene transcription and consequently the flavonol production. The accumulation of these compounds was reduced when the bacterial flagellin peptide elicitor flg22 was concurrently applied. At the same time, flg22 enhanced the production of defence-related compounds (phytoalexin, camalexin, scopoletin and lignin). Flavonols, lignin and scopoletin all derive from phenylalanine. When both stresses (UV-B and flg22) are simultaneously applied, the pathway for defence-related compounds increased at the expense of flavonol synthesis (Schenke et al., 2011; Zhou et al., 2017).

3.4 Post-transcriptional regulation of MYBs by miRNAs in the crosstalk

TF activities are finely regulated at various steps in diverse cellular signaling networks for optimal growth and survival under different growth conditions (Yamaguchi-Shinozaki et al., 2006). Well-established molecular and biochemical mechanisms underlying regulation of TF activities include gene transcriptional regulation, post-transcriptional regulation of RNA metabolism, protein translation, post-translational modifications, and controlled protein turnover. In particular, the existence of different mechanisms of post-transcriptional control of mRNA metabolism is a key molecular scheme that modulates the TF activities in plant responses to environmental cues (Yun et al., 2008).

In Arabidopsis, a study has been carried out to functionally characterized miR858a, which putatively targets R2R3-MYB transcription factors involved in flavonoid biosynthesis (Sharma et al., 2016). Overexpression of miR858a in Arabidopsis resulted in the downregulation of several MYB TFs involved in flavonoid biosynthesis pathway, hence decrease the flavonoid production. In contrast, knockdown of miR858a by target mimic led to plant growth reduction and delayed flowering (Sharma et al., 2016). In addition, MYB TF was found to be upregulated in response to high temperature in cotton. Like the previous study (Guan et al., 2014), MYB TF was targeted by miR828a and miR858 (Wang et al., 2016). From this finding, they suggest MYB TF and miR828 and miR858 may have dual role in cotton, during fiber development and adaptation against high temperature. In Arabidopsis, Reyes and Chua (2007) reported that ABA-induced accumulation of miR159 was a homeostatic mechanism to direct MYB33 and MYB101 transcript degradation to desensitize hormone signaling during seedling stress responses. Moreover, in potato, a recent study identified three novel miR159 family members (stu-miR159a, stu-miR159b and stu-miR159c) and their targeted GAMyb-like genes (StGAMyb-like1, StGAMyb-like2.1 and StGAMyb-like2.2), putatively involved in drought stress response (Yang et al., 2014). The expression level of the three stu-miR159 members significantly decreased after 25 days of drought stress treatment, whereas the expression of their targeted GAMyb-like genes greatly increased, suggesting that stu-miR159s negatively regulated the expression of potato GAMyb-like genes. These results give a new insight into the role of GAMyb-like genes, which seem to be involved not only in ABA response during germination, but also in the response to water stress. Very recently, Tirumalai et al. (2019) demonstrated that miR828 and miR858 regulate VvMYB114 to promote anthocyanin and flavonol accumulation in grapes. MYB family genes were targeted by miR828, which is associated with purple tuber skin and flesh color, and involve in anthocyanin regulation in potato (Bonar et al., 2018). In our study, mutant analysis indicated flg22-reduction and UV-B-induction of CHS were impeded in mutants *myb11* and *myb12*, respectively. Moreover, mutant *myb111* indicated the CHS expression of UV-B-upregulation was abolished, compared with wild-type plants. In addition, no notable difference of flg22-suppression and UV-B-induction between wild-type plants and triple mutant *myb11/12/111.* In mutant *myb75*, the suppression of CHS by flg22 is to some content attenuated. Taken together, MYB family (MYB11, MYB12, MYB111 and MYB75) may be

involved in the crosstalk, suggesting a role of MYBs in forming a complex and highly interconnected regulatory network in the flg22/UV-B signal cascades.

4 A functional model

Since decades, it is known that one of the defense mechanism of plants to acclimate to UV-B radiation is biosynthesis of UV-B screening compounds such as flavonoids. This accumulation is found in the entire upper epidermis of the leave implying they would attenuate UV-B radiation before it reaches the mesophyll, where the photosynthesis reaction happens (Wilson and Greenberg, 1993). The suppression of UV-B induced-flavonoid production by elicitor treatment has been described in many species, including the model plant *Arabidopsis thaliana* in both cell culture and seedlings (Schenke et al., 2011; Zhou et al., 2017). This interference in the respective signaling cascade is known as the “crosstalk”. Investigation of this crosstalk can be achieved by utilizing chalcone synthase (CHS) as a marker gene. MYB transcription factors have been proposed as main positive and negative regulator, regulated by miRNAs on post-transcriptional level. Most importantly, function identification of miRNAs from this study will provide a new insight into the crosstalk between flg22 and UV-B induced signaling pathways in plants. Based on our data presented in this study, we could propose a function model for miRNA-target interactions to illustrate how the miRNA and their targets are involved in regulation of the CHS-pathway via the crosstalk between flg22 and UV-B induced signal cascades in Arabidopsis. The model includes 13 miRNA families representing 15 miRNAs (Group I), which were upregulated by flg22 and downregulated by UV-B, while 4 miRNA families representing 5 miRNAs (Group II), which are downregulated by flg22 and upregulated by UV-B. Antagonistic effects of flg22 and UV-B on the expression of miRNAs in/between 2 groups as well as an antagonistic regulation of TFs between 2 groups are essential and constitute a multiple and complex regulatory layer in plant response to flg22 and UV-B induced signal cascades. This leads to re-programming of FPGs expression, ensuring plants to effectively activate the PTI against pathogen infection. A great challenge remains to dissect the mechanism how miRNAs are regulated in response to diverse environmental and endogenous stimuli, e.g. by flg22 and UV-B as well as their crosstalk in plants.

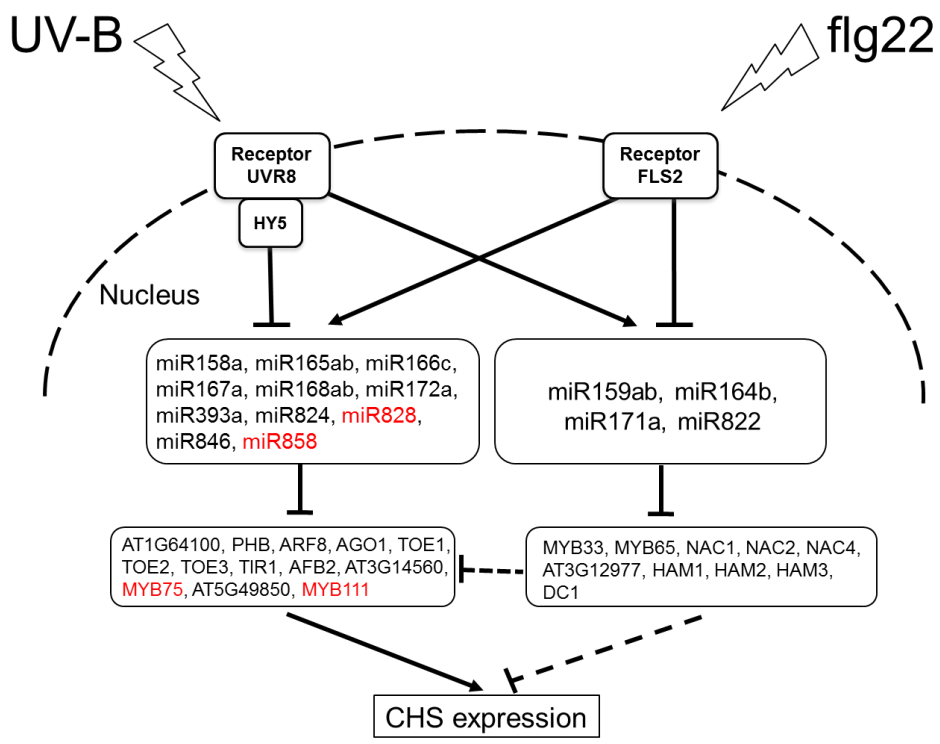

Figure 4 A functional model for miRNA-target interactions involved in regulation of the crosstalk between flg22 and UV-B in Arabidopsis. The miRNAs regulate the TFs and finally FPGs. Antagonistic effects of flg22 and UV-B on the expression of miRNAs in/between 2 groups as well as an antagonistic regulation of TFs between 2 groups are essential in the crosstalk between flg22 and UV-B induced signal cascades and constitute a multiple and complex regulatory layer of CHS gene expression. Positive interactions are represented with arrows, and negative interactions with bars.

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Summary

In their environment, plants are continuously exposed to various stresses of abiotic and biotic factors, and have evolved different counter strategies. In case of UV-B irradiation, plants defend themselves with the production of sinapoylmalate and flavonoids, which function as natural sunscreens. Since more than two decades, it is known that such UV-B-induced flavonol pathway genes (FPGs) are attenuated when concomitant elicitation by pathogen or by application of elicitors (simulating biotic stress), e.g. the bacterial peptide flg22, a defined Microbe/Pathogen Associated Molecular Patterns (MAMP/PAMP) occurs. The suppression of flavonoid production during MAMP Triggered Immunity (MTI) is believed to allow the plant focusing its metabolism on the pathogen defense by directing phenylalanine resources from UV-B protective flavonol production towards production of phytoalexins and cell wall fortification by lignin incorporation during MTI. Although this kind of "crosstalk" of plant responses to biotic (flg22) and abiotic (UV-B) induced signal cascades has been intensively investigated in the cell cultures, underlying mechanisms remains so far unsolved. Thus, understanding and dissection of signal pathways governing this crosstalk are of great scientific and practical significance. In this thesis in addition to general introduction (Chapter I) and discussion (Chapter V), three research chapters are presented aiming to elucidation of molecular mechanisms underlying this crosstalk with focus on the role of plant-derived microRNAs (miRNAs).

Chapter II: "Investigation of the crosstalk between the flg22 and the UV-B induced flavonol pathway in *Arabidopsis thaliana* seedlings". In the cell culture, it has been shown that flavonoid accumulation depends on expression of the Arabidopsis FPGs, which are upregulated by UV-B irradiation but repressed by the bacterial elicitor flg22 during MTI. Here we extend our observations made initially in cell cultures to whole plant seedlings, and demonstrate that such signal crosstalk is fully functional in Arabidopsis in planta. However, we observed some differences in the expression patterns of MYB transcriptions factors (TFs) as compared to data from the cell culture system. Thus, we present an updated working model how this crosstalk might function in Arabidopsis in planta. This system based on seedlings of the model plant *Arabidopsis thaliana* constitutes a valuable platform for further dissection of the underlying molecular mechanism, e.g. by deploying gain/loss-of-function of candidate genes.

Chapter III: "Identification and characterization of microRNAs involved in the crosstalk between flg22 and UV-B induced signal cascades in *Arabidopsis thaliana*". We proposed that plant-derived miRNAs are crucial regulators in the crosstalk between flg22 and UV-B induced signal cascades. By deep sequencing we identified 217 miRNAs representing 204 conserved and 13 novel miRNAs involved in the crosstalk. Among them, e.g. 106 miRNAs were upregulated by flg22 and downregulated by UV-B. Furthermore, a set of specific interactions between miRNAs and their targets were confirmed showing reciprocal changes in their expression levels, simultaneously. As revealed by GO and KEGG analysis *in silico*, predicted miRNA target genes participate in a series of plant biological and molecular processes as well secondary metabolism pathways. Two modulations of miRNA-target interactions were obtained in the crosstalk. The first one consisting of miR158, miR165, miR166, miR167, miR168, miR172, miR391, miR393, miR447, miR824, miR828, miR846 and miR858, which were all repressed by UV-B irradiation, while upregulated by flg22, and the second one constitute miR159, miR164, miR171 and miR822, being repressed by flg22 and upregulated by UV-B. Both miRNA sets display converse regulation with the change in transcript levels of their targets. Furthermore, we demonstrated that knockdown of miR858 (Group I) in Arabidopsis goes along with increased the chalcone synthase (CHS) expression while its overexpression results in its depression. Vice versa, knockout of miR164b (Group II) depresses the CHS gene expression while its overexpression strongly elevated the CHS gene expression, providing the first genetic evidence that miRNAs identified in this study constitute an additional layer in regulating the crosstalk between flg22 and UV-B induced signaling cascades.

Chapter IV: "miR858 and miR828 are master regulators for UV-B- and flg22-signal crosstalk in *Arabidopsis thaliana*". Identification of two highly conserved miRNAs (miR858 and miR828) being involved in the crosstalk and their targets MYB111 and MYB75 that proved to play an important role in regulation of FPGs and the flavonoid accumulation provoked us to assume that miR858-MYB111 and miR828-MYB75 interactions play an important role in the crosstalk. Here, we demonstrate that both miR858 and miR828 are regulated by UV-B and flg22 in a UVR8 and FLS2 receptors-dependent manner and relying on the respective signaling pathways. Comparison between miR858/MYB111-promoter-GUS and their transcript levels evidences that the MYB111 is regulated not only at the transcriptional level, but also suffered from post-transcriptional modification in response to the flg22 and UV-B challenges, in which miR858 acts as a determinant

regulator. Following this, we conclude that the post- transcriptional regulation mediated by plant-derived miRNAs constitutes the crosstalk between the flg22 and UV-B induced signal cascades in Arabidopsis. This allows an extension of the crosstalk model between plant responses to biotic (flg22) and abiotic (UV-B) stresses in Arabidopsis.

Taken together, this work shows, besides an overall high miRNA importance, that some newly identified plant-derived miRNAs play an important role in regulating the crosstalk between biotic (flg22) and abiotic (UV-B) stress responses, largely broaden our knowledge about the regulatory role of miRNAs. In addition, these results constitute a promising starting point for understanding the mode of action of the crosstalk and it potential application in the practice, e.g. by interfering with the regulatory network using the CRISPR technology aiming at improvement of plant defense mechanisms towards various a/biotic stresses.

Zusammenfassung

Rolle von miRNAs auf die Regulation der Flavonolpfad-Gen Expression in *Arabidopsis thaliana* und ihr möglicher Einfluß auf das Wechselspiel zwischen UV-B und flg22 Signaltransduktionskaskaden

Pflanzen sind in ihrer natürlichen Umgebung verschiedenen Stressen abiotischer und biotischer Natur ausgesetzt und haben entsprechende Anpassungsstrategien entwickelt. Im Falle von UV-B Strahlung schützen sich Pflanzen durch die Produktion von Sinapoylmalat und Flavonoiden, welche als natürlicher Sonnenschutz fungieren. Seit mehr als 20 Jahren ist bekannt, dass die durch UV-B induzierte Expression der Flavonolpfad-Gene (FPGs) durch die zeitgleiche Applikation sogenannter Elizitoren, welche biotischen Stress simulieren unterdrückt wird. Zu diesen Elizitoren gehört auch das bakterielle Peptid flg22, ein hochkonserviertes Mikroben/Pathogen-Assoziiertes Molekulares Muster (engl.: MAMP/PAMP), welche die MAMP-Induzierte Immunität (engl.: MTI) in der Pflanze auslösen. Die Unterdrückung der Flavonoid-Produktion während der MTI dient der Umsteuerung des pflanzlichen Metabolismus, um so den Phenylpropanoid-Stoffwechselweg für die Produktion anti-mikrobieller Abwehrmoleküle, wie Phytoalexinen und Zellwandverstärkungen durch Lignifizierung, zu nutzen. Obwohl dieses Wechselspiel (eng.: „crosstalk“) zwischen den abiotischen (UV-B) und biotischen (flg22) Signaltransduktionskaskaden intensiv in Zellkulturen untersucht wurden, blieben die zugrundeliegenden Mechanismen weitestgehend unbekannt. Daher ist ein tieferes Verständnis und eingehende Analyse der für den Crosstalk verantwortlichen Signal-Wege von großer wissenschaftlicher, aber auch praktischer Bedeutung. In dieser Arbeit werden zusätzlich zur allgemeinen Einleitung (Kapitel I) und Diskussion (Kapitel V), drei Kapitel vorgestellt, in denen die molekularen Mechanismen, welche dem Crosstalk unterliegen näher beleuchtet werden, mit dem Fokus auf der Rolle pflanzlicher mikro-RNAs (miRNAs).

Kapitel II: “Investigation of the crosstalk between the flg22 and the UV-B induced flavonol pathway in *Arabidopsis thaliana* seedlings” ist eine Originalpublikation, in welcher die Funktionalität des Crosstalks in Arabidopsis Keimlingen dargestellt wird. In pflanzlichen Zellkulturen wurde bereits gezeigt, dass die von der Expression von FPGs abhängige Produktion

der Flavonoide durch UV-B induziert, aber durch den bakteriellen Elizitor flg22 während der MTI unterdrückt wird. Hier erweitern wir diese Beobachtung auf Arabidopsis Keimlinge und zeigen so, dass der Crosstalk auch *in planta* funktioniert. Im Vergleich zum Zellkultursystem beobachteten wir einige Unterschiede im Expressionsmuster von MYB Transkriptionsfaktoren, sodass wir ein aktualisiertes Arbeitsmodel des Crosstalks *in planta* vorstellen konnten. Ein auf Keimlingen basierendes System in der Modellpflanze Arabidopsis stellt ein wertvolles Werkzeug dar, um den Mechanismus des Crostalks aufzuklären, z.B. durch die Analyse von Überexpressions- oder Funktionsverlust-Mutanten in Kandidatengenen.

Kapitel III: "Identification and characterization of microRNAs involved in the crosstalk between flg22 and UV-B induced signal cascades in *Arabidopsis thaliana*" stellt ein Manuskript dar, in welchem wir Arabidopsis miRNAs identifizieren, die während des Crosstalks differenziell reguliert werden. Wir vermuten, dass diese pflanzlichen miRNAs eine wichtige Rolle im Crosstalk zwischen den flg22 und UV-B induzierten Signaltransduktionskaskaden spielen. Insgesamt wurden im Crosstalk durch Tiefen-Sequenzierung 217 miRNAs identifiziert, davon sind 204 konserviert und 13 bisher unbeschrieben. Von diesen sind z.B. 106 miRNAs durch flg22 hoch- und durch UV-B herunterreguliert. Weiterhin konnten spezifische Interaktionen zwischen einigen dieser miRNAs und ihren Ziel-RNAs durch reziproke Regulation ihrer Expression demonstriert werden. Die durch *in silico* GO und KEGG-Analyse vorhergesagten miRNA Zielgene sind in einer Reihe biologischer und molekularer Prozesse involviert, darunter auch dem Sekundärmetabolismus. Zwei miRNA-Zielgen Regulationsmodule wurden im Crosstalk identifiziert: die erste Gruppe besteht aus miR158, miR165, miR166, miR167, miR168, miR172, miR391, miR393, miR447, miR824, miR828, miR846 und miR858, die durch UV-B unterdrückt und durch flg22 hochreguliert werden, während die zweite miR159, miR164, miR171 und miR822 beinhaltet, welche durch UV-B induziert und durch flg22 unterdrückt werden. Beide miRNA Gruppen zeigen eine ihren Zielgenen entgegengesetzte Regulation auf Transkriptebene. Außerdem konnte durch Ausschalten der miRNA858 (Gruppe I) in Arabidopsis eine Expressionssteigerung des FPG Schlüsselenzyms Chalcone Synthase (CHS) gezeigt werden, während miRNA858 Überexpression zur Expressionsunterdrückung führte. Umgekehrt führte der Verlust von miRNA164b (Gruppe II) zur Expressionsunterdrückung der CHS, währen die Überexpression einen positiven Einfluss ausübte. Damit wurde der erste genetische Beweis erbracht, dass die in dieser Studie identifizierten

miRNAs eine zusätzliche Rolle in der Regulierung des Crosstalks zwischen den flg22 und UV-B induzierten Signaltransduktionskaskaden spielen.

Kapitel IV: "miR858 and miR828 contribute to crosstalk between UV-B- and flg22-signalling in *Arabidopsis thaliana*" ist ein Manuskript, in welchem die Funktion von miR858 und miR828 im Crosstalk näher beleuchtet wird. Die Identifizierung zweier hoch-konservierter miRNAs (miRNA858 und miRNA828) und ihrer Zielgene, MYB111 bzw. MYB75, welche beide im Crosstalk an der Regulation der FPGs und damit der Flavonoid Akkumulation beteiligt sind, legt nahe, dass die miR858-MYB111 und miR828-MYB75 Interaktionen eine wichtige Rolle im Crosstalk spielen. Hier zeigen wir, dass beide miRNAs durch UV-B und flg22 in Abhängigkeit der zugehörigen Rezeptoren (UVR8 bzw. FLS2) und den durch diese aktivierten Signaltransduktionskaskaden reguliert werden. Ein Vergleich zwischen der Expression von miR858/MYB111-promoter-GUS Konstrukten und den nativen Transkriptmengen zeigt, dass MYB111 nicht nur transkriptional reguliert ist, sondern auch post-transcriptional durch miRNA858 in Abhängigkeit von UV-B oder flg22 Behandlung. Daraus lässt sich folgern, dass die post-transkriptionale Regulation durch pflanzliche miRNAs direkt zum Crosstalk zwischen der flg22 und UV-B vermittelten Signaltransduktion beiträgt. Dies erlaubt eine Erweiterung des Crosstalk-Modells zwischen der pflanzlichen Reaktion auf biotischen (flg22) und abiotischen (UV-B) Stress in Arabidopsis.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit neben der allgemeinen Bedeutung von miRNAs, dass einige neu identifizierte pflanzliche miRNAs eine wichtige Rolle im Crosstalk zwischen der biotischen (flg22) und abiotischen (UV-B) Stressantwort spielen, was unser Verständnis über ihre regulatorische Funktion enorm erweitert. Zusätzlich bieten diese Ergebnisse eine gute Erklärung den Mechanismus dieses Crosstalks zu verstehen und so eine praktische Anwendung zu ermöglichen, z.B. durch Manipulation des regulatorischen Netzwerkes mittels CRISPR Technologie, um so die pflanzliche Abwehr gegen verschiedene (a) biotische Stresse zu verbessern.

Material and Methods

This chapter includes detailed protocols for the main procedures used in this study.

1 Materials used in this thesis

1.1 Machines

Machines	Model	Company
Agarose gel electrophoresis	Mini-Sub® Cell GT	Bio-Rad
Autoclave	VX-75	Systec
Centrifuge (30 tubes)	5417R	Eppendorf
Centrifuge (8 PCR stripes)	SN10010417	VWR
Centrifuge (6 tubes)	SN07030982	VWR
Centrifuge (96-well plate)	Multifuge X3R	Thermo Fisher Scientific
Electronic Balance (d=0.1 mg)	ABJ80-4M	KERN
Electronic Balance (d=0.01 g)	KB1200-2	KERN
Gel documentation	Gel Doc ™ XR+	Bio-Rad
Incubator	Excellent UFE 400-800	Memmert
Incubator shaker	Certomat® IS	Sartorius
Magnetic stirrer	Combimag REO	IKA WERKE
Microscope	Stereo Discovery.V20	Zeiss
Microscope	TCS SP1	Leica Biosystems
NanoVue	106726	GE Healthcare
PCR maschine	TProfessional Standard Gradient	Biometra
PCR maschine	C1000	Bio-Rad
pH meter	WTW inoLab®pH720	EYDAM
Photo camera	DX 3000	Nikon
Pipettes	2.5 µl, 20 µl, 200 µl, 1000 µl	Eppendorf
Realtime PCR maschine	CFX96	Bio-Rad
Spectrophotometer	UVmini-1240	SHIMADZU
Sterile workbench	Herasafe	Thermo Fisher Scientific
Thermocell Cooling&Heating Block	HB-202	BIOER
Vortex	Vortex Genie 2	Scientific Industries
Water bath	1160S	VWR
Water bath, shaking	1083	GFL Gesellschaft für Labortechnik GmbH

1.2 Chemicals

Chemical	Article number	Company
40% Acrylamide	1610144	Bio-Rad
Acetic acid	3738.2	Roth
Acetone	7328.1	Roth
Acetosyringone	2196105	Fluka
Ammonium acetate	7869.2	Roth
Ampicillin	K0291.1	Roth
APS (Ammonium peroxodisulphate)	9592.2	Roth
Aqua-Roti-Phenol	A980.1	Roth
Bacto™ Agar	214010	BD
Biozym LE-Agarose	840004	Biozym
Boric acid	6943.1	Roth
Bromophenol blue	61335-100	Fluka
$CaCl_2$ (Calcium chloride)	CN93.2	Roth
$CaCl_2O_2$ (Calcium hypochlorite)	5164.3	Roth
Chloramphenicol	3886.1	Roth
Chloroform	3313.2	Roth
CTAB (Cetyltrimethylammonium bromide)	9161.2	Roth
Daishin-Agar	D1004.1000	Duchefa Biochemie
DEPC (Diethyl pyrocarbonate)	K028.1	Roth
DMSO (Dimethylsulfoxid)	4720.2	Roth
D(+)- Sucrose	4621.2	Roth
EDTA (Ethylenediamine tetraacetic acid)	8043.1	Roth
Ethanol	9065.5	Roth
Ethidiumbromide	21251	Boehringer
GeneAmp dNTP Blend, 100 mM	N8080261	Invitrogen
GeneRuler 1 kb DNA Ladder	SM0314	Thermo Fisher Scientific
GeneRuler 100 bp DNA Ladder	SM1313	Thermo Fisher Scientific
Gentamicin	G0124.0025	Duchefa Biochemie
Glycerol	3783.2	Roth
IPTG	2316.3	Roth
Isoamyl alcohol	8930.1	Roth
Isopropanol	6752.4	Roth
Kanamycin	K0126.0025	Duchefa Biochemie
KCl (Potassium chloride)	6781.1	Roth
KH_2PO_4 (Potassium dihydrogen phosphate)	3904.1	Roth
KOH (Potassium hydroxide)	6751.3	Roth
MES	4256.2	Roth
$MgCl_2$ (Magnesium chloride)	8.147.330.100	Merck
MOPS	6979.3	Roth
Murashige & Skoog (MS) Salze (including MES/Vitamins)	M0254.0050	Duchefa Biochemie
NaAc (Sodium acetate)	6773.1	Roth

NaCl (Sodium chloride)	3957.2	Roth
Na_2HPO_4 (Di-sodium hydrogen phosphate)	X987.2	Roth
NaH_2PO_4 (Sodium Di-Hydrogen Phosphate Monohydrate)	K300.2	Roth
NaOH (Sodium hydrate)	6771.2	Roth
Rifampicin	R0146.0005	Duchefa Biochemie
RNase A	R1253	Thermo Fisher Scientific
SDS (Sodium lauryl sulfate)	4360.1	Roth
Silwet Gold	AM6A12W004	Spiess Urania
Spectinomycin	S4014-5g	Sigma
TEMED	2367.3	Roth
Tris-hydrochlorid (HCl)	9090.3	Roth
Tris-Ultrapure	5429.3	Roth
Triton X 100	3051.3	Roth
Tryptone/Peptone	8952.2	Roth
TRIzol® Reagent	15596018	Invitrogen
Tween-20	9127.1	Roth
Ultra Low Range 10 bp	SM1211	Thermo Fisher Scientific
Water	3175.2	Roth
X-Gal	2315.4	Roth
X-GlcA	X1405.1000	Duchefa Biochemie
Yeast Extract	2363.4	Roth
β-mercaptoethanol	4227.1	Roth

1.3 Enzymes and Kits

Kit and Enzyme	Article number	Company
DNase I	EN0521	Thermo Fisher Scientific
DreamTaq DNA Polymerase (5 U/µl)	EP0704	Thermo Fisher Scientific
Gateway™ BP Clonase™ II Enzyme mix	11789020	Invitrogen
Gateway™ LR Clonase™ II Enzyme mix	11791020	Invitrogen
GeneRacer™ Kit	L150201	Invitrogen
Maxima SYBR Green/ROX qPCR Master Mix (2X)	733-1383	VWR
NucleoSpin Gel and PCR Clean-up	740609250	Macherey-Nagel
NucleoSpin Plasmid Kit	740588.250	Macherey-Nagel
pGEM®-T Vector Systems	A3600	Promega
Phusion High-Fidelity DNA Polymerase (2 U/µl)	F530S	Thermo Fisher Scientific
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Fisher Scientific

1.4 Software

Software	Source
BLAST	http://blast.ncbi.nlm.nih.gov/Blast.cgi
Minitab	https://www.minitab.com/
miRBase	http://www.mirbase.org/
psRNATarget	http://plantgrn.noble.org/psRNATarget/
The Arabidopsis Information Resource (TAIR)	http://www.Arabidopsis.org/

1.5 Culture media

LB medium (solid)	Amount
Tryptone/Peptone	10 g
Yeast Extract	5 g
Bacto™ Agar	15 g
ddH_2O	to 1L
NYGA medium (solid)	
Tryptone/Peptone	5 g
Yeast Extract	3 g
Glycerol	20 ml
Bacto™ Agar	15 g
ddH_2O	to 1L
½ MS medium (solid), pH 5.8 (adjust with KOH)	
MS Salze MES/Vitamins	2.45 g
Sucrose	10 g
Daishin Agar	8 g
ddH_2O	to 1L

1.6 Plant and bacteria material

Arabidopsis thaliana	**Decription**
wild-type Col-0	Produced by this laboratory
wild-type Ler-0	Produced by this laboratory
UVR8 mutant *uvr8* (in Ler-0)	Lack 15 amino acids of receptor region
HY5 T-DNA *hy5* (in Col-0)	SALK_096651C
FLS2 T-DNA mutant *fls2* (in Col-0)	SALK_062054
UVR8/FLS2 mutant *uvr8*×*fls2* (in Col-0)	Genetic cross
BAK1 T-DNA mutant *bak1* (in Col-0)	SALK_034523
BIK1 T-DNA mutant *bik1* (in Col-0)	SALK_005291C
PLC2 T-DNA mutant *plc2* (in Col-0)	SALK_152284C
RbohD T-DNA mutant *rbohD* (in Col-0)	SALK_109396C
MPK3 T-DNA mutant *mpk3* (in Col-0)	SALK_151594
MPK6 T-DNA mutant *mpk6* (in Col-0)	SALK_127507
STTM858 (in Col-0)	Addgene plasmid #84177
STTM828 (in Col-0)	Addgene plasmid # 84157
miR164b knockout line (in Col-0)	SALK_136105.53.60.x
miR164b overexpression line (in Col-0)	Lab stock
MYB4 T-DNA mutant *myb4* (in Col-0)	CSHL_ET3967
MYB11 T-DNA mutant *myb11* (in Col-0)	SALK_077068
MYB12 T-DNA mutant *myb12* (in Col-0)	myb12-1f
MYB111 T-DNA mutant *myb111* (in Col-0)	GK291D01
MYB11/MYB12/MYB111 T-DNA mutant *myb11/12/111* (in Col-0)	Produced by crossings of myb12-1f, GABI-Kat291D01 and SALK_077068
MYB75 T-DNA mutant *myb75* (in Col-0)	pst16228
Nicotiana tabacum	
Nicotiana tabacum	Lab stock
Pseudomonas syringae* pv. *Tomato	
DC3000	Lab stock
hrpL	Lab stock
hrcQ	Lab stock
Escherichia coli	
DH5α	Lab stock
Agrobacterium tumefaciens	
GV3101	Lab stock

2 Methods used in this thesis

2.1 Isolation of genomic DNA

Genomic DNA (gDNA) was isolated from each single plant using CTAB (cetyltrimethylammonium bromide) buffer (Rogers and Bendich, 1985) (Table 1). Each step was described as following:

1) Plant material is grounded in liquid nitrogen and collected into 2 ml tube.
2) Add 1,000 µl CTAB and 3 µl β-mercaptoethanol into each tube and incubate at 65 °C for 1 h.
3) After cooling down, 1µl RNase is added and incubate for another 15 min at 37 °C incubator. Then add 500 µl Chloroform/Isoamylalcohol (24:1) and shake for 10 min.
4) Centrifuge at 14,000 rpm for 15 min at room temperature. Then transfer the upper aqueous phase (~700 µl) into a new 1.5 ml tube. Add 700 µl Isopropanol and mix gently.
5) Incubate the samples at 4 °C for 1 h.
6) The samples are centrifuged for 15 min at 14,000 rpm at room temperature and discard the supernatant.
7) The pellets are washed in 70% and 100% ethanol for 5 min, respectively.
8) The pellets are dried for 10 min and dissolved in 50 µl H_2O.
9) DNA was checked on the 1.2% agarose gel.

Table 1 Solution buffer used in gDNA isolation

Solution	Composition
2x CTAB (1 L)	0.2 M Tris (pH 7.5)
	1.4 M NaCl
	0.02 M EDTA (pH 8.0)
	20 g CTAB
Washing Solution I	76% Ethanol (v/v)
	0.2 M Ammoniumacetat
Washing Solution II	76% Ethanol (v/v)
	10 mM Ammoniumacetat
TE buffer	0.01 M Tris-HCl (pH 8.0)
	1 mM EDTA (pH 8.0)

2.2 Isolation of total RNA

Total RNA was isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Each step was describe as following:

1) Plant tissue is grounded in liquid nitrogen and placed into a 2 ml tube.
2) Add 1 ml TRIzol® Reagent. The samples are intensively vortexed for 5 min and incubated at room temperature for 5 min.
3) Add 200 µl Chloroform into each tube and shake tube vigorously by hand or vortex for 30 s. Then incubate for 2 min at room temperature.
4) Centrifuge the samples at 12,000 rpm for 15 min at 4 °C. Then remove the aqueous phase (normally 500 µl) into a new 1.5 ml tube.
5) Add 500 µl Isopropanol into the new tube and softly invert. The samples are incubated at 4 °C for 1 h.
6) Centrifuge the samples at 12,000 rpm for 15 min at 4 °C.
7) The supernatant is removed and the pellets are carefully washed in 80% DEPC-ethanol and 100% ethanol, respectively.
8) The pellets are dried and dissolved in 50 µl DEPC-H_2O. The quality of RNA is controlled by using of NanoVue (GE Healthcare).

2.3 Plasmid isolation

Plasmid isolation from bacterial cells is performed in the cloning experiments. Bacterial strains used for plasmid isolation are usually grown in the appropriate sterile liquid culture overnight before performing the protocol. Each step was describe as following:

1) Cultivate and harvest bacterial cells. Use 1-5 ml of a saturated bacteria LB culture, and pellet cells in a centrifuge for 30 s at 11,000 x g. Discard the supernatant and remove as much of the liquid as possible.
2) Cell lysis. Add 250 µl Resuspension Buffer A1. Resuspend the cell pellet completely by vortexing or pipetting up and down.

3) Add 250 µl Lysis Buffer A2. Mix gently by inverting the tube 6-8 times. Do not vortex to avoid shearing of genomic DNA. Incubate at room temperature for up to 5 min until lysate appears clear.
4) Add 300 µl Neutralization Buffer A3. Mix thoroughly by inverting the tube 6-8 times until blue samples turn colorless completely.
5) Clarification of lysate. Centrifuge for 5 min at 11,000 x g at room temperature.
6) Bind DNA. Place a column in a collection tube (2 ml) and pipette a maximum of 700 µl of the supernatant onto the column. Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the column back into the collection tube.
7) Wash silica membrane. Add 600 µl Wash Buffer A4. Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place column back into the empty collection tube.
8) Dry silica membrane. Centrifuge for 2 min at 11,000 x g and discard the collection tube.
9) Elute DNA. Place the column in a 1.5 ml tube and add 50 µl Elution Buffer AE. Incubate for 1 min at room temperature. Centrifuge for 1 min at 11,000 x g. Samples were stored at 4 °C for short term storage and for long term storage at -20 °C.
10) For plasmid quality control, 1:10 diluted samples (2 µl of the sample in the dilution) were run in a 1% agarose gel at 100 V for 30 min. Isolated plasmid samples could be used for cloning, sequencing, PCR, transformation, restriction analysis.

2.4 Reverse Transcription (cDNA Synthesis)

For cDNA synthesis of genes, 1 µg total RNA treated by DNase I (Table 2) in 20 µl volume using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) at 42 °C in 1 h (Table 3).

For cDNA synthesis of miRNAs, 1 µg total RNA treated by DNase I (Table 2) was used for further step using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Mature miRNAs expression analysis was performed by stem-loop RT-PCR described by Chen et al. (2005). The mature miRNA sequence appended with two adenines on the 3’ end was used as forward primer sequence to ensure correct binding of the primer to the poly(T) region of the mature miRNA cDNA and preclude potential binding to the miRNA precursor. Reaction mix, including DNase I treated RNA and cDNA synthesis RT primer, was incubated at 65 °C for 5 min (Table 4). Chilled on ice,

spun down and placed back on ice. Above product from each sample was reversely transcribed with reagent provided according to the manufacturer's instructions (Table 5). The transcript of miRNA was quantified using a forward primer specific to miRNA precursor and the universal reverse primer. *U6* small nuclear RNA was used as an internal control for miRNA gene expression normalization.

Table 2 Reaction composition of DNA digestion for gene

Reagent	**Volume**
Total RNA (< 1 µg)	5 µl
10× DNase Buffer	1 µl
DNase I	1 µl
ddH_2O	3 µl
Total	10 µl

Table 3 Reaction composition of cDNA synthesis of gene

Reagent	**Volume**
DNase treated RNA (< 1 µg)	10 µl
5X Reaction buffer	4 µl
10 mM dNTP mix	2 µl
Ribolock RNase inhibitor (20 U/µl)	1 µl
RevertAid M-MuLV RT (200 U/µl)	1 µl
Oligo $(dT)_{18}$ primer	1 µl
ddH_2O	1 µl
Total	20 µl

Table 4 Reaction composition of cDNA synthesis of miRNA (I)

Reagent	**Volume**
DNase treated RNA (< 1 µg)	5 µl
cDNA synthesis RT primer	1 µl
ddH_2O	6 µl
Total	12 µl

Table 5 Reaction composition of cDNA synthesis of miRNA (II)

Reagent	Volume
Above product	12 µl
5X Reaction buffer	4 µl
10 mM dNTP mix	2 µl
Ribolock RNase inhibitor (20 U/µl)	1 µl
RevertAid M-MuLV RT (200 U/µl)	1 µl
Total	20 µl

2.5 Expression analysis of genes and miRNAs by real time PCR

For expression analysis of genes, 1 µl of 1:1 cDNA dilution of the synthesized cDNA were used for the gene transcripts by RT-qPCR (Table 6). RT-qPCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (2X) (VWR). Real-time PCR was performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following program: 10 min 95 °C; 40 × 15 s 95 °C, 30 s 59 °C, 30 s 72 °C; 10 s 95 °C, melting curve from 65 °C to 95 °C. Normalization of the expression levels was carried out using *Actin2* (AT3G18780) as internal reference gene.

For expression analysis of miRNAs, 1 µl of 1:20 and 1:5 cDNA dilutions for miRNAs reference gene *U6* and mature miRNAs, respectively, were used. RT-qPCR was carried out using Maxima SYBR Green/ROX qPCR Master Mix (2X) (VWR). The PCR reactions were performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the following program: 10 min 95 °C; 50 × 10 s 95 °C, 10 s 59 °C, 10 s 72 °C; 10 s 95 °C, melting curve from 65 °C to 95 °C. The PCR products were then exposed to a temperature ramp to generate the dissociation curves and determine amplification specificity. PCR products were checked by 12% polyacrylamide gel electrophoresis (PAGE) (Table 7) and defined by 10 bp DNA ladder (Thermo Fisher Scientific).

The relative expression change folds of miRNAs and related genes were both calculated using comparative Ct method (Livak and Schmittgen, 2001). Statistical analysis was carried out using a 2-way ANOVA or Student's *T*-test according to Minitab software (MINITAB, 2000).

Table 6 Reaction composition of RT-qPCR

Reagent	Volume
Maxima SYBR Green/ROX qPCR Master Mix (2X)	5 µl
Forward primer (1 mM)	1 µl
Reverse primer (1 mM)	1 µl
Diluted cDNA	1 µl
ddH_2O	2 µl
Total	10 µl

Table 7 Composition of polyacrylamide gel electrophoresis (PAGE)

Composition	Volume
40% acrylamide	7.5 ml
10x TBE	2 ml
10% APS	120 µl
TEMED	9.2 µl
DEPC-H2O	2 µl
Total	20 ml

2.6 RLM-5' RACE

5´-rapid amplification of cDNA ends (RACE) was carried out for analysis of target genes of miRNAs using the GeneRacer™ Kit (Invitrogen) following the manufacturer´s instruction with 2 µg total RNA. The first both steps of dephosphorylating RNA and removing of mRNA cap structure were ignored and started with ligation of the RNA oligo to the mRNA followed by the reverse transcription of mRNA by using the oligo $(dT)_{18}$ primers. For amplifying cDNA ends, gene specific primer was designed. Each step was described as following:

1) Ligate the RNA oligo to mRNA. Add 2 µg RNA to the tube containing the prealiquoted, lyophilized GeneRacer™ RNA Oligo. Centrifuged briefly to collect the fluid in the bottom of the tube. Add the reagents in to the tube and mixed gently (Table 8). Incubated at 37 °C for 1 h and centrifuged briefly.
2) Add the following reagents in to the RNA oligo ligation tube and mixed gently: 50 µl Chloroform/Isoamylalcohol (24:1), 50 µl Phenol and 90 µl DEPC-H_2O. Vortex and centrifuge at 14,000 rpm for 10 min at room temperature.

3) Transferred supernatant (~120 µl) to a new tube add the same amount Chloroform/Isoamylalcohol (24:1). Vortex and centrifuge at 14,000 rpm for 10 min at room temperature.
4) Transfer supernatant (~100 µl) to a new tube add 1/10 Volume 3M NaAc and 2.5 Volume 100% ethanol, and mix and incubate overnight at -20 °C.
5) Centrifuge at 14,000 rpm for 20 min at 4 °C, and wash the pellet with 70% ethanol. Centrifuge at 14,000 rpm for 4 min at 4 °C, and resuspend the pellet in 12 µl DEPC-H_2O.
6) 12 µl above RNA to perform cDNA synthesis according to the manufacturers recommendation by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific).
7) Touchdown PCR for amplifying cDNA ends (Table 9). Temperature cycling conditions for touchdown PCR were as follows: 3 min at 95 °C, followed by 5 cycles of 30 s at 95 °C and 30 s at 70 °C, 5 cycles of 30 s at 95 °C and 30 s at 68 °C, and 25 cycles of 30 s at 95 °C, 30 s at 65 °C and 30 s at 72 °C and finally 10 min at 72 °C.
8) One µl of this initial touchdown PCR was template for the following nested PCR (Table 10). Touchdown PCR program is: 3 min at 95 °C, followed by 5 cycles of 30 s at 95 °C, 30 s at 64 °C and 30 s 72 °C, 5 cycles of 30 s at 95 °C, 30 s at 62 °C and 30 s at 68 °C, and 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C and finally 10 min at 72 °C.
9) The reaction products were separated on a 1.2% agarose gel, purified with the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and cloned into the pGEM®-T Vector Systems (Promega) by heatshock for sequencing.

Table 8 Reaction composition of RNA oligo ligation

Reagent	Volume
RNA Oligo (250 ng/µl)	1 µl
Template RNA	2 µg
10x T4 Ligase buffer	1 µl
ATP (10 mM)	1 µl
T4 RNA liagase (10 U/µl)	1 µl
DEPC-H_2O	To 11 µl

Table 9 Reaction composition of RACE PCR

Reagent	Volume
Gene Racer 5' primer (10 mM)	3 µl
Reverse Gene Specific primer (10 mM)	1 µl
RT template	1 µl
10x DreamTaq buffer	5 µl
dNTP (10 mM)	1 µl
DreamTaq DNA Polymerase (5 U/µl)	0.5 µl
H_2O	38.5 µl
Total	50 µl

Table 10 Reaction composition of RACE nested PCR

Reagent	Volume
Gene Racer 5' nest primer (10 mM)	1 µl
Reverse Nested Gene Specific primer (10 mM)	1 µl
Initial PCR template	1 µl
10x DreamTaq buffer	5 µl
dNTP (10 mM)	1 µl
DreamTaq DNA Polymerase (5 U/µl)	0.5 µl
H_2O	40.5 µl
Total	50 µl

2.7 Vector preparation via gateway clone GUS constructs

The GUS constructs of miR858, miR828, MYB111 and MYB75 were prepared by Gateway cloning using the Gateway™ BP Clonase™ II Enzyme mix (Invitrogen) and Gateway™ LR Clonase™ II Enzyme mix (Invitrogen). Each step was describe as following:

1) Bacterial strain (DH5α) containing the vectors are used in gateway cloning (pDONR201, pGWB series vectors) were first isolated from the overnight stock cultures.

2) gDNA is used to amplify the desired inserts for cloning using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) (Table 11).
3) PCR reactions for all the desired inserts were A-tailed by DreamTaq Polymerase (Table 12) and ligated into the pGEM®-T Vector Systems (Promega), according to the manufacturer's description (Table 13). Incubated at 16 °C overnight.
4) T-Vectors containing the inserts are used as templates for the gateway PCR. Phusion High-Fidelity DNA Polymerase is used for the amplification. The inserts of interest are firstly amplified using primers containing *att*B attachment site from the Gateway™ BP Clonase™ II Enzyme mix (Invitrogen) (Table 14).
5) In a 1.5 ml tube, BP recombination reaction is performed. An *att*B-flanked DNA fragment and an *att*P-containing donor vector (pDONR201) are used to generate an entry clone. BP Clonase™ enzyme is mixed briefly then 2 µl is added into the tube and mixed well by vortexing briefly twice (Table 15). Incubate at 25 °C overnight.
6) 1 µl of 2 µg/µl Proteinase K solution is added to the mix and incubate at 37 °C for 10 min to stop the reaction.
7) Competent *E. coli* DH5α is transformed by heat-shock method and selected for kanamycin resistant entry clones.
8) Plasmid isolation (mentioned in 2.3) from the positive clones which are further confirmed by PCR using vector primers.
9) LR recombination reaction is performed (Table 16). Selected positive entry clone is mixed with 1 µl of the desired destination vectors (pGWB433, GUS gene under the control of interest genes/miRNAs upstream promoter region, $Spec^R$ + Kan^R). LR Clonase™ enzyme is mixed briefly then 2 µl is added into the tube and mixed well by vortexing briefly twice. Incubate at 25 °C overnight.
10) 1 µl of 2 µg/µl Proteinase K solution is added to the mix and incubate at 37 °C for 10 min to stop the reaction.
11) Competent *E. coli* DH5α is transformed by heat-shock method and selected for kanamycin and spectinomycin resistant destination clones.
12) Plasmid isolation (mentioned in 2.3) from the positive clones which are further confirmed by PCR using vector primers.

13) The destination vector containing the desired fragment is then used to transform *A. tumefaciens* (GV3101).
14) Positive clones are used to transform Arabidopsis (Col-0) plants by floral dip method.

Table 11 Reaction composition of PCR (Phusion High-Fidelity DNA Polymerase)

Reagent	**Volume**
5x Phusion HF Buffer	4 µl
Forward primer (1 mM)	1 µl
Reverse primer (1 mM)	1 µl
Template gDNA	2 µl
dNTP (10 mM)	0.4 µl
Phusion DNA Polymerase (2 U/µl)	0.2 µl
H_2O	11.4 µl
Total	20 µl

Table 12 Reaction composition of A-tailing

Reagent	**Volume**
PCR product	7.8 µl
10x DreamTaq buffer	1 µl
dATP (10 mM)	0.2 µl
DreamTaq DNA Polymerase (5 U/µl)	1 µl
Total	10 µl

Table 13 Reaction composition of T-clone

Reagent	**Volume**
2x Rapid Ligation Buffer	5 µl
pGEM®-T Easy Vector	1 µl
DNA Ligase	1 µl
PCR product (50 ng/µl)	3 µl
Total	20 µl

Table 14 Reaction composition of gateway PCR

Reagent	**Volume**
10x DreamTaq buffer	2 µl
*att*B forward primer (1 mM)	1 µl
*att*B reverse primer (1 mM)	1 µl
PCR product	2 µl
dNTP (10 mM)	0.4 µl
DreamTaq DNA Polymerase (5 U/µl)	0.1 µl
H_2O	13.5 µl
Total	20 µl

Table 15 Reaction composition of BP reaction for gateway clone

Reagent	Volume
*att*B-PCR product (50 ng/µl)	3 µl
pDONR201 (supercoiled, 150 ng/µl)	1 µl
BP Clonase™ enzyme	2 µl
H_2O	4 µl
Total	10 µl

Table 16 Reaction composition of LR reaction for gateway clone

Reagent	Volume
Positive entry clone (100 ng/µl)	3 µl
pGWB433 (150 ng/µl)	1 µl
LR Clonase™ enzyme	2 µl
H_2O	4 µl
Total	10 µl

2.8 Floral dip transformation of *Arabidopsis thaliana*

Floral dip (Clough and Bent, 1998) was used to generate transgenic Arabidopsis expressing interest miRNAs and genes. Grow Plants in pots, 4 plants per pot, 6 pots for each transformation in short daylight for 5-6 weeks until good rosette growth. Transfer to long daylight to induce flowering. Arabidopsis plants with numerous immature buds and few siliques are used for inoculation. Each step was describe as following:

1) Inoculate 4 ml for each strain from -80 °C stock and incubate overnight at 28 °C in 200 rpm shaker.
2) Inoculate 100 ml with 100 µl overnight cultures and incubate again overnight at 28 °C in 200 rpm shaker.
3) Spin down 100 ml of the cultures in two 50 ml Falcon tubes at 5,000 rpm for 10 min at room temperature.
4) Resuspend each Falcon tube with 30 ml 5% sucrose solution.
5) Add 60 µl Silwet Gold to 240 ml 5% sucrose solution and mix with resuspended *Agrobacteria* in a 2.5-liter beaker.

6) Invert plant pot and place into beaker ensuring all flowerheads are submerged. Leave for 10 s and agitate slightly to release air bubbles. Remove plants and then repeat dipping as before.
7) Remove excess inoculum by shaking onto paper roll so flowerheads are well separated.
8) After infiltration, plants are kept in the darkness, out of sunlight for two days to provide moist conditions for the bacterial propagation.
9) Transfer to long daylight in green house to set seeds. Flowering stems are covered from the paper bag once the pods emerge to prevent shattering of the seeds.
10) Seeds are allowed to dry on plants. Stems harboring the seed bags are removed from the plants for seed collection.

2.9 Seed sterilization and germination

1) Seeds are disinfected using 5% $Ca(ClO)_2$ for 5 min.
2) Add 1 ml of 70% EtOH and vortex for 3 min.
3) Seeds are washed twice with the sterilized distilled water. Remaining water is removed from the seeds by passing them through the sterilized filter paper.
4) Seeds are allowed to dry on the filter paper. Dried seeds are stored at 4 °C in a sterile small petri dish sealed by parafilm and labeled according to the corresponding insert.
5) Single seeds are carefully spread on ½ MS medium plates (Murashige and Skoog, 1962) contained kanamycin for selection of transgenic seedlings.
6) After one week, only the seedlings growing successfully in the presence of antibiotic on ½ MS plates are carefully transferred to the soil in small pots. Pots are covered with plastic foil to keep the small seedling safe from external stress. Seedlings are allowed to grow, first under short day conditions. After the formation of sufficient rosette, transfer plants under long daylight conditions to set seeds.

2.10 Infiltration of *Nicotiana benthamiana* for transient expression via *Agrobacterium*

Transient expression in tobacco plant (*Nicotiana benthamiana*) was used to determine the subcellular location of a protein of interest when tagged with a reporter such as GUS gene. The root tumor bacteria, *Agrobacteria* strain GV3101, were used to introduce the target gene

expression cassette into benthamiana mesophyll cells (Li, 2011). Each step was describe as following:

1) Inoculate one single colony of *Agrobacterium* in 5 ml LB with appropriate antibiotics. Grow overnight at 28 °C.
2) Use 100 µl of the overnight culture to inoculate 25 ml LB medium (with same antibiotics, plus 20 µM acetosyringone should be added after autoclaving and immediately before use) and grow overnight.
3) Precipitate the bacteria (5,000 x g, 15 min), resuspend the pellet in Infiltration Solution (Table 17). Leave on the bench (room temperature) for overnight before infiltration. The final A_{600} should be adjusted to 0.4.
4) Prepare dilution 1:1 of Infiltration Solution for working solution, including miR828 overexpression suspension solution and Infiltration Solution, TS1 suspension solution and Infiltration Solution, TS2 suspension solution and Infiltration Solution, MT1 suspension solution and Infiltration Solution, MT2 suspension solution and Infiltration Solution, miR828 overexpression suspension solution and TS1 suspension solution, miR828 overexpression suspension solution and TS2 suspension solution, MT1 suspension solution and TS1 suspension solution, as well as MT2 suspension solution and TS2 suspension solution.
5) Perform the infiltration with 5 ml syringe for each combination. Simple press the syringe (no needle) on the underside of the leaf, and exert a counter-pressure with finger on the other side. Successful infiltration is often observed as a spreading “wetting” area in the leaf.
6) After 3 days, 10 leaf discs per plant with a diameter of 3 cm are punched out and collected in 50 ml Falcon tubes. Add 5 ml 90% Acetone solution at 4 °C for overnight.
7) Discard Acetone solution. Add PBS buffer (Table 18) to wash for twice.
8) GUS staining.

Table 17 Composition of Infiltration Solution

Composition	**Amount**
$MgCl_2$	10 mM
MES-K (pH 5.6)	10 mM

Acetosyringone (fresh)	100 µM

Table 18 Composition of PBS Solution

Composition	**Amount**
NaCl	127 mM
KCl	2.7 mM
Na_2HPO_4 (pH 7.4)	10 mM
KH_2PO_4	2 mM

2.11 GUS staining

Leaves from the transformed plants or infiltration of tobacco were gently removed and kept in 2 ml or 50 ml Falcon tubes for GUS testing. Interest insertions contain GUS gene which will constitutively express and produce β-glucuronidase only in the successfully transformed. Each step was describe as following:

1) X-Gluc solution (Table 19) was added in the tubes according to the size of the leaves.
2) Leaves immersed in the X-Gluc solution were incubated at 37 °C for 45 min to overnight.
3) Leaves were taken out of the X-Gluc solution and placed in 70% ethanol for chlorophyll extraction making it easier to observe the blue staining.

Table 19 Composition of X-Gluc solution

Composition	**Volume**
X-Gluc (0.1 g/ml DMSO)	300 µl
NaH_2PO_4 (0.2 M, pH 7.0)	38 ml
Na_2HPO_4 (0.2 M, pH 7.0)	62 ml
Triton 100	1 drop
Total	~100 ml

2.12 Bacteria cultivation

Three strains of *Pseudomonas syringae* were applied in this experiment. Details of each strain listed in the Table 20. Three strains of *Pseudomonas syringae* DC3000, *hrpL*, *hrcQ* were cultured on NYGA solid medium. Those three strains of bacteria were grown in NYGA solid with different

antibiotics as following, for DC3000 (50 mg/l kanamycin and 50 mg/l rifampicin), for *hrpL* (100 mg/l kanamycin), and for *hrcQ* (50 mg/l spectinomycin).

Table 20 Overview of *Pseudomonas syringae* strains used in this study

***Pseudomonas syringae* strains**	**Description**
DC3000	Wild-type, control
hrpL	Mutant lacks on its transcriptional factor for T3SS genes
hrcQ	T3SS-deficient-mutant, not able to translocate its effectors

2.13 Infection system

1) *Pseudomonas syringae* inoculum is prepared by picking one single colony from the stock plate and grown them in NYGA medium with the appropriate antibiotic in 28 °C for 2 days.
2) On the following day, the bacterial suspensions are made by adding 5 ml of 10 mM $MgCl_2$ into the plate contains fresh bacteria.
3) After scrapping out the bacteria and mixing them well, the suspension is transferred into a 15 ml tube and centrifuged at 5,000 rpm for 5 min.
4) Discard the supernatant and the bacterial pellet is resuspended with $MgCl_2$. The concentration of the bacterial suspension is determined by using a spectrophotometer at OD_{600} = 0.2, resulted a dense bacterial suspension containing approximately 1 x 10^8 colony forming units/ml (cfu/ml).
5) After addition of 0.04% Silwet Gold the suspension is mixed thoroughly, of which 30-40 ml are sprayed onto the plants until all leaves are moistened. The plants are covered with a plastic lid.
6) 4 days after infection, 3 leaf discs per plant with a diameter of 6 mm are punched out and shaked for 1 h in 500 µl 10 mM $MgCl_2$ with 0.01% Silwet Gold.
7) After that, a 1:10 serial dilution from 10^{-1} to 10^{-5} is prepare with 10 mM $MgCl_2$ in 96-well plates. Of each dilution 10 µl are plated on NYGA plates with appropriate antibiotics and incubated at 28 °C for 2 days.
8) Estimating the density of the bacteria would give a clear distinction in each strain symptom development in Arabidopsis leaf. The colony number of each dilution is counted and the average number of colony forming units (cfu) per cm^2 leaf area is calculated. The

quantification of *Pst* growth could be done by a DNA-based method such as quantitative real-time PCR as well.

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Declaration

Herewith, I declare that the work is my genuine work and nothing but the references and tools mentioned in my thesis have been used to conduct this work.

The submitted written version of this work corresponds to the one on the electronic storage medium.

Besides this, I declare that the thesis had not been submitted elsewhere in an examination procedure.

Kiel, den 4. Dec. 2019

Zheng Zhou

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