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SHAM TO DECEIVE (STD), A FUNGAL EFFECTOR AND ITS FUNCTIONAL STUDIES

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PREFACE

The following thesis focuses on the functional studies of Mlp124478, a poplar leaf rust effector. The study described here will contribute to the functional genomic studies to unravel the interaction between effectors and their targets.

The main body of the thesis consists of six different chapters: introduction, materials and methods, results, discussion, conclusion and references. The first chapter starts with the general introduction of poplar leaf rust *M. larici-populina*, its sequenced genome, preliminary studies on genomics and transcriptomics, candidate effector discovery, importance of heterologous model systems in plant-pathogen interaction studies, and subcellular localization of effectors inside plant tissues.

Since Duplessis et al (2011) published the whole genome sequence of *M. larici-populina*, it gave an access to further study the candidate effector proteins at molecular level and investigate their role in pathogenesis. We chose an effector, Mlp124478 for the functional genomic studies. To this end, we used *in planta* pathogen assays, genotyping, live-cell imaging, comparative transcriptomics, protein-nucleic acid interaction and yeast two-hybrid assay to infer the functional nature of Mlp124478. The detailed materials and methods are discussed in chapter two. In the third chapter, we explained our findings, and discuss on those findings in chapter four. To our knowledge this is the first attempt at using transcriptomics of transgenic *Arabidopsis* plants expressing rust fungal effector (*M. larici populina* effector) to understand the expression pattern of genes differentially expressed in presence of an effector.

We published a review on relationship between virulence function of effectors and their subcellular accumulation in the host cells in the journal "Virulence" which is presented in Annex B. I carried out two additional research projects, (I) localization of *Arabidopsis* TAF15b and its role in plant immunity, and (II) nuclear protein components participating in MAMP-triggered immunity. The manuscripts were published in

Molecular Plant-Microbe Interactions (MPMI) and Plant Signaling and Behavior, respectively, which are presented in Annex C and D. The manuscript describing my doctoral research on Mlp124478 is to be submitted to "Nature Scientific Reports" and is presented in Annex E.

In conclusion, the studies presented in the following thesis provide novel insights into Mlp124478 functions in the plant, such as target in the host cell, remodelling host transcription process to alter plant gene expression for the benefit of the pathogen.

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RÉSUMÉ

La rouille du peuplier est causée par le basidiomycète *Melampsora larici-populina,* qui infecte les tissus des feuilles et y sécrètent, via des structures d'alimentation spécialisées appelées haustoriums, des protéines effectrices, aussi nommés effecteurs. Les mécanismes par lesquels les effecteurs de la rouille du peuplier favorisent la virulence du pathogène sont mal connus. L'effecteur Mlp124478 a été sélectionné comme sujet d'étude de cette thèse parmi un groupe de 1184 petites protéines sécrétées par *M.a larici-populina.* Le gène codant pour Mlp124478 appartient à la famille CPG2811, qui comporte neuf membres qui sont spécifiques à l'ordre des Pucciniales. Son expression est fortement augmentée lors de l'infection et peut atteindre des niveaux 50 fois plus élevés que le niveau basal 96 h après le début de l'infection, un moment qui correspond, selon la cinétique d'infection par *M. larici-populina,* au stade de croissance biotrophique à l'intérieur des cellules du mésophylle. Mlp124478 est le seul membre de sa famille à posséder une séquence prédite de localisation nucléolaire (NoLS).

Nous avons utilisé les plantes modèles *Arabidopsis thaliana* et *Nicotiana benthamiana* pour déterminer que Mlp124478 se retrouve principalement dans les noyaux et dans les nucléoles et qu'il favorise la croissance de l'oomycète pathogène *H. arabidopsidis*. L'expression constitutive de Mlp124478 chez *A. thaliana* altère la morphologie des feuilles de rosette, qui acquièrent une apparence ondulée, et diminue l'expression des gènes impliqués dans la réponse immunitaire. Nos résultats indiquent en effet que les gènes surexprimés en présence de Mlp124478 ne sont pas des gènes spécifiquement impliqués dans défense des plantes mais que ces derniers sont plutôt parmi les plus sous-exprimés. En somme, nos résultats suggèrent que Mlp124478 manipule les plantes en ciblant les compartiments nucléaires, où il altère la transcription par sa liaison au promoteur TGA1a afin de supprimer la réponse transcriptionnelle à l'exposition aux agents pathogènes et d'augmenter l'expression de gènes qui ne sont pas impliqués dans les réponses de défense. Étant donné l'effet trompeur que cet effecteur

exerce sur l'immunité et la réponse aux pathogènes, nous proposons de le renommer SHAM TO DECEIVE (STD) et c'est ce nom qui sera utilisé pour cette thèse. Nos résultats actuels concordent avec la présence de STD dans le noyau, nous ne pouvons cependant pas écarter que cet effecteur ait une fonction distincte dans le nucléole.

À notre connaissance, il s'agit de la première utilisation de données de transcriptomique obtenues à partir de plants d'*A. thaliana* transgéniques exprimant un effecteur de *Melampsora-larici populina* pour étudier les patrons d'expression différentielle. Cette étude transcriptomique a le potentiel d'augmenter considérablement nos connaissances sur les patrons de régulation de l'expression des gènes en réponse aux pathogènes biotrophes, et n'aurait pas pu être réalisée en utilisant le modèle d'expression transitoire dans *N. benthamiana*.

ABSTRACT

The basidiomycete *Melampsora larici-populina* causes poplar leaf rust, invades leaf tissue and secretes effector proteins through specialized feeding structures known as haustoria. The mechanisms by which rust effectors promote pathogen virulence are poorly understood. Out of 1184 small secreted proteins, Mlp124478 has been chosen in this study, it belongs to the CPG2811 gene family which has 9 members specific to the Pucciniales. *Mlp124478* expression is strongly enhanced during infection and reaches 50-fold induction at 96 h after infection. Given the kinetics of *M. larici-populina* infection, this corresponds to the biotrophic growth stage in mesophyll cells. Mlp124478 is the only one in its family which contained predicted nucleolar localization sequence (NoLS).

We investigated the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* and established that Mlp124478 accumulates in the nucleus and nucleolus, and promotes growth of the oomycete pathogen *Hyaloperonospora arabidopsidis*. Stable constitutive expression of *Mlp124478* in *A. thaliana* altered leaf morphology, observed through increased waviness of rosette leaves and repressed expression of genes involved in immune responses. Our results indicate that the list of up-regulated genes did not specifically contain genes involved in plant defence, but rather genes involved in defence were among the most repressed. Taken together, our results suggest that Mlp124478 manipulates plants by targeting nuclear compartments, and remodeling transcription via binding to TGA1a promoter to suppress the transcriptional response to pathogens, and mislead the host into up-regulating the expression of genes unrelated to defence. Therefore, we suggest to rename (as use thereafter) this gene as *SHAM TO DECEIVE (STD)*, to reflect its effect on plant immunity. While our current results are consistent with STD in the nucleus, we cannot rule out that it could also have a distinct function in the nucleolus.

To our knowledge, this is the first attempts at using transcriptomics of transgenic *Arabidopsis* plants expressing *M.-larici populina* effector to understand the expression pattern of genes differentially expressed in presence of effector. Since Mlp124478 accumulates in the nucleus and nucleolus, the study of transcriptome of transgenic plants over-expressing effector might boost our understanding of the regulatory pattern of gene expression in response to biotrophs, which could not as easily be performed using transient expression in *N. benthamiana*.

Keywords: Mlp124478, effectors, nucleolus, biotroph, transcription, transcription factor-binding sites

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LIST OF ABBREVIATIONS AND ACRONYMS

ABRC	Arabidopsis Biological Resource Center
ALSD	Almond leaf scorch disease
ARL	*ARGOS-like*
AtMLO12	*MILDEW RESISTANCE LOCUS O 12*
AVR	Avirulent
bZIP	Basic region/leucine zipper motif
CAZys	Carbohydrate active enzymes
CC	Coiled-Coil
CC-NBS-LRR	Coiled-coil Nucleotide-Binding Site Leucine-Rich Repeat
cfu	Colony forming unit
CgEP1	*Colletotrichum graminicola* Effector Protein 1
ChIP	Chromatin Immunoprecipitation Assay
ChIP-PCR	Chromatin immunoprecipitation-polymerase chain reaction
CoIP	Coimmunoprecipitation
CRK21	Cysteine-rich receptor-like protein kinase 21
CRN	Crinkler
CSEPs	Candidate secreted effector proteins
CVC	Citrus variegated chlorosis
DIG	Digoxigenin
Dpi	Days post infiltration
DS	Double-stranded
EDS1	*ENHANCED DISEASE SUSCEPTIBILITY* 1/ /
eds1-1	*Enhanced disease susceptibility 1-1*

EHM	Extrahaustorial matrix
EM	Extrahaustorial membrane
EMSA	Electrophoretic mobility shift assay
ESM1	Epithiospecifier modifier 1
EST	Expressed sequence tag
ETI	Effector-Triggered Immunity
ETS	Effector-triggered susceptibility
GFP	Green Fluorescent Protein
GO	Gene Ontology
H. a Noco2	*Hyaloperonospora arabidopsidis* Noco2
HD-ZIP	Homeodomains associated with the Leucine Zipper
HESPs	Haustorially Expressed Secreted Proteins
HMG	High mobility group
HR	Hypersensitive response
HSP1	Heat shock protein 20 like protein 1
IBIS	Institut de Biologie Integrative et des Systems
I_N	Nuclear intensity
I_{No}	Nnucleolar intensity
IP	Immunoprecipitated
K	Lysine
LB	Luria-Bertani
LCM	Laser Capture Microdissection
LRRs	Leucine Rich Repeats
MAMP	Microbes associated molecular pattern
MAPK	Mitogen-Activated Protein Kinases

Mlp	*Melampsora larici-populina*
MS	Mass Spectrometry
N	Nnucleus
NBS-LRR	Nucleotide-Binding Site Leucine-Rich Repeat
NDR1	*NONRACE SPECIFIC DISEASE RESISTANCE* 1
NLS	Nuclear Localization Signal
No	Nucleolus/nucleoli
NoLS	Nucleolar Localization Sequnce
NPR1	*NONEXPRESSOR OF PR-1*
NTP	NAC transcription factors
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PAD4	*PHYTOALEXIN DEFICIENT* 4
PAMP	Pathogen associated molecular pattern
PCD	Programmed cell death
PD	Pierce's disease of grapevine
PRR	Pathogen recognition receptors
*Pst*DC3000ΔCEL	*Pseudomonas syringae* pv. *tomato* strain DC3000ΔCEL
pth	*P*athogenicity gene
PTI	PAMP-Triggered Immunity
R	Arginine
R	Resistance
RFP	Red Fluorescent Protein
RIN4	RPM1 Interacting Protein 4
RLKs	Receptor-Like Kinases

RLPS	Receptor-Like Proteins
ROS	Reactive Oxygen Species
RT	Room temperature
SA	Salicylic acid
SAG101	*SENESCENCE ASSOCIATED GENES* 101
SAP11	Secreted AY-WB Protein 11
SLH1	*SENSITIVE TO LOW HUMIDITY* 1
snc1	Suppressor of npr1
SPs	Secretory proteins
SSPs	Small secreted protein
T3SS	Type three secretion system
TAL	Transcription Activator-Like
TCP	Teosinte branched 1/Cycloidea/Proliferating
TFBSs	Transcription factor-binding sites
TFs	Transcription factors
TIR	Toll/Interleukin-1 Receptor
TIR-NBS-LRR	Toll/Interleukin-1 Receptor Nucleotide-Binding Site Leucine-Rich Repeat
TSS	Transcription start site
Y2H	Yeast two-hybrid
YEP	Yeast extract peptone

CHAPTER I

INTRODUCTION

1.1 Plant Pathogens-constraint to world food supply

Plants provide world's food supply as well as fuel, shelter, medicine and transportation. Like animals, plants are under continuous attack of pathogens impending threat to food security. Plant pathogens belongs to various taxa such as nematodes (Jones et al. 2013), virus (Scholthof et al. 2011), bacteria (Mansfield et al. 2012), oomycetes (Kamoun et al. 2015), fungi (Dean et al. 2012), viroids (Owens and Hammond 2009) and parasitic plants (Hibberd and Dieter Jeschke 2001). Plant diseases are threat to world's crop production. Among food crops, disease epidemics have driven to famines and huge migrations over the history, such as Irish potato famine of 1845-1849. Since potato was the main crop in Ireland, *Phytophthora infestans* the causal agent of potato blight disease created extensive damages throughout Ireland. As a result of the disaster, approximately 1 million Irish people died, 1.3 million people migrated and 300,000 births did not take place (Boyle and Grádo 1986). However, fundamental components of plant disease epidemics comprise abundance and susceptibility of crops, abundance and virulence of pathogen and favorable environments (Fig. 1.1) (Francl 2001). Decline of pathogenic inoculum, suppressing pathogen virulence and increasing of crop genetic assortment may minimize plant diseases which eventually sustain our steady food supply (Strange and Scott 2005). Hence, molecular understanding of pathogenicity, disease resistance and plant-pathogen interactions are indispensable to control plant pathogens and retain plants healthy for food security.

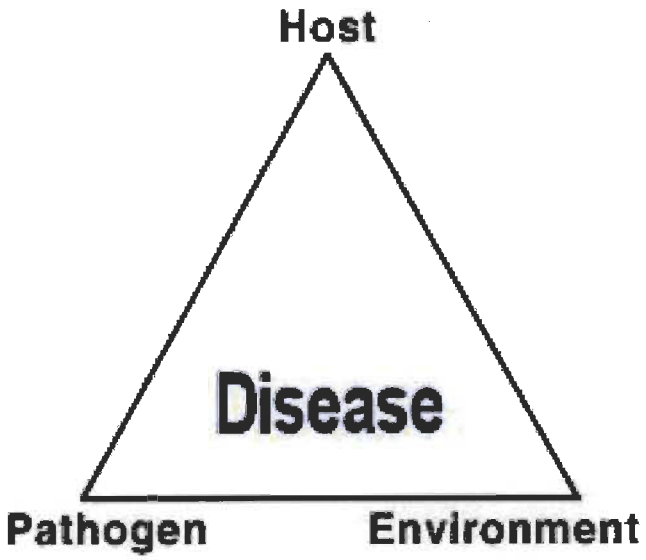


Fig. 1.1 Disease triangle.
Three causal factors are necessary for causing disease: host, pathogen and environment. Successful disease development requires the presenc of a biotic agent with an interaction of a susceptible host, a virulent pathogen and favourable environment. On the other hand, upon the exclusion of interaction of any of these three component plant disease is prevented. (Adapted from Stevens, 1960.)

1.2 Plant defence systems

Plants are constantly challenged by their surrounding environments such as biotic (virus, bacteria, oomycetes, fungi, nematodes, etc.) and abiotic factors (drought, temperature, nutrient deficiency, lack of oxygen, UV radiation or pollution). Plants have a wide array of defence mechanisms which allows them to fight against diverse biotic and abiotic stresses (de las Mercedes Dana, Pintor-Toro and Cubero 2006, Guest and Brown 1997). Plants have evolved an amazingly rich array of defence mechanisms, which include both passive and active mechanisms, to respond to biotic and abiotic stresses.

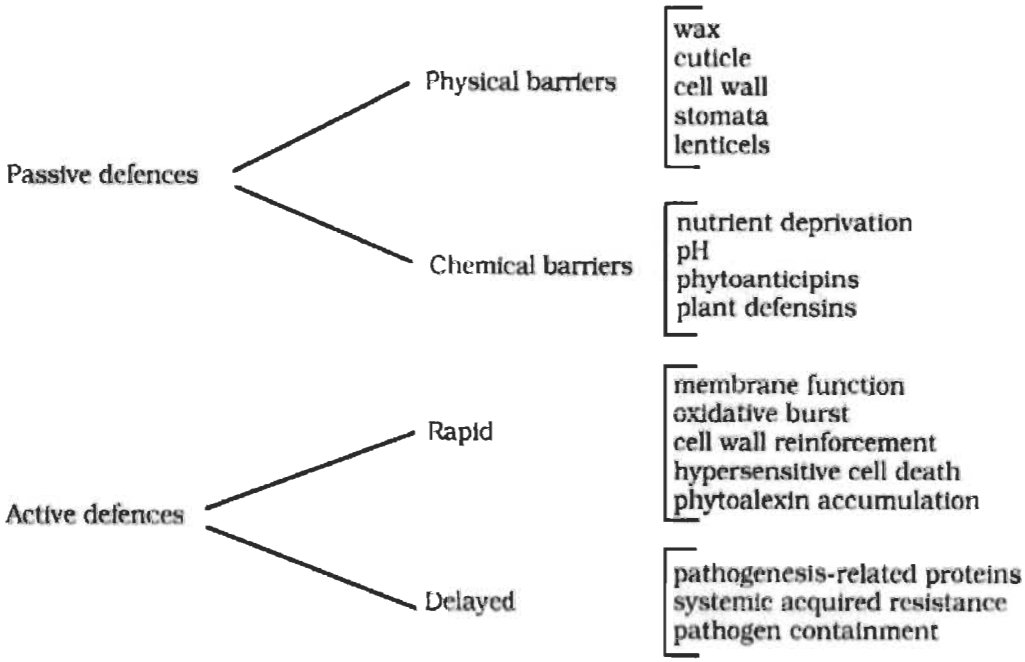


Fig. 1.2 Outline of plant defence systems against different pathogens.
Plant defence systems has been classified into two major systems: passive and active defences. Passive defences include physical and chemical barriers, and active defences includes rapid and delayed mechanisms. (Source: Guest and Brown 1997.)

1.2.1 Passive defence

Constitutive barriers are the passive barrier of plants involving physical and chemical defence. Largely physical barriers involve the specialized plant surface which comprise cell wall, bark, waxy layer, cuticle and stomata. Thickness and chemical composition and molecular mechanism underlying plant cell walls are daunting for some pathogens (Malinovsky, Fangel and Willats 2014). Waxy layered cuticular surface on top of epidermal cells covering plant tissues protects plants from many pathogenic attack (Göhre and Robatzek 2008).

Plants possess some physiological access sites, for instance stomata and wounds or hydathodes. Sometimes pathogens can easily enter through these access points, but once the pathogens are inside the host cells, they are antagonized by punitive environments such as uncomfortable pH or antimicrobial compounds (Göhre and Robatzek 2008).

Plants can respond via the production of chemical compounds or secondary metabolites such as phytoanticipins to the exterior environment which can stop pathogens development. For example, dead cells of brown onion skins excreted quinones catechol and protocatechuic acid preventing spore germination of *Botrytis cinerea* (neck rot pathogen) and *Colletotrichum circinans* (smudge pathogen) (Guest and Brown 1997). Saponins (found abundantly at the surface of quinoa grains) are glycosides based phytoanticipins which are toxic to pathogens having sterols in the membranes, since it binds to sterols in cell membrane of pathogens and subsequent degradation of membrane integrity (Guest and Brown 1997). Plant defensins are another type of chemical compounds produced by some plants, for example proteinase- and polygalacturonase-inhibitors or lectins which restrict pathogen nutrient uptake and impede their development. Anti-feeding activity of defensins may provide protection in response to insect transmitted virus (Guest and Brown 1997).

1.2.2 Active defences

Plants may quickly respond to pathogens through various active defence mechanisms such as changes in membrane functions, oxidative burst (generating Reactive Oxygen Species, ROS), hypersensitive response (HR) and reinforcement of cell wall (Guest and Brown 1997). Upon the attack of pathogens, plants inhibit further growth of infection by rapid death of cells at the infection surroundings visualized as HR, a mechanism similar to programmed cell death (PCD) in animals. HR starts with the efflux of potassium and hydroxide ions from the cells and influx of hydrogen and calcium ions. As a result, cells undergoes rapid response of respiration i.e. oxidative burst by generating ROS, such as hydrogen peroxide, nitrous oxide and super oxide ions (Heath 2000). ROS distresses cell membrane by inducing lipid damage which eventually affects cells and is visualised as local cell death or lesions. Simultaneously, callose or lignin deposits around the cell walls strengthens the cells adjacent to the pathogenic infection.

The defence mechanisms presented above is contingent on pathogen detection and molecular activation of defence pathways. Although plants lack an adaptive immune system like animals, they have acquired the ability to detect and protect themselves against infectious microorganisms via a two-layer immune system, PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) (Fig. 1.3) (Jones and Dangl 2006).

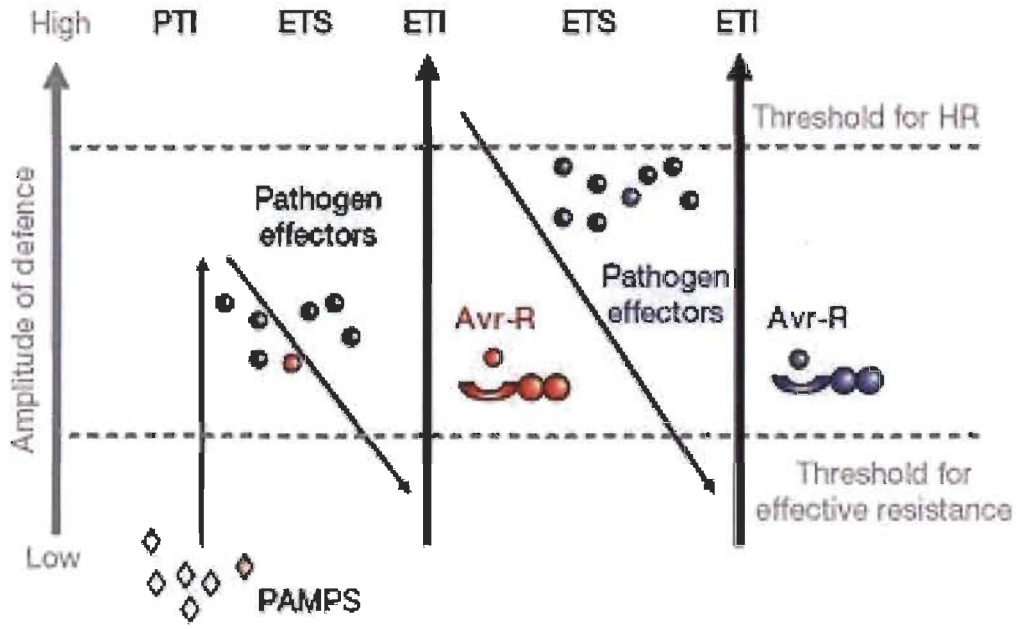


Fig. 1.3 The zigzag model of the plant immune system.
In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs (Pathogen Recognition Receptors) to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue) - these can help pathogens to suppress ETI. (Source: Jones and Dangl, Nature, 2006.)

1.2.2.1 PAMP-Triggered Immunity (PTI)

The first layer of innate immunity is termed as P/MAMP (Pathogen/Microbe-Associated Molecular Patterns) Triggered Immunity (PTI). PTI is activated by the recognition of PAMP by pathogen recognition receptors (PRR) located at plant cell surface. Plant PRR includes Receptor-Like Kinases (RLKs) or Receptor-Like Proteins (RLPs) which have an extracellular domain, Leucine Rich Repeats (LRRs) (Shiu and Bleecker 2003). P/MAMPs are conserved microbial features such as bacterial flagellin, Elongation factor-Tu and chitin (Jones and Dangl 2006, Bittel and Robatzek 2007, Boller and Felix 2009, Gómez-Gómez and Boller 2000, Kaku et al. 2006, Zipfel et al. 2006). P/MAMP are integral pathogen protein required for pathogen survival, hence they are under selective pressure and evolve slowly enabling their detection by plants. Thus, PTI is specific to conserved pathogen molecules which means that it is not specific to a bacterial species or genus. Upon the attack of pathogens, P/MAMP activate PRR in the host leading to a downstream activation of a Mitogen-Activated Protein Kinases (MAPK) signaling cascade and trigger PTI (Fig. 1.4) (Nitta, Ding and Zhang 2014, Pieterse et al. 2009, Asai et al. 2002). Activation of MAPK signaling activate the transcriptional rewiring of more than 1200 genes (Zipfel et al. 2004). The importance of MAPK cascade and downstream transcription factors were well illustrated in *Arabidopsis* using bacterial flagellin (Asai et al. 2002). Recognition of flagellin by FLS22 kinase promote the activation of MAPK cascade, MEKK1, MKK4/MKK5 and MPK3/MPK6, which activate downstream transcriptional factors WRKY22/WRKY29 for triggering defence resistances (Asai et al. 2002). However, PTI responses are slow and weak because of low specificity of pathogen recognition. So PTI can pause further colonization of pathogens.

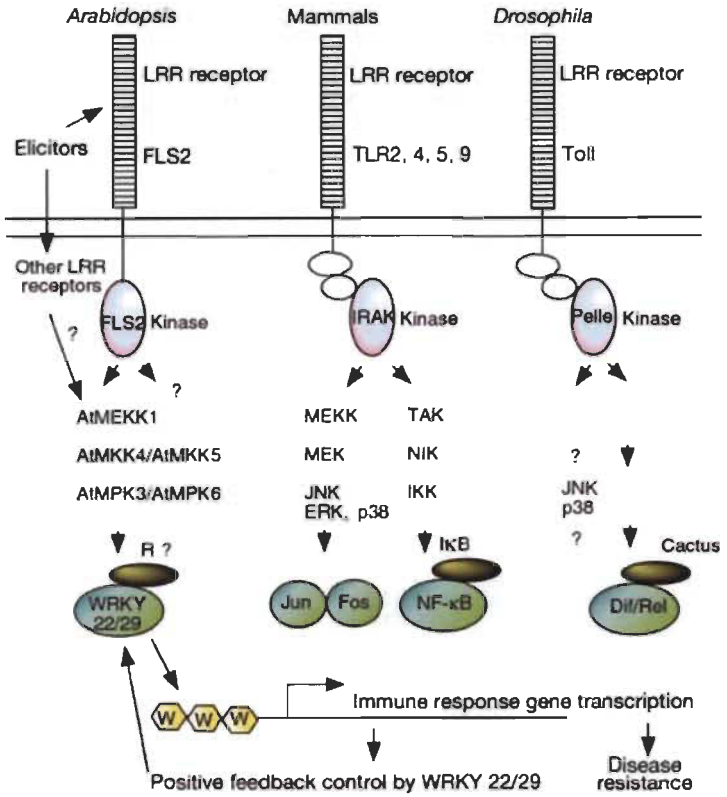


Fig. 1.4 Model of MAPK signaling pathway and PTI.
Innate immunity signalling is activated with the recognition of PAMP by LRR receptors in *Arabidopsis*, mammals and *Drosophila*. It triggers MAPK cascades (AtMEKK1, AtMKK4/AtMKK5, AtMPK3/AtMPK6 in *Arabidopsis*; MEKK, MEK, JNK, ERK, TAK, NIK and IKK in mammals and JNK, p38 in *Drosoplila*) which activate transcription factors (WRKY 22/29 in *Arabidopsis*; Jun Fos and NF-kB in mammals, and Dif/Rel in *Drosophila*) in the downstream of signaling cascades to trigger disease resistances. But a putative repressor (R) could control the activity of transcription factors WRKY22 and WRKY29 because their overexpression circumvents the recruitment of elicitors. Figure adapted from Asai et al. 2002.

1.2.2.2 Effector-triggered immunity (ETI)

To suppress PTI, pathogens have developed an arrangement of effectors to evade this defence layer by delivering effectors/virulence factors into the host. Effectors can restrict PTI, resulting in Effector Triggered Susceptibility (ETS) (Fig. 1.3). Effector are detected in plants by a set of intracellular immune receptors, coined resistance

proteins (R). R proteins can directly or indirectly recognize specific effectors (avirulence) of invading pathogens leading to activation of Effector-Triggered Immunity (ETI), the second layer of plant immune system. ETI is extremely specific to a race or strain of pathogen as it recognizes a virulence determinant or its effect, it is fast and a strong defence response leading to a HR to restrict further pathogenic infection (Mur et al. 2008). If the host does not contain suitable receptors (R proteins), then effectors suppress the host defence and subsequent ETS occurs (Fig. 1.3) (Jones and Dangl 2006, Pieterse et al. 2009, Ausubel 2005).

1.3 Resistance proteins

Over the course of evolution, plants have evolved copious resistance mechanisms which involves R proteins. Regardless of the diversity of pathogens, genes encoding R proteins belongs to the Nucleotide-Binding Site Leucine-Rich Repeat (NBS-LRR) family (synonyms: NB-ARC-LRR, NB-LRR), which includes the central nucleotide-binding site (NB/NBS) and C-terminal leucine-rich repeat (LRR) domains (Meyers 2003, Tameling and Takken 2008). Furthermore, depending on the N-terminal domain, these NBS-LRR proteins can be subdivided into two subclasses: (i) one class contains significant homology to the cytosolic portion of the Toll/Interleukin-1 Receptor (TIR) domain (i.e. TIR-NBS-LRR); (ii) other class contains non-TIR-NBS-LRR, which has a Coiled-Coil (CC) domain (i.e. CC-NBS-LRR) instead of TIR domain (Dangl and Jones 2001, Tameling and Takken 2008) (Table 1.1). Apart from CC-NBS-LRR, non-TIR-NBS-LRR also comprises other families such as ED-NBS-LRR proteins and NBScc-LRR proteins lacking an additional N-terminus domain. In poplar, another group of protein family has been reported, which is called as mixed protein family containing both TIR and CC domains (TIR-CC-NBS-LRR) (Table 1.1). Dynamic nuclear trafficking of R proteins has been demonstrated as important to achieve ETI (Cheng et al. 2009, Liu and Coaker 2008). R proteins localize to different subcellular compartments (from inner plasma membrane to the cytosol and nucleus), but nuclear localization are associated with substantial reprogramming of transcription. It has been reported that some plant R proteins accumulate in the nucleus upon the recognition of

effector (Cheng et al. 2009, Shen et al. 2007, Wirthmueller et al. 2007). In response to effectors, host transcriptional reprogramming include transcription factors (TFs) such as WRKY (Eulgem 2005). For example, MLA10, a powdery mildew effector translocates to the nucleus, interacts with both WRKY (transcriptional repressor) and MYB6 (transcriptional activator) and activate defence responses. Consequent to pathogen effectors invasion, NBS-LRR activate and trigger nuclear associated immune responses that implicates shuttling of proteins through the nuclear membrane; mRNA export from nucleus and activation/repression of transcription occur (Shen and Schulze-Lefert 2007).

Table 1.1. Major classes of resistance (R) proteins.

Domain structure	Domain arrangement	Example	References
TIR-NBS-LRR 	TIR-NBS-LRR	N receptor L6 receptor	Mestre and Baulcombe (2006) Howles et al (2005)
	TIR-NBS-LRR-WRKY	RRS1-R receptor	Deslandes et al (2003) Noutoshi et al (2005)
	$NBS_{(TIR)}$-LRR	2 Arabidopsis*	Meyers et al (2003)
	CC-NBS-LRR	I-2 RPS5	Tameling et al (2006) Ade et al (2007)
non-TIR-NBS-LRR 	$NBS_{(CC)}$-LRR	4 Arabidopsis*	Meyers et al (2003)
	BED-NBS-LRR	Poptr_1:787192	Kohler et al (2008)
Mixed 	TIR-CC-NBS-LRR	2 *Populus**	Kohler et al (2008)

* Represents only the number of NBS-LRR gene sequences are available.

According to the nibbler (named after their central nucleotide-binding, NB and leucine-rich repeat, LRR domains) model of R proteins function (Fig. 1.5), the interaction between effectors and NBS-LRR modifies the configuration of the LRR domain (Takken and Tameling 2009). Upon the perception of effector by C-terminus LRR domain, alteration occurs at the edge between ARC2 (NB-ARC) subdomain and N-terminus LRR domain. Hereinafter, nucleotide exchange occurs at NB (similar as NBS domain mentioned earlier section 1.3). However, second confirmational change occurs due to ADP/ATP convertion which alters the interaction between downstream TIR/CC and LRR domains resulting in formation of an active signalling complex (ON state) (Fig. 1.5). In the "ON" state, NBS subdomain expose to preliminary defence signalling. In contrast, hydrolysis of ATP rearranges the protein complex to its ADP-bound automated inhibited mode (OFF state) (Fig. 1.5).

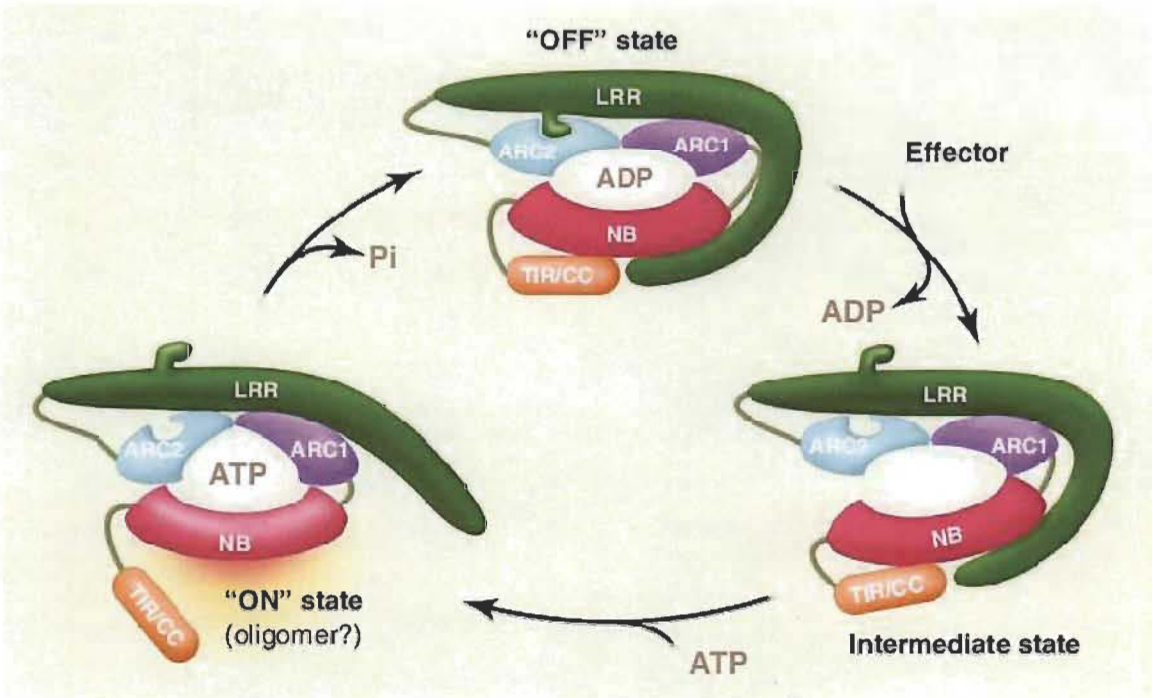


Fig. 1.5 Generalized model of R protein function and activation.
Biochemical studies revealed that the interaction between effectors and NBS-LRR modifies the configuration of the LRR domain. Upon the preception of effectors by C-terminal part of LRR changes the interface between N-terminal part and the ARC2 domain, thus creating an open confirmation of R protein which is prone to nucleotide exchange. The exchange between ADP/ATP triggers a second conformational change,

modifying the interaction between the NB-ARC, N-terminal TIR/CC and the C-terminal LRR domains, which results in the ON state of the function. In absence of pathogen, R proteins (NB-LRR) exist in an autoinhibited, ADP-bound OFF state which is stabilized by LRR domain. On the other hand, in the activated state, the NB subdomain becomes exposed to initiate defence signaling. ATP hydrolysis resets the protein into its ADP-bound autoinhibited OFF state. In this figure NB is similar as NBS domain mentioned earlier section 1.3. (Source: Takken and Tameling, 2009.)

Resistance protein pathway is mediated by either TIR-NB-LRR (/TIR-NBS-LRR) or CC-NB-LRR(/CC-NBS-LRR) (Germain and Seguin 2011) (Fig. 1.6), 93 TIR-NB-LRR and 51 CC-NB-LRR has been reported in *A. thaliana* Col-0 (Meyers 2003). TIR-NB-LRR resistance pathway generally signals through the gene complex of *EDS1/PAD4/SAG101* (*ENHANCED DISEASE SUSCEPTIBILITY* 1/*PHYTOALEXIN DEFICIENT* 4/*SENESCENCE ASSOCIATED GENES* 101). EDS1 is cytosolic and forms nuclear protein complex with PAD4 and SAG101. On the other hand, CC-NBS-LRR pathway generally signals through *NDR*1 (*NONRACE SPECIFIC DISEASE RESISTANCE* 1) gene localizing in plasma membrane. TIR-NBS-LRR and CC-NBS-LRR pathways mingle for the synthesis of defence responsive hormone salicylic acid (SA). SA is a necessary signal for Systematic Acquired Resistance (SAR) which is mediated by *NPR1* (*NONEXPRESSOR OF PR-1*) genes (Fig. 1.6).

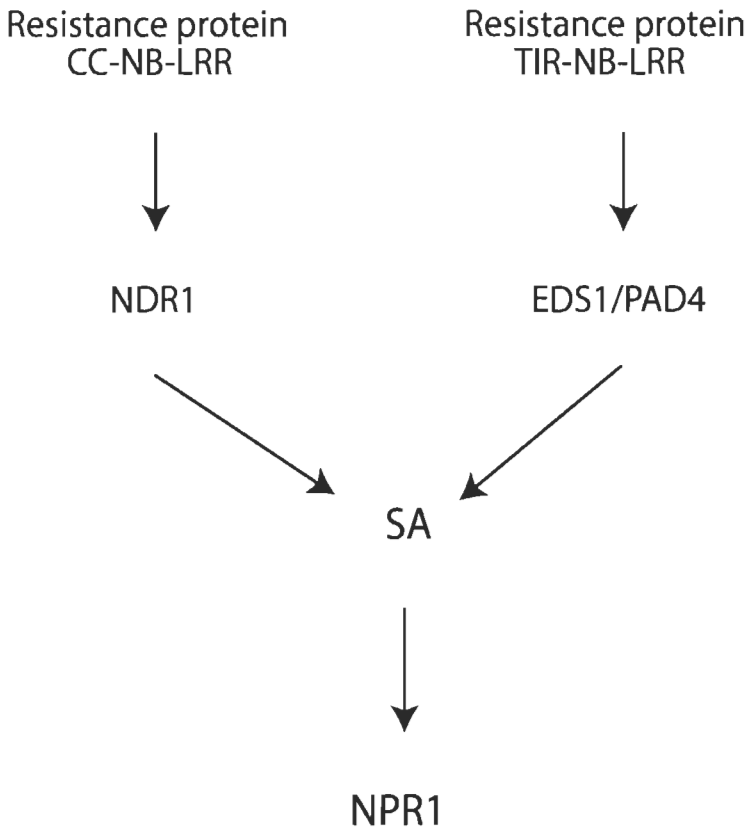


Fig. 1.6 R protein pathways in *A. thaliana*.
Different resistance pathways with the requirements of salicylic acid (SA) as signalling molecule, which lead to the induction of defense-related gene have been identified in *A. thaliana*. *PAD4* and *EDS1* gene complex functions upstream of SA signalling pathway in resistance controlled by TIR-NBS-LRR type *R* genes. In contrary, resistance conditioned by CC-NBS-LRR type *R* genes activates independently and requires *NDR1*. Both *NDR1* and *PAD4/EDS1* gene complex combined signals for SA and resistance triggers via NPR1. EDS1: *ENHANCED DISEASE SUSCEPTIBILITY* 1; PAD4: *PHYTOALEXIN DEFICIENT* 4/*SENESCENCE ASSOCIATED GENES* 101; NDR1: *NONRACE SPECIFIC DISEASE RESISTANCE* 1; SA: Salicylic acid and NPR1: *NONEXPRESSOR OF PR-1*. (Source: Germain and Seguin 2011.)

NBS-LRR mediated resistance mechanisms are still poorly understood. Therefore, understanding of the immune activation mechanism mediated by R and its cognate avirulent effector (AVR) is an important topic of research in the field of plant-pathogen interactions.

1.4 Molecular models of plant-pathogen recognition

The relationship between effectors and host receptors is defined by the gene-for-gene model (Flor 1971) which encompasses the direct effect of an unambiguously recognized effector by the receptor i.e. one-on-one relationship between an avirulence protein (Avr) and a resistance protein (R) (Fig. 1.7A). Sometimes support of additional proteins in the host is necessary for the initiation of resistance response, which prompted the second model which is an extension of gene-for-gene model and termed the guard model (Fig. 1.7B). According to the guard model, the target of the effector protein (the guardee) in the host is an essential element which explains indirect perception mechanism of effectors by an appropriate guard protein, a NBS-LRR receptor (R protein). This model assumes that R proteins act by guarding the effectors target and the modification of this target by the guardee results in activation of the R protein that triggers resistance in the host (Van Der Biezen and Jones 1998, Dangl and Jones 2001). The guard model was experimentally verified to explain the mechanism of *P. syringae* AvrPto perception by the tomato resistance proteins Pto and Prf (Van Der Biezen and Jones 1998) which were later on generalized for the perception of other effectors (Dangl and Jones 2001). Direct detection of the effectors does not occur in this model, but the R protein detect modification of the guardee; therefore, the guard monitors a host structural and/or functional changes (Jones and Dangl 2006, Tameling and Takken 2008).

Depending on the presence or absence of the *R* gene, the guardees are subjected to selection forces: (I) to promote the perception of guardees i.e. strong interaction, and (II) to evade manipulation by the guardee i.e. weaker interaction. So these two contradictory selection forces on the same effector interaction surface of the guardee results in an evolutionary unstable situation. This selection force could be eased upon the evolution of a host protein, termed as decoy. Thereafter, it focuses in perception of the effector by the R protein which has no function either in the disease development or resistance. As a result, in the decoy model (Fig. 1.7C), the decoy mimics effectors targets to sham the pathogen into a recognition event without participating to the pathogen fitness in absence of R protein (van der Hoorn and Kamoun 2008). The decoy

model was experimentally verified in pepper, tomato an *Arabidopsis* where pBs3 (Zhou and Chai 2008), RCR3 (Dixon et al. 2000) and RIN4 (Kim et al. 2005) were identified as decoy respectively.

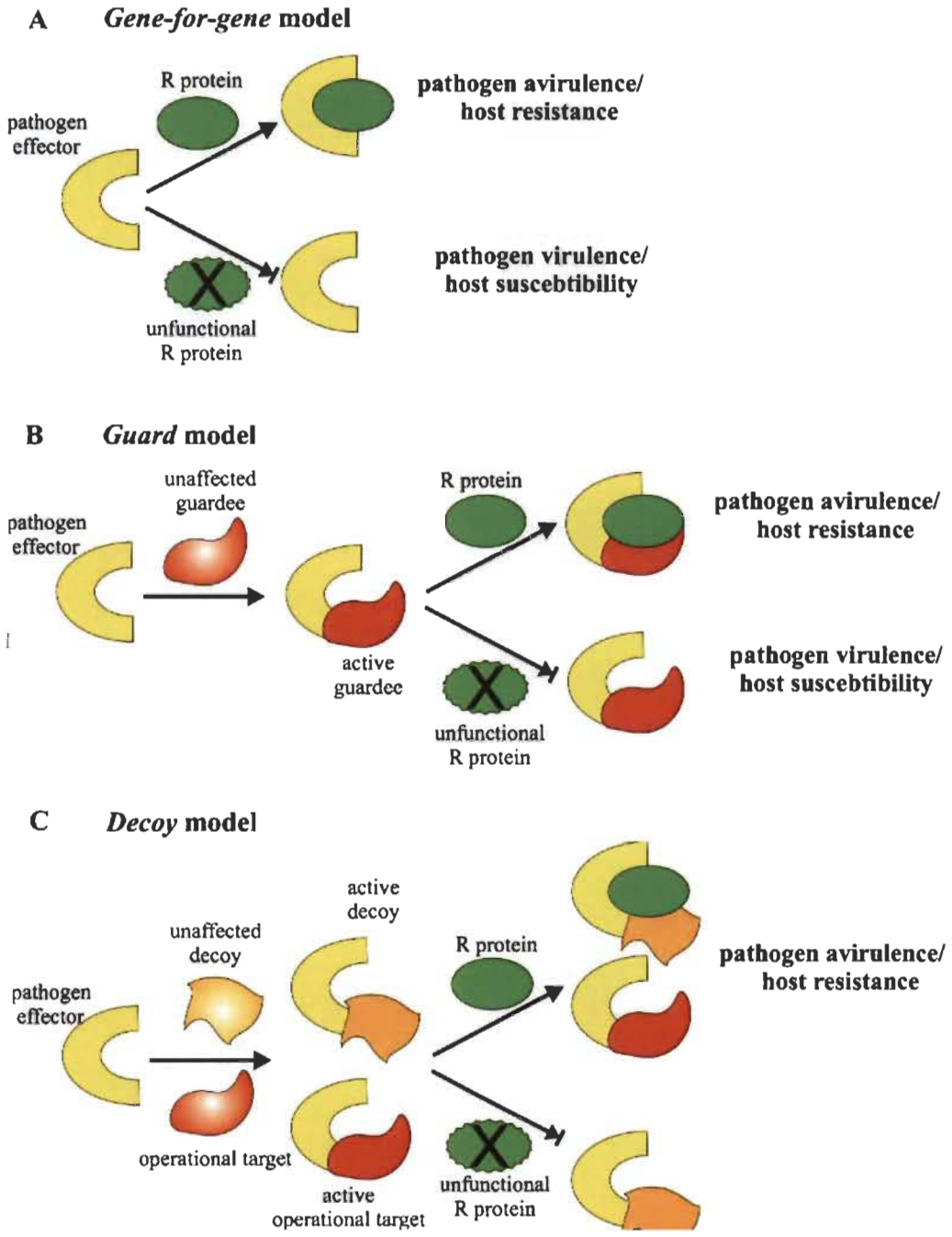


Fig. 1.7 Molecular models of pathogen recognition.
(A) The gene-for-gene model. It involves the direct effect of a specified recognition of effector on the receptor site. (B) The guard model:

the target protein of the effector (guardee) is guarded by an appropriate guard protein, NBS-LRR receptor. (C) The decoy model: specific proteins which are similar to the protein targeted by pathogenic effectors are generated by the host plant, whose only function is to bind those effectors and acts as a mediator in the interactions with R proteins. Details are in the text). (Source: Glowacki et al 2011.)

1.5 Plant pathogens

Plants are continuously under the attack from diverse microbial pathogens through multiple ways. Some pathogens colonize intra- or inter-cellular spaces of various plant tissues (roots, leaves etc.), settle down on surfaces while some others travel through vascular bundles (Yadeta and BP 2013). Pathogens perceive nutritional benefits from the host plants. Some pathogens (biotrophic pathogens) depend on living tissues for nutrient uptake. There are also pathogens (necrotrophic) that feed on dead tissues. Regardless of the feeding style, some pathogens are devastating for crop plants, and few types of pathogens are being extensively studied to understand the interactions of molecular plant-pathogen interaction.

1.5.1 Viral pathogens

Viruses are very small particles restricted to specific type of host and spread plant diseases causing massive economic losses. Plant virologists in association with molecular plant pathologists ranked the top ten plant viruses in importance to economy and scientific research (Scholthof et al. 2011) enlisted in Table 1.2.

Table 1.2. Top ten viral pathogens in plants.

Rank	Virus	Common diseases	Author of virus description
1	*Tobacco mosaic virus* (TMV)	Mosaic and necrotic diseases on tobacco, tomato and other solanaceous plants	Karen-Beth G. Scholthof
2	*Tomato spotted wilt virus* (TSWV)	Spotted wilt in tomato and some other vegetables	Scott Adkins
3	*Tomato yellow leaf curl virus* (TYLCV)	Leaf curl in tomato	Henryk Czosnek
4	*Cucumber mosaic virus* (CMV)	Mosaic disease on tomato, tobacco, pepper	Peter Palukaitis
5	*Potato virus Y* (PVY)	Dark mosaic or tuber necrotic ringspot of potato	Emmanuel Jacquot
6	*Cauliflower mosaic virus* (CaMV)	Mosaic in cauliflower	Thomas Hohn and Barbara Hohn
7	*African cassava mosaic virus* (ACMV)	Cassava mosaic disease	Keith Saunders
8	*Plum pox virus* (PPV)	Sharka disease of stone fruits	Thierry Candresse
9	*Brome mosaic virus* (BMV)	Mosaic on barley and other grass family	Paul Ahlquist
10	*Potato virus X* (PVX)	Mosaic or necrotic in potato, tomato, pepper	Cynthia Hemenway

Most plant virus are single stranded positive sense RNA. Compatible interaction of host, viral particle and environmental factors may cause severe diseases and plant death. The defence mechanism described above are effective against viruses (Scholthof et al. 2011).

1.5.2 Bacterial pathogens

While some bacteria can actually be beneficial to plants, some are pathogenic. Pathogenic bacteria cause numerous serious diseases in plants, although numbers are fewer than viruses and fungus and cause comparatively less damages and economic losses (Kennedy and Alcorn 1980). Regardless of beneficial bacteria, over 200 bacterial

species are threat to plants. In consonance with economic and scientific importance, recently molecular plant pathologists ranked top ten bacterial plant pathogens (Table 1.3) (Mansfield et al. 2012).

Table 1.3. Top ten bacterial plant pathogens.

Rank	Bacterial pathogen	Common diseases	Author of bacterial description
1	*Pseudomonas syringae* pathovers	Bacterial speck of tomato, bleeding cancer of horse-chestnut, blight disease of bean	John Mansfield
2	*Ralstonia solanacearum*	Bacterial wilt of tomato, eggplant, tobacco and some ornamental plants, potato brown rot and banana Moko disease	Stephane Genin
3	*Agrobacterium tumefaciens*	Crown gall tumor	Shimpei Magori, Vitaly Citovsky
4	*Xanthomonas oryzae* pv. *oryzae*	Bacterial blight	Malinee Sriariyanum, Pamela Ronald
5	*Xanthomonas campestris* pathovers	Black rots of crucifers infecting all cultivated brassicas	Max Dow
6	*Xanthomonas axonopodis*	Angular leaf spot of cotton	Valerie Verdier
7	*Erwinia amylovora*	Fire blight disease of several fruit plants (apple, quince, pear, blackberry, raspberry) and several wild and cultivated rosaceous ornamental plants	Steven V. Beer
8	*Xylella fastidiosa*	Citrus variegated chlorosis (CVC), almond leaf scorch disease (ALSD), Pierce's disease of grapevine (PD)	Marcos A. Machado
9	*Dickeya* (*dadantii* and *solani*)	Necrosis, blight and soft rot of potato tubers, bulbs of vegetables and ornamental crops	Ian Toth
10	*Pectobacterium carotovorum* (and *P. atrosepticum)*	Soft rot diseases, potato blackleg disease in the temperate areas	George Salmond

1.5.3 Oomycete pathogens

Because of their similar filamentous structure and feeding habits, oomycetes were once considered within the fungal kingdom. The development of molecular phylogenetic studies and advanced understanding of evolutionary relationships revealed them to constitutes a separate class of organisms, closer to diatoms and brown algae. Now it constitutes an individual class of pathogens, oomycota. Pathogenic effect of oomycetes on plants allowed molecular plant pathologists to identify most devastating oomycetes according to their importance in pathology and economic importance (Kamoun et al. 2015). Based on the recent publication (Kamoun et al. 2015), the top ten oomycete pathogens in plant molecular pathology are summarized in Table 1.4.

Table 1.4. Top ten oomycete plant pathogens.

Rank	Species	Common diseases
1	*Phytophthora infestans*	Late blight
=2	*Hyaloperonospora arabidopsidis*	Downy mildew
=2	*Phytophthora ramorum*	Sudden oak death; Ramorum disease
4	*Phytophthora sojae*	Stem and root rot
5	*Phytophthora capsici*	Blight; stem and fruit rot; various others
6	*Plasmopara viticola*	Downy mildew
7	*Phytophthora cinnamomi*	Root rot; dieback
=8	*Phytophthora parasitica*	Root and stem rot; various others
=8	*Pythium ultimum*	Damping off; root rot
10	*Albugo candida*	White rust

The '=' sign before the ranking indicates that the species tied for that position. Number of papers published in 2005-2014 is based on searches of the Scopus database (http://www.scopus.com) using the species names as a query. For *H. arabidopsidis*, a search for the alternative name Peronospora parasitica was also performed and the combined number is shown.

Among the top ten, *Phytophthora* and *Pythium* are hemibiotrophic and necrotrophic, respectively whereas *Hyaloperonospora arabidopsidis* is an obligate biotrophic pathogen (Stassen and Van den Ackerveken 2011). Complete genome sequencing of *H. arabidopsidis* (Coates and Beynon 2010), *P. infestans* (Haas et al. 2009), *P. sojae*, *P. ramorium* (Tyler et al. 2006), and *P. ultimum* (Lévesque et al. 2010) unwrapped the identification of huge catalog of effector proteins for enriched understanding of oomycete-plant interactions.

1.5.4 Fungal pathogens

According to the comprehensive phylogenetic classification, fungi are divided into six groups-Ascomycota, Basidiomycota, Glomeromycota, Zygomycota, Chytridiomycota and Microsporidia (Hibbett et al. 2007). Plants and fungi association is primitive and fungi are a vital group of plant pathogens. But less than 10% of all known fungi can inhabit with living plants (Knogge 1996). Mostly fungi are decomposers, living on the relics of plants or other organisms for food supply. Another group of fungi (mycorrhizal fungi) are associated with plant roots and both plants and fungus enjoy the mutual benefits.

In terms of feeding habit, fungi are divided into biotrophs, necrotrophs and hemibiotrophs. Biotrophic fungi uptakes nutrients from living tissues. Necrotrophs survives on dead tissue of host, whereas hemibiotrophs feeds on both living and dead tissues, which is characterized by usually having an initial biotrophic stage followed by a necrotrophic stage. They feed on living tissues for certain period of time and later on continue on dead tissues. Recently plant pathologists have ranked the top ten fungal plant pathogens according to their economic and scientific importance (Dean et al. 2012). Recently (Dean et al. 2012), the top ten fungal pathogens in plant molecular pathology (Table 1.5) and disease symptoms has been published (enlisted in Fig. 1.8). *Magnaporthe oryzaye* ranked first in the top ten pathogenic fungus list. *M. oryzae* is a filamentous ascomycetes fungus and causal agent of blast diseases of rice (Ou 1980) which is the primary caloric source for the half of the world's population (Khush 2005).

Table 1.5. Summary of the top ten fungal pathogens in molecular plant pathology.

Rank	Fungal pathogen	Type	Causal agent	Author of fungal description
1	*Magnaporthe oryzae*	Filamentous ascomycetes	Rice blast	Ralph Dean
2	*Botrytis cinerea*	Grey mould	Soft fruits and ornamentals	Jan A. L. van Kan
3	*Puccinia* spp.	Obligate, biotrophic basidiomycetes	Wheat stem rust by *Puccinia graminis* f. sp. *tritici*; stripe rust by *P. striiformis* f. sp. *Tritici*); leaf rust by *P. triticina*	Zacharias A. Pretorius
4	*Fusarium graminearum*	Ascomycetes	Hexaploid wheat	Kim Hammond-Kosack
5	*Fusarium oxysporum*	Ascomycetes	Melon, tomato, cotton and banana	Antonio Di Pietro
6	*Blumeria graminis*	Ascomycetes	Powdery mildews of grasses	Pietro Spanu
7	*Mycosphaerella graminicola*	Ascomycetes	Blotch of wheat	Jason J. Rudd
8	*Colletotrichum* spp.	Ascomycetes	Anthracnose spots and blights of aerial plant parts and post-harvest rots.	Marty Dickman
9	*Ustilago maydis*	Basidiomycetes, biotrophic	Corn smut	Regine Kahmann
10	*Melampsora lini*	Basidiomycetes	Flux and linseed rust	Jeff Ellis

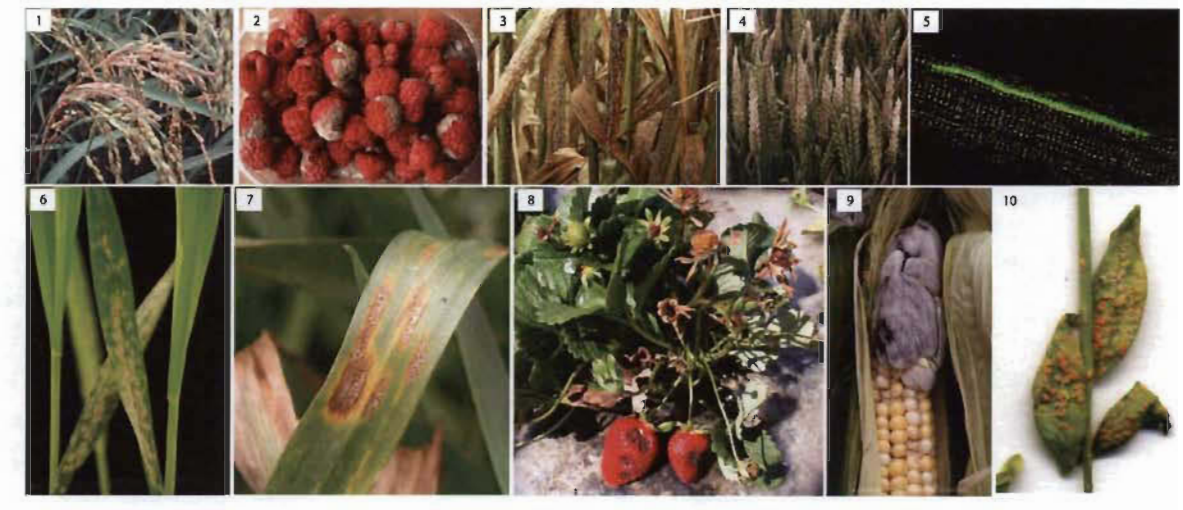


Fig. 1.8 Disease symptoms of top ten fungal pathogens.
(1) Rice blast with *Magnaporhe oryzae*; (2) Gray mould on raspberry with *Botrytis cinerea*; (3) Stem rust of wheat by *Puccinia gramins* f. sp. *tritici*; (4) Head blight of wheat by *Fusarium graminearum*; (5) Growth of *Fusarium oxysporum* hyphae on roots of tomato; (6) Powdery mildew on barly leaves by *Blumeria graminis* f. sp. *Hordei*; (7) Blotch of wheat caused by *Mycosphaerella graminicola*; (8) *Colletotricum acutatum* infected strawberry; (9) Corn smut of maize with *Ustilago maydis*; (10) Rust of flax with *Melampsora lini*. (Source: Dean et al. 2012.)

1.5.4.1 Rust fungus as pathogen

Rust fungi are filamentous biotrophic, eukaryotic plant pathogens belonging to the Pucciniales family under the phylum Basidiomycota. Rust fungi cause devastating diseases in native and commercial crop plants including wheat, maize, bean, pine, poplar and other cereals (Duplessis et al. 2011a, Helfer 2014, Aime et al. 2006). Most pathogenic rust fungi manipulate host physiology and cellular functions according to their own interest which eventually induces a number of diseases. Comparative interaction studies of plant-pathogens have demonstrated the significance of several molecules of pathogens such as effectors, carbohydrate active enzymes (CAZys) and secondary metabolites (Stergiopoulos et al. 2013, O'Connell et al. 2012). Alike oomycetes, rust fungi develop a specialized feeding structure called haustoria (Fig. 1.9). Following contact with the host, the urediospore develops a germ tube, forms appressorium intercellularly, penetrates throughout the host cell wall, pushes the plasma membrane, and eventually forms a bubble-like structure, the haustoria (Catanzariti,

Dodds and Ellis 2007, Hahn and Mendgen 2001). In addition to nutrient uptake, rust pathogens secrete effectors proteins inside into the host cell via haustoria and manipulate host cellular processes and suppress host defences (Voegele and Mendgen 2003).

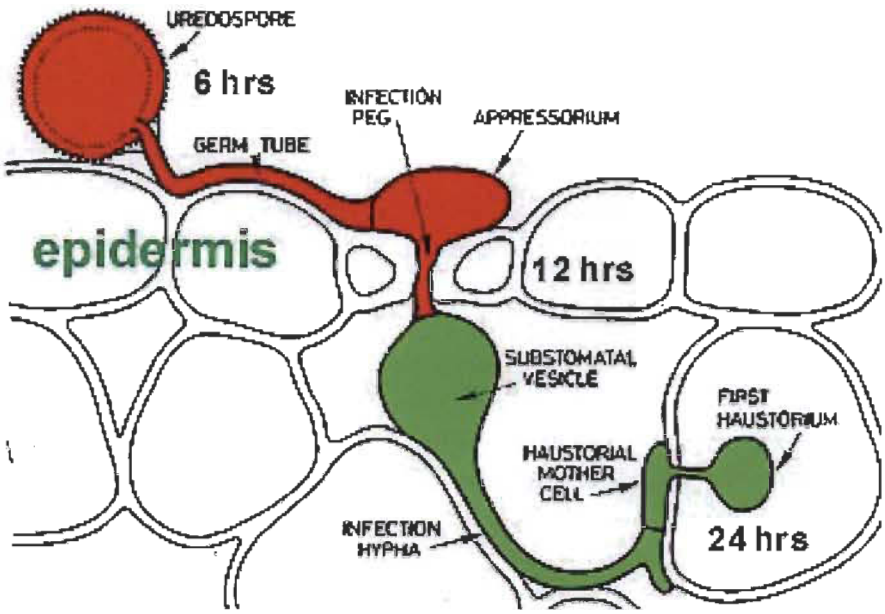


Fig. 1.9 Cartoon showing haustoria formation.
Following contact with the host, the urediospore develops a germ tube, form an appressorium intercellularly. Then penetrates throughout the host cell wall and pushes the plasma membrane, form infection hyphae and haustoria throughout epidermal cells. (Drawn by K. Mendgen, from Introductory Mycology from John Wiley & Sons, Inc.)

1.6 Plant pathosystems

A pathosystem is a subsystem of an ecosystem termed by the parasitism phenomenon of a pathogen. In a plant pathosystem, the pathogen has the ability to colonize within host tissues, and the host may have a resistance or defence system to protect itself from this invasion. (Robinson 1977). It is a multidisciplinary concept that conveys diverse field of plant biology including plant breeding, pathology, nematology and entomology. A good model pathosystem is required in order to improve our understanding of plant-pathogen interactions (Meng et al. 2014); specifically the cellular and molecular understanding of PAMPs perception, PTI, ETI, transport of vesicles,

transcriptional networks and reciprocity between hormone signaling and disease resistance (Nishimura and Dangl 2010).

1.7 Effectors

A molecule or secreted protein from a plant associated organisms which colonize and modify cellular structure or functions in the host is termed as "effector" (Hogenhout et al. 2009). Plant pathogens secrete molecules known as effectors into the plant tissues to promote parasitic growth. Gram-negative bacteria (for instance *P. syringae*) secrete effectors using type three secretion system (thereafter T3SS) for delivery inside the host cells (Abramovitch, Anderson and Martin 2006). The very presence of effector is what defines microbes as pathogenic or not pathogenic (Hacquard et al. 2016b). Some of these secreted proteins can trigger an HR in resistant plants (i.e. avirulence property), while the same proteins were later on found to promote virulence (i.e. virulence property) in susceptible plants (plants lacking the cognate R proteins). Since the same protein display dual activities (avirulence/virulence) in the case of incompatible or compatible interactions, thereafter the term "effector" alleviate the conceptual limitations of avirulence and virulence terminology (Hogenhout et al. 2009). Recently the word effector has been adopted by a broad range of microbiologist/pathologists and now is being preferentially used in the field of fungi and oomycete as well (Hogenhout et al. 2009). Effectors target a variety of host cell compartments and molecules such as proteins and DNA, and modulate their location, stability, or function to the advantage of the pathogen (Chaudhari et al. 2014, Lewis, Guttman and Desveaux 2009, Win et al. 2012). Recently, the term effector has also been used to denote secreted proteins of microbes that establish symbiotic relationship with plants (Plett et al. 2011, Kloppholz, Kuhn and Requena 2011).

1.7.1 Rust effectors

Recently, genome sequences of four rust fungi (two Pucciniaceae and two Melampsoraceae) became available. Genomic and transcriptomic analyse revealed a

wide range of small secreted proteins known as candidate secreted effector proteins (CSEPs) (Petre, Joly and Duplessis 2014). Haustoria delivers effectors/CSEPs into the host cells and also rewires molecular trafficking between the host and the parasite (Rafiqi et al. 2012). Six effectors have been reported from three rust species to be secreted via haustoria and translocated into host cells (Table 1.6) (Kemen et al. 2005, Petre et al. 2014, Upadhyaya et al. 2014, Ellis, Catanzariti and Dodds 2006). Nowadays, plant molecular pathologists utilize effectors as probes to identify and understand plant processes targeted by pathogens in order to develop resistant crop plants.

Table 1.6. List of rust effector proteins identified so far.

Effector protein	Aa residues (mature)	Signal peptide	Expression	Localization in infected tissues	Avr property (immune receptor)	Biochemical function
AvrM	284-347	Yes	Haustorium[a]	Haustorium, EHMx, plant cytosol[a]	Yes (M)	nd
AvrL567	127	Yes	Haustorium	Pant cytosol	Yes (L5, L6, L7)	nd
AvrP123	94	Yes	Haustorium	Plant nucleus	Yes (P, P1, P2, P3)	nd
AvrP4	65	Yes	Haustorium	Plant cytosol	Yes (P4)	nd
RTP1	201	Yes	Haustorium[a]	Haustorium/ EHMx/plant cytosol/ plant nucleus[a]	nd	Protease inhibitor/ filament-formation
PGTAUSPE-10-1	np	np	Haustorium	nd	Yes[b]	nd

Role of virulence has not been determined for any one of these effector proteins.
Avr=Avirulence; aa=amino acid; EHMx=extra-haustorial matrix; nd=not determined; np=not published.
[a] *Direct evidence of the presence of the protein acquired by immunolocalization.*
[b] *a host-specific toxic effect was detected.*

Although AvrM and RTP1 have been shown to translocate into host cells from haustoria during infection, host plant immune receptors detect AvrM and AvrL567 which ultimately proves the internalization of effector protein in the host cells (Ellis, Dodds and Lawrence 2007). However, pathogen-free assessments recommend the autonomous entry of AvrM, AvrL567 and AvrP4 effector proteins into the plant cells (Catanzariti et al. 2006, Rafiqi et al. 2010, Kale et al. 2010). In addition, it has been showed that regardless of diverse rust effectors, signal in the N-terminal regions mediate the accumulation of AvrM and AvrL567 effector proteins into the host cells (Rafiqi et al. 2010).

1.7.2 Effectors and respective helpers and targets

Overall it is crucial to assess how the effector proteins functions inside the host cells, whether they targets proteins in the host cell compartments or protein(s) in the host cell evolved as helper or elicitor of the effector (Win et al. 2012). It is thought that effectors activity for colonization in the host and manipulation of cellular processes could significantly evolve (Win et al. 2012). Some effectors are enzymes, such as some of T3SS delivered effectors which biochemically alter host molecules, such as HopZ1a has been reported to be physically interact with GmHID1 (2-hydroxyisoflavone). The interaction of HopZ1a and GmHID1 leads to degredation of GmHID1 and thereby leading to enhanced bacterial multiplication (Deslandes and Rivas 2012, Cunnac, Lindeberg and Collmer 2009). Few effectors do not convey enzymatic activity; however, they bind to host proteins to alter host cellular functions. In addition to this, such effectors hinder some enzymes activity (kinases, glucanases, peroxidases and proteases) (Song et al. 2009, Tian et al. 2004, Tian et al. 2007). Groups of effectors modify gene expression by binding to nucleic acids, the penultimate example of effector affecting transcription are Transcription Activator-Like (TAL) effectors which directly binds to promoter motifs of host genes to modify host transcription (Gu et al. 2005, Yang, Sugio and White 2006, Boch et al. 2009).

As the identity of effector targets in host remains unknown for a large number of uncharacterized or unidentified effectors from diverse group (pathogens/microbes/symbionts), biochemical mechanisms of how effectors manipulate host cellular functions are still poorly understood. Regardless of specific target of effectors, now it has been reported that a single effector can target multiple proteins or can disturb diverse cellular processes in the host (Fig. 1.10A) (Win et al. 2012). For example, AvrPto and AvrPtoB effectors of *P. syringae* secreted in tomato and *Arabidopsis*, directly bind to several immune receptor kinases to abrogate their function and impede PTI signaling pathways (Abramovitch et al. 2006, Xiang et al. 2008). Secreted AY-WB Protein 11 (SAP11), a phytoplasma effector, binds to class I and II of Teosinte branched 1/Cycloidea/Proliferating (TCP) transcription factors (TF), but subverts the class II type TCP TF (Sugio et al. 2011); hence one of the main function of effectors is the suppression of immunity.

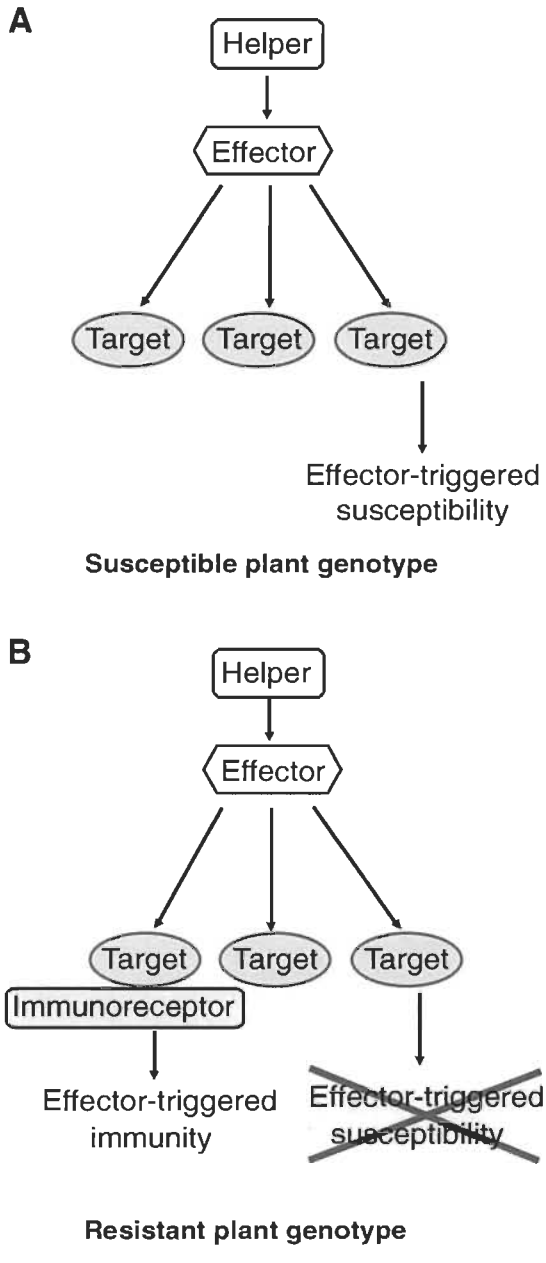


Fig. 1.10 Effectors and their host-cell helpers and targets.
This genetic model defines the position of pathogen effectors and their respective host-cell helpers or targets in the signaling pathways leading to susceptibility (A) and resistance (B). Effector targets and helpers are distinct plant susceptibility factors. Pathogen effectors recruit host helper proteins and cooperate for proper function. Activated effectors bind cognate targets, manipulate them, and form active effector-target complexes. In a susceptible interaction, the effector-target complex is not recognized and results in an altered cellular state of effector-triggered susceptibility (ETS). In a resistant interaction, this complex triggers host recognition by cognate immune receptors leading to effector-triggered immunity (ETI). (Source: Win et al. 2012.)

Regardless of proteins used as effectors target, some proteins in the host cytoplasm may modify effector proteins by binding to it or it forms an association with the effector and acts as cofactors, which forms an active complex. Therefore, host proteins that facilitate effectors translocation into different subcellular localization, are known as effectors helper proteins (Fig. 1.10B) (Win et al. 2012). Classical example of host effector helper is cyclophilin (a chaperone). AvrRpt2, a cysteine protease is secreted as an inactive form by *P. syringae*, but as soon as it is inside the host cell, it interacts with cyclophilin. The association of AvrRpt2-cyclophilin accelerates folding of proteins and cyclophilin activates self-processing of AvrRpt2, aggravating the breakdown of RPM1 Interacting Protein 4 (RIN4), the target protein of AvrRpt2 (Coaker, Falick and Staskawicz 2005).

Conceptually host proteins used as target or helper for the effectors are host susceptibility factors, because both target and helper proteins are inside the host cell which are prejudiced by the invading organism to launch effectors function within the host cells, and could eventually become targets for the establishment of ETI (Win et al. 2012).

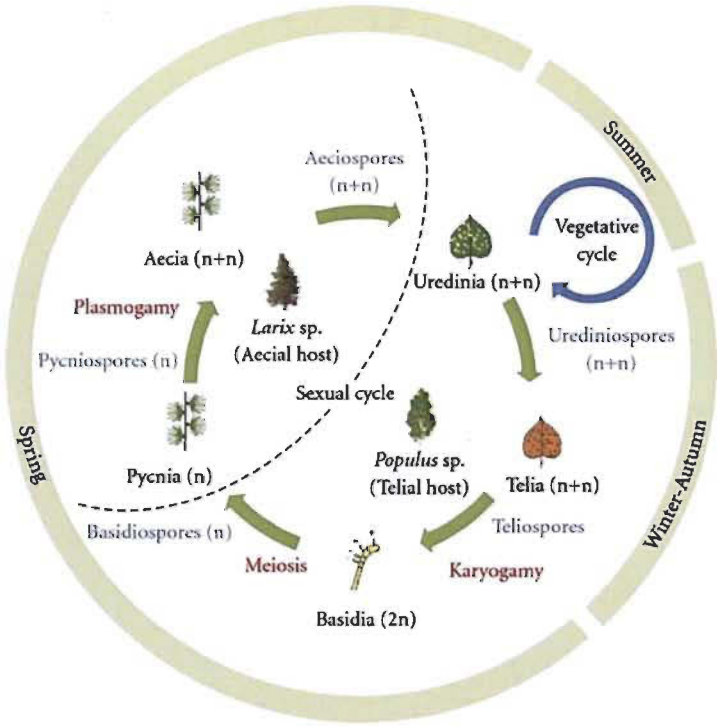
1.8 Biology of poplar-*Melampsora larici-populina* interactions

The high degree of damage caused to plants by rust fungi (order Pucciniales) conveyed them as the most studied obligate biotrophic fungal pathogens (Dean et al. 2012). *M. larici-populina* (*Mlp*) is a leaf rust caused by a biotrophic pathogen belonging to the Pucciniales order of Basidiomycetes, a threat to tree plantation worldwide. Its heterocious macrophytic life style demand two different hosts: *Populus* sp (poplar) for asexual reproduction and *Larix* sp (Larch) for sexual reproduction. *M. larici-populina* comprises two cycles: biological cycle and vegetative cycle (Fig. 1.11) (Hacquard et al. 2011a). The biological cycle involves five different spore forms: teliospores, basidiospores, pycniosopores, aeciospores and urediniospores. Diploid teliospores (2n) of *M. larici-populina* hibernates on dead leaves of poplar over the winter. Once spring starts, telieospores go through karyogamy and meiosis and yields

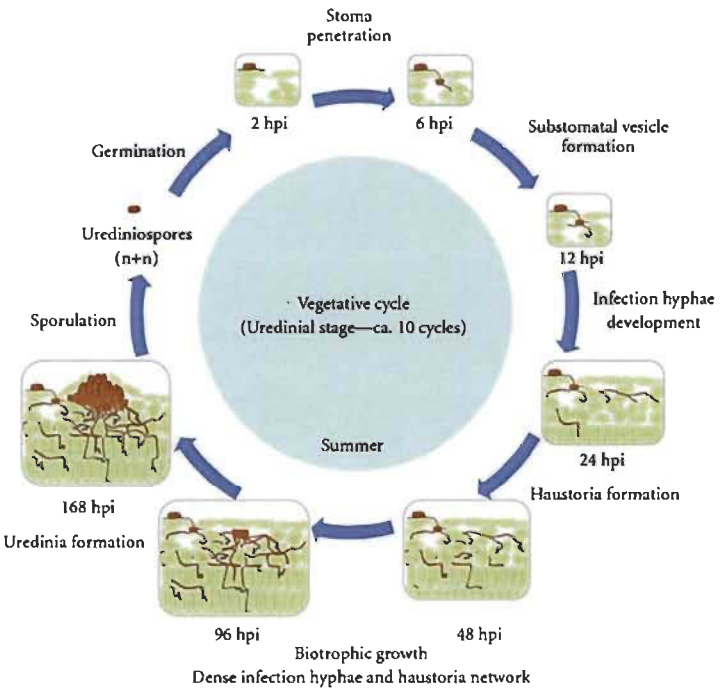
windborne haploid basidiospores (n) which infect larch needles. Henceforth, within few days, pycniospores (n) are produced on needles of larch and plasmogamic fusion of opposite mating type pycniospore generate aeciospores (n+n). At the end of the spring, aeciospores infect poplar leaves and produce another yellow-orange pustule-like sporulation (uredinium) on the abaxial side of mature leaves. Uredinium release uredinospores (n+n) (asexual phase) and can spread over large distances, and function as inoculum for further infection for poplar epidemics. Numerous infectious vegetative cycles emerged throughout summer and autumn. However, at the end of summer/autumn, telia (black pustule) forms on poplar leaves which again produce teliospores (hybernating/overwintering spores) and complete the life cycle of *M. larici-populina* (Fig. 1.11a) (Hacquard et al. 2011a).

As most pathogenic observations materialize during the asexual developmental phase of the vegetative cycle (uredinospore formation and spreading) of *M. larici-populina* (Fig. 1.11b), this phase drew the attention of molecular pathologists concentrating on functional characterization of poplar-*M. larici-populina* interactions (Hacquard et al. 2011a). Microscopic studies of *M. larici-populina* colonization on poplar leaves uncovered the foremost developmental transitions under precise environmental conditions (Boyle et al. 2010, Duplessis et al. 2011b, Rinaldi et al. 2007, Hacquard et al. 2010). Uredinospores starts germination in 2 hours post-inoculation (hpi) and within 6 hpi, its germ tube starts to penetratethrough stomata (Fig. 1.11b). Immediately upon the formation of substomatal vesicle at 12 hpi, it develops first haustoria at 17 hpi. Haustoria penetrate throughout mesophyll cells within 24 hpi. Compatible interaction provokes increased fungal biomass formation (>30 folds) between 48 hpi to 96 hpi (Boyle et al. 2010, Hacquard et al. 2010, Rinaldi et al. 2007, Hacquard et al. 2011b), and develops compact haustorial and hyphal network within mesophyll cells (Rinaldi et al. 2007). After around 168 hpi (7 days) massive hyphal network of *M. larici-populina* produces new uredinum which release fresh uredinospores on the leaf surfaces (Rinaldi et al. 2007).

Contingent of incompatible interaction, robust defence mechanisms of plant arrest the growth of rust fungus (Hacquard et al. 2011b, Rinaldi et al. 2007, Laurans and Pilate 1999). For instance, cytological interpretations uncovered the formation of strong HR with monolignols accumulation, and collapsed infected cells nearby the infected cells within 48 hpi (Laurans and Pilate 1999, Rinaldi et al. 2007). Despite all the efforts taken for studying the pathosystem of the obligate biotroph *M. larici-populina*, the inadequacy of efficient genetic transformation of hybrid poplar compatible with rust infections, the impossibility to transform the rust and the long generation time of poplar are severe bottleneck for molecular understanding of poplar-poplar rust interactions.



(a)



(b)

Fig. 1.11 Life cycle of *M. larici-populina*.

M. larici-populina has a complex heteroecious macrocyclic lifestyle comprising two different cycle on the leaves of poplar: (a) biological

macrocyclic cycle; and (b) vegetative cycle. Fig. (a) shows biological cycle (a) is completed on two diferent hosts (poplar and larix) and involvs five different forms of spores. In early spring, overwintered diploid teliospores undergoes on karyogamy and meiosis producing haploid basidiospores. Basidiospores disseminated by wind and infect needles of larch plant, and produce haploid pycniospores. Due to the mating of opposite type pycniospores, generates dikaryotic aeciospores. Later on aeciospores comes on poplar leaves via wind and generates a sporulation structure, uredinium on the abaxial side of mature leaves. Urediniospores are released and dispersed over large distances. Multiple vegetative cycle (b) can be completed on poplar leaves during spring and summer. In autumn, teliospores grows in black telia pustules on poplar leaves. Fig. (b) shows poplar-poplar rust interactions at different time points. Urediniospores germinate on the abaxial epidermis, produce germ tubes, and penetrates through stomata at 6 hpi (hours postinoculation). Subsequent infection hyphae has been developed at 12 hpi and first haustoria is developed at 17 hpi. Fungal biomass strongly increases and dense network of infection hyphae and haustoria forms within mesophyll cells between 48 and 96 hpi. At 168 hpi fungal pressure leads to form new urediniospores. (Source Hacquard et al. 2011).

1.9 Post-genomic approaches for CSEPs

Genome sequencing is a powerful tool that provide more efficient access to gene sequences. The application of genome sequencing in plant-microbe interaction research abilities to shorten the overall time for development of molecular genetic information required for functional studies (Cantu et al. 2011). Until now genome sequences of four rust fungus have been available: the wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*) (Cantu et al. 2011), the wheat stem rust (*P. graminis* f. sp. *tritici*) (Duplessis et al. 2011a), the poplar leaf rust (*Melampsora larici-populina*) (Duplessis et al. 2011a) and flax rust (*M. lini*) (Nemri et al. 2014). Secreted proteins from different rust fungal pathogens were considered for different *in silico* analysis for the prediction of candidate effector proteins to better understand the functionalities of he pathogen in host plant; such as hundreds of CSEPs encoding genes have been revealed from genome-wide effector mining of these four rust species. For example, 1,088 potential CSEPs in *P. striiformis* f. sp. *tritici* (Cantu et al. 2011); 1,106 CSEPs in *P. graminis* f. sp. *tritici* (Duplessis et al. 2011a); 1,184 CSEPs have been revealed in *M. larici-populina* (Duplessis et al. 2011a) and 762 priority CSEPs in *M. lini* (Nemri et al. 2014).

Since *M. larici-populina* has been established as a model for tree-microbe interactions, understanding of the molecular mechanisms behind the pathogenicity on plants appears to be crucial for the management of tree plantations. Therefore, prediction of secretome, genome-wide analysis of gene families and transcriptome of *M. larici-populina*, identification of Secreted Proteins (SPs), and systematic identification and prioritization of CSEPs has became essential for the functional studies of *M. larici-populina* (Hacquard et al. 2011a, Lorrain, Hecker and Duplessis 2015). Recently a distinctive pipeline (Lorrain et al. 2015) for CSEPs prioritization in *M. larici-populina* has been sketched based on the findings of two independent groups (Hacquard et al. 2012, Saunders et al. 2012), which is represented in Fig. 1.12. The effectoromics pipelines can be divided in four main steps. The first step is genome-wide prediction of *M. larici-populina* secreted proteins. Both group used similar prediction tools such as SignalP3.0, TargetP1.1 and TMHMM for the secretome prediction of *M. larici-populina* (Fig. 1.12). SignalP3.0 used for the prediction of signal peptide from the proteome, which drives the effector proteins outside the fungal cells. TargetP1.1 was used to identify proteins probably retained inside the fungal cells, and TMHMM was used to exclude proteins carrying transmembrane α-helix domains. Similarity based Markov clustering TribeMCL has been used to group SPs in tribes to assess multiple gene families in *M. larici-populina* and *P. graminis* f. sp. *tritici* (Saunders et al. 2012). On the contrary, second study added a further annotation with expert curation of genes corresponding to SPs in addition to TribeMCL based clustering, which directed to the characterization of SP gene families (Duplessis et al. 2011a, Hacquard et al. 2012). In the second step, Small Secreted Proteins (SSPs) has been predicted from a wide range of secretome (Fig. 1.12). Saunders et al. 2012 considered functional annotation, detection of novel effector motifs and annotation of effector features for SPs. Functional annotation allows SPs slelction with no conserved protein domain families (PFAM) with the exception of avirulence proteins which may have such domains. Then they applied MEME tool to detect *de novo* conserved patters over SPs in rust. The *Melampsora Genome Consortium* (Duplessis et al. 2011a, Hacquard et al. 2012) considered a manual curation of SSPs (i.e. <300 amino acids) and intron/exon structural homology and conserved cysteine patterns, and revealed 1,184 SSPs.

In the third step, criteries has been considered for the selection of CSEPs from a wide range of SSPs (Fig. 1.12). Therefore, features such as expression pattern during infection or haustoria formation, protein size, cysteine residues, presence of signatures homology to previously reported rust Haustorially Expressed Secreted Proteins (HESPs), organization in gene families were taken into account to prioritize CSEPs (Hacquard et al. 2012, Lorrain et al. 2015, Petre et al. 2014, Saunders et al. 2012). A common criterion over all rust genomes is that they encode genes for SPs, but investigations with potential criteria were considered for being CSEPs. Alike oomycetes effectors conserved motif (RXLR), a common motif ([YFW]xC) has been reported within sequence of *P. graminis* f. sp. *tritici* (Godfrey et al. 2010). [YFW]xC motif is also present within CSEPs sequences in *M. larici-populina (Duplessis et al. 2011a, Hacquard et al. 2012)*. However, functional, structural illustration and role in translocation for [YFW]xC has not been demonstrated so far (Petre et al. 2014). In the fourth step, criteries has been considered for the top priority CSEPs (Fig. 1.12). To this end, hierarchical clustering was performed for ranking the tribes with the highest priority of containing CSEPs (Saunders et al. 2012). By doing so, Saunders and collaborators were able to derive four clusters with the most promising SP tribes that could possibly correspond to CSEPs for further studies. One of the largest tribe consists 92 members in one of those clusters is specific to *M. larici-populina*. This tribe containing large proportion of SPs and corresponds to the largest poplar rust SSPs family with 111 members as reported by Duplessis et al. (2011a). Recently Petre et al (2015) used effectoromics pipeline and identified priority *M. larici-populina* CSEPs for *in planta* expression studies in *Nicotiana benthamiana* as a powerful heterologous model system to study their subcellular acculation in plant, and to identify potential interactors in plant. Petre and collaborators gave stronger weight to some of the criteria used by two studies reported earlier (Duplessis et al. 2011a, Saunders et al. 2012). Redundant family members were removed to emphasis on orphan and lineage-specific CSEPs, given that pathogenicity mechanisms indicate highly specific functions. This criterion directed to a subset of 24 priority CSEPs from initialy identified 1,184 SSPs of *M. larici-populina* (Petre et al. 2015b). All 24 prioritized CSEPs are essential for the further functional

characterization to unravel the molecular interaction between *M. larici-populina* and plant.

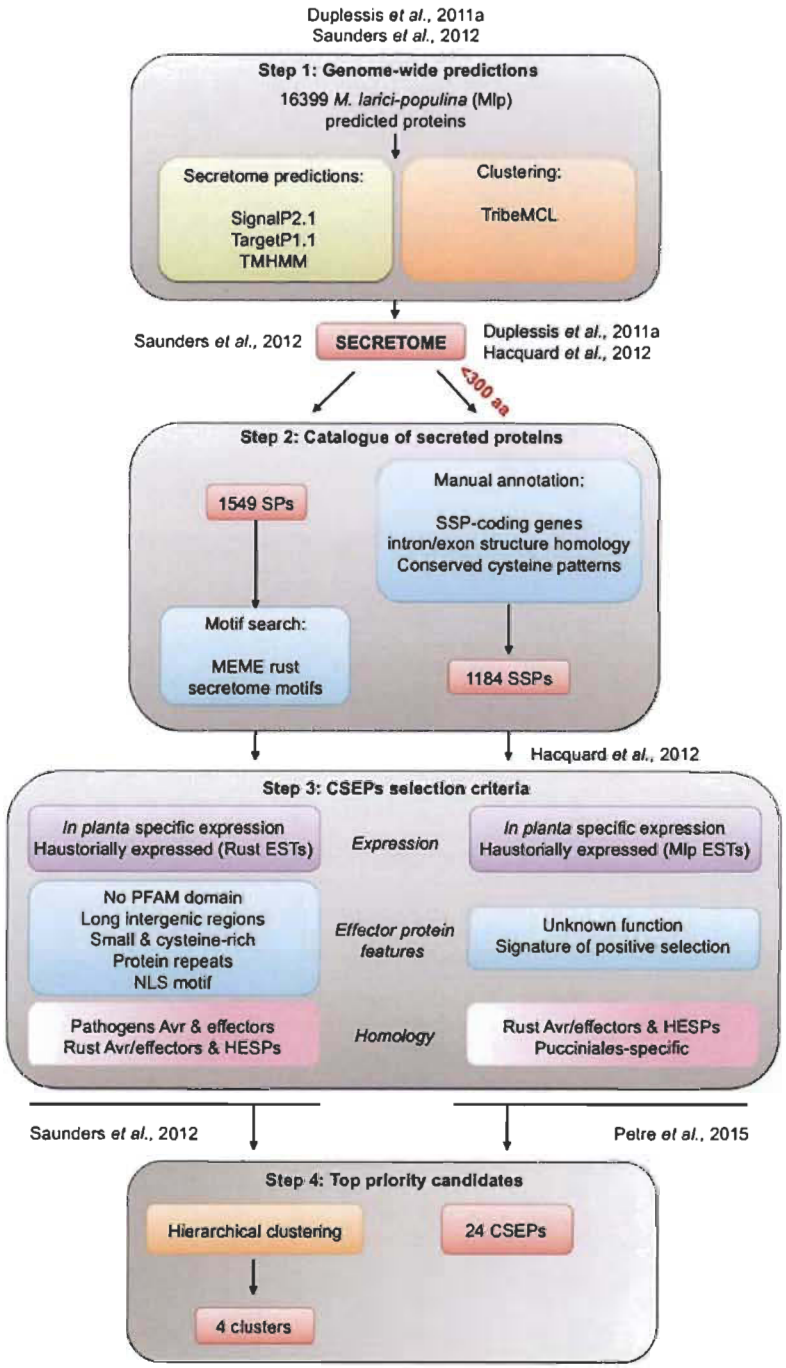
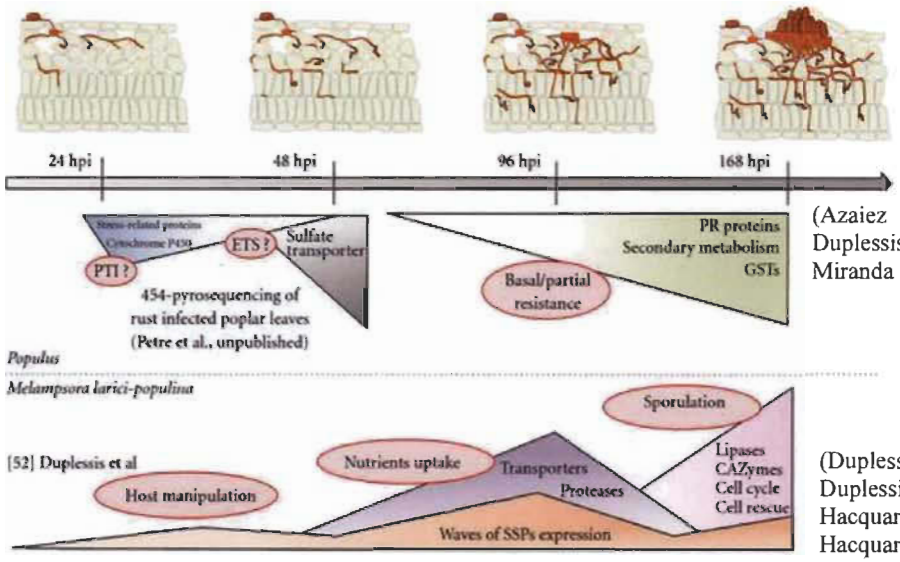


Fig. 1.12 Pipelines of effector mining of *M. larici-populina* for prioritizing CSEPs. *M. larici-populina* effector mining pipeline comprises four major steps: step 1: *Genome-wide predictions* identify the secretome of *M. larici-populina* using prediction tools (green) and clustering of gene families with TribeMCL

(orange); step 2: *Catalog of secreted proteins* identifies a set of secreted proteins; step 3: *CSEPs selection criterie* identifies CSEPs by different characteristics; and step 4: *Top priority candidates* prioritizes CSEPs for further functional studies. Avr=avirulence protein; EST=expressed sequence tag; SP=secreted protein; SSP=small secreted protein; HESP=haustorially expressed secreted protein. (Adapted from Lorrain et al. 2015.)

1.10 Know-how from the genome and transcriptome of poplar and *M. larici-populina*

Genome sequencing of *M. larici-populina* (Duplessis et al. 2011a) provided access to 1,184 small secreted peptides and DNA sequences which enables functional characterization of potential CSEPs (Hacquard et al. 2012). Several research groups (Azaiez et al. 2009, Boyle et al. 2010, Miranda et al. 2007, Rinaldi et al. 2007, Levée et al. 2009) have studied the transcription profiling on poplar-*M. larici-populina* interactions. These studies revealed that for the period of incompatible interaction (resistant plant), early induction of defence responses occurred, i.e. host-specific resistance. Conversely, during compatible interaction, late induction of defence responses ensued, i.e. partial resistance (Fig. 1.13) (Azaiez et al. 2009, Boyle et al. 2010, Miranda et al. 2007, Rinaldi et al. 2007, Levée et al. 2009).



(Azaiez et al. 2009, Duplessis et al. 2011b, Miranda et al. 2007)

(Duplessis et al. 2011a, Duplessis et al. 2011b, Hacquard et al. 2010, Hacquard et al. 2011b, Joly et al. 2010)

Fig. 1.13 Major transcriptome regulations in a compatible interaction of Poplar-*M. larici-populina*.

Transcriptome studies on poplar-poplar rust. A set of induced fungal genes broadly differs during colonization in host with preferential transcript expression at early time points (24-48 hpi, haustoria formation); intermediate time points (48-96 hpi, biotrophic growth phase), and late time points (96-168 hpi, biotrophic phase, uredinia formation and sporulation stage). Triangles indicate genetic programs assembled by Poplar (top) and *M. larici-populina* (bottom). Red circles represent concomitant biological functions. PTI=PAMP-Triggered Immunity; SSPs=Small Secreted Proteins; CAZymes=Carbohydrate-Active Enzymes. (Source: Hacquard et al. 2011a.)

Whole genome oligoarray and transcriptome profiling of *M. larici-populina* during asexual stages on poplar leaves at different time course of infection revealed dynamic gene expression pattern concomitant with virulence or host-specific resistance (Duplessis et al. 2011b). Duplessis et al. (2011b) reported that 76% of *M. larici-populina* transcripts were detected during leaf infection stages and 20% were only detected *in planta* which includes few transporters and small secreted proteins (SSPs). In the case of compatible interactions, transcription profiling of poplar leaves infection revealed suppression of some genes encoding defence and secondary metabolism enzymes at 18 hpi, 24 and 48 hpi. Aforesaid feature could imitate ETS which upholds *M. larici-populina* virulence through suppressing PTI. However, maximum ten genes were reported to be induced, but level of sulphate transporter gene was increased

(Fig. 1.13). This was surprising because sulphate pathway is compromised in rust fungi (Duplessis et al. 2011a). It opens perspectives to study transport, assimilation and metabolism of sulphate in poplar-*M. larici-populina* compatible interaction.

Laser Capture Microdissection (LCM) for poplar-*M. larici-populina* interaction was a key breakthrough which allowed to isolate haustoria and hyphae from the infected tissues (Hacquard et al. 2010). LCM-coupled transcriptome revealed highly expressed transcripts in *M. larici-populina* structures and nearly all encrypted SSPs. However, maintenance of *M. larici-populina* biotrophy might require this unexpected highly expressed number of candidate effectors during infection. Wide-ranging transcripts were reported as induced using LCM-isolated uredinia comprising diverse cell cycle and cell rescue related transcripts (Hacquard et al. 2010). Cell cycle and cell rescue related transcripts have specific role in defence mechanisms. Stimulation of cell cycle related transcripts reinforce the cell division activity around micro dissected sporulation area; on the other hand, transcripts related to cell rescue and defence imitate active defence during late activation of poplar responses, such as defence gene expressions or oxidative burst while poplar-*M. larici-populina* are in a compatible interaction (Fig. 1.12) (Boyle et al. 2010, Duplessis et al. 2009). Surprisingly, transcriptomics time course study identified some transcripts which are primarily distinguished in the sporulation area at 168 hpi (Duplessis et al. 2011b). However, some genes encrypting CSEPs and expressed in micro-dissected infected mesophyll cells were primarily expressed in infection hyphae and haustoria at 96 hpi (Duplessis et al. 2011b). Above-mentioned annotations support the transcriptional swapping between cell types in the infected host tissues during formation of uredinia and uredinospores. Therefore, combinational observations comprising genomics, transcriptomics and LCM established a comprehensive understanding of rust fungus transcriptome (like *Mlp*) from *in planta* expression.

1.11 Heterologous model system for effector studies

Heterologous system comprises the expression of gene in a host organism other than the original source, which is simpler than the natural source (Yesilirmak and Sayers

2009). *Arabidopsis thaliana* appeared as a model organism over three decades ago (Meyerowitz 1989, Meyerowitz 2001, Ossowski et al. 2008, Redei 1975) and its role in genetics resulted as a powerful model system in molecular biology (Koornneef and Meinke 2010) and in molecular plant-microbe interactions. *Nicotiana benthamiana* is another notorious heterologous model plant which is susceptible to various plant pathogens such as virus, bacteria, oomycete and fungi. Simplified and efficient transfection method enabled *N. benthamiana* for transient expression of proteins which attracted plant biologist rapidly (Chapman, Kavanagh and Baulcombe 1992, Escobar 2003, Goodin et al. 2005, Goodin et al. 2007, Goodin et al. 2002, Goodin et al. 2008).

Recently several groups have reported on the use of heterologous systems to investigate the function, localization, or interaction partners of effectors from biotrophic pathogens (Caillaud et al. 2012a, Caillaud et al. 2012b, Du et al. 2015, Gaouar et al. 2016, Petre et al. 2015a, Petre et al. 2016, Petre et al. 2015b, Kunjeti et al. 2016). Multiple effectors (HaRxLs) of *H. arabidopsidis* have been assessed on *Arabidopsis* to verify whether they manipulate host defence or not are illustrated in Fig. 1.14 (Fabro et al. 2011). Fabro and his colleagues developed transgenic *Arabidopsis* plants constitutively expressing HaRxLs effectors and infiltrated with bacterial pathogen *P. syringae* ΔavrPto/ΔavrPtoB, and spray inoculated with conidiospores of *H. Arabidopsis* isolate Noco2 (Fig. 1.14A & B). They quantified pathogenic responses to assess virulence (Fig. 1.14). They found that transgenic *Arabidopsis* expressing different HaRxLs effectors showed increased susceptibility to *P. syringae* ΔavrPto/ΔavrPtoB (Fig. 1.14A). Seven transgenic lines expressing effectors showed enhanced susceptibility to *H. arabidopsidis* isolate Noco2 (Fig. 1.14B) (Fabro et al. 2011). Moreover, HaRxLs effectors have also been stably expressed *in planta* in *Arabidopsis* and their nuclear accumulation promoted diverse phenotypes, and suppress nuclear processes mediated plant immunity (Caillaud et al. 2012b).

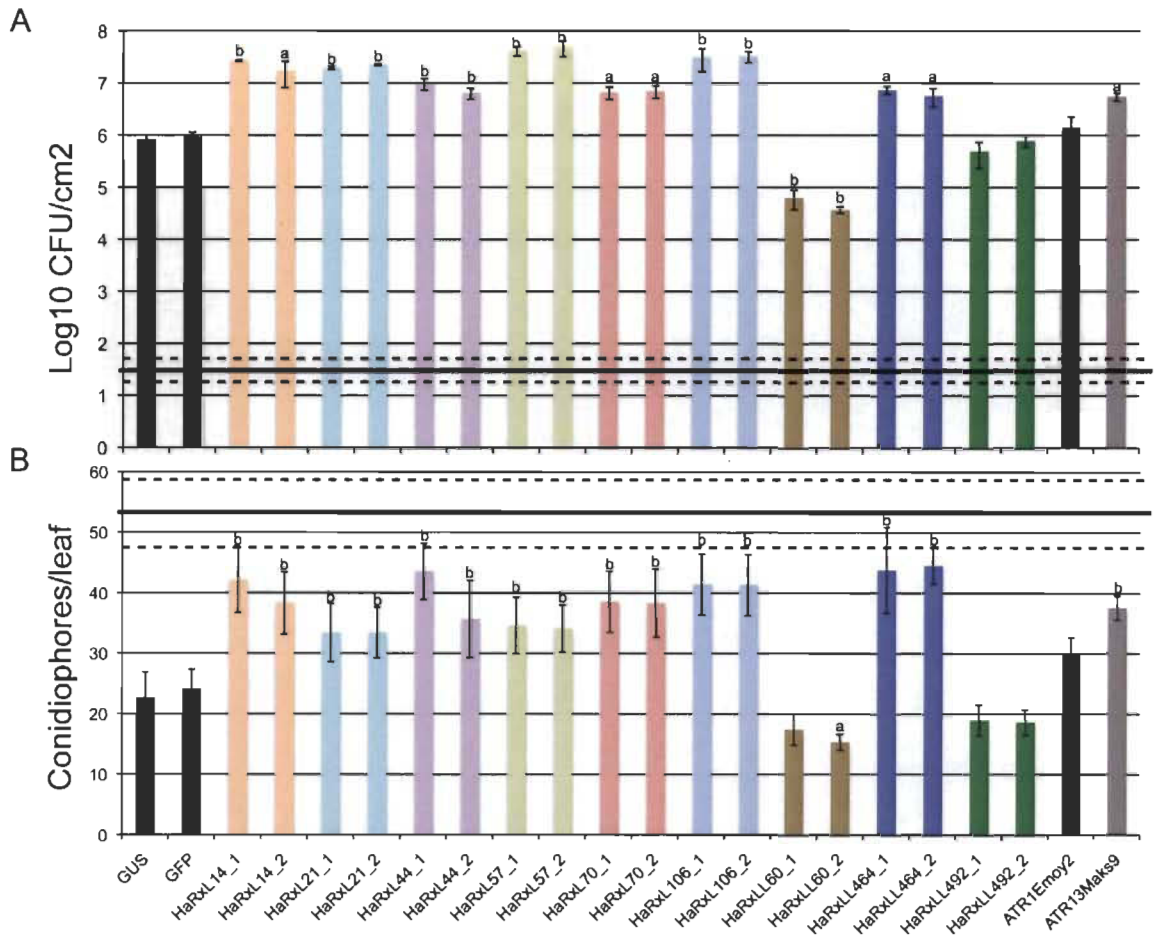


Fig. 1.14 ***Arabidopsis*** **plants expressing HaRxLs and response to pathogens.** Transgenic *Arabidopsis* Col-0 plants expressing HaRxLs effectors and their response against *P. syringae* ΔavrPto/ΔavrPtoB and *Hpa* isolate Noco2. (A) Two independent transgenic lines per HaRxL were infiltrated with *Pst*-ΔavrPto/ΔavrPtoB (OD_{600}=0.0005) and bacterial populations counted at 0 and 3 dpi (days postinoculation). (a) T-test p value<0.05, (b) T-test p value<0.01. (B) Seedlings were spray inoculated with a suspension of 1×10^4 conidiospores per ml of *Hpa* isolate Noco2. The number of conidiophores per leaf was counted in 4 leaves per seedling at 6 dps. The horizontal black and dashed lines represent the average ±2 × SE of the number of conidiophores per leaf found in the hyper-susceptible mutant Col-0 eds1-2. (a) T-test p value<0.01, (b) T-test p value<0.05. (Source: Fabro et al. 2011.)

Molecular understanding of effectors and resistance proteins accumulation and interaction is essential for unraveling plant-pathogen interactions. Hence, subcellular localization studies of *P. infestans* RXLR effector AVR1 and potato late blight resistance protein R1 has been studied in *N. benthamiana*. Both R1 and AVR1

accumulates in nucleus and cytoplasm and arbitrate R1 mediated resistance (HR) when R1/AVR1 in a close proximity in the nucleus (Du et al. 2015). Using *N. benthamina*, twenty candidate effectors from *M. larici-populina* have been tested for subcellular localization and protein interactions (Petre et al. 2015b). *M. larici-populina* target diverse subcellular compartments and coimmunoprecipitation (CoIP) assay coupled with Mass Spectrometry (MS) identified 606 interacting proteins in *N. benthamiana* (Petre et al. 2015b). Sixteen candidate effectors from *P. striiformis* f. sp. *tritici* have been considered for subcellular localization and protein-protein interaction studies using *N. benthamiana* as a heterologous model (Petre et al. 2016).

Using *A. thaliana* virulence assay of Mlp124202, a *M. larici-populina* effector confirmed that it does not modify the susceptibility of *A. thaliana* to *P. syringae* pv. *tomato* DC3000. Green Fluorescent Protein (GFP) tagged Mlp124202 accumulated within membranes and cytoplasm of *A. thaliana* and *N. benthamiana* and was hypothesized to be involved in vesicle mediated cellular trafficking (Gaouar et al. 2016).

1.12 Subcellular localization of effectors

Subcellular localization of effectors is certainly essential for the cellular reprogramming and to modify immunity. Since subcellular accumulation of all rust effector proteins has not been completely understood, a model has been sketched to draw better understanding of subcellular accumulation of oomycetes effectors (Fig. 1.15) (Catanzariti et al. 2007). Upon attachment onto host, pathogen forms appressorium throughout apoplastic spaces and then develop haustoria which cross cell wall but not cell membrane. First effector proteins are being secreted into the extrahaustorial matrix (space between haustorial membrane and plasma membrane). Later on effectors translocate into the host cellular compartments in different ways, such as (I) it directly cross extrahaustorial membrane, (II) or use vesicles in the cell membrane as cargo. Either ways, once effector proteins are inside the host cell, it may manipulate host metabolism or trigger host defence. Some other effectors may target another subcellular compartments or may further translocate into the nucleus and alter

transcription. (III) effectors secreted in the apoplastic spaces may enter adjacent cell via an unknown mechanism and may be recognized by resistance protein to trigger defence.

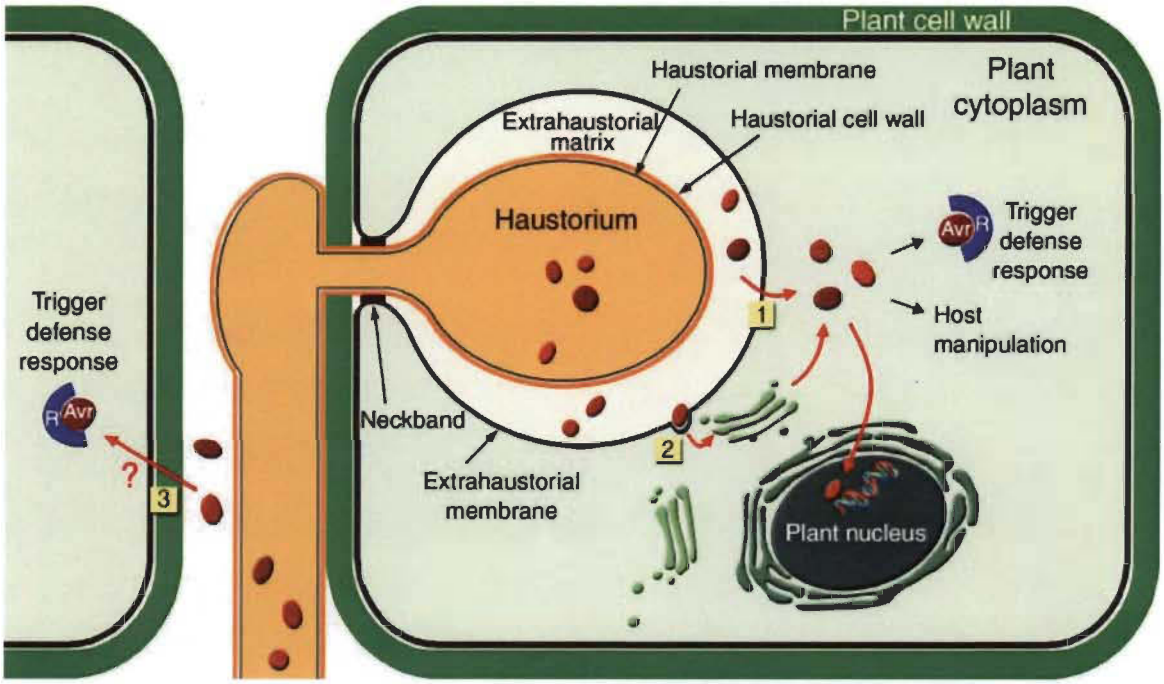


Fig. 1.15 Schematic diagram showing host, haustoria and effectors distribution. Being secreted from haustoria, effectors comes to extrahaustorial matrix (EHM) and transported within host cell in several ways: (1) effectors directly cross the extrahaustorial membrane (EM); (2) pass EM using vesicles. Once effectors are in the cytoplasm, it may trigger defence responses or manipulate host metabolism. Effectors may be recognized by resistance proteins (R) which are known as avirulence proteins (Avr) which triggers a defence response. Conversely, some effectors may be translocated into other cellular organelles such as nucleus which might modify host transcription; (3) occasionally effectors are secreted into apoplast and can arrive host cell over an unknown mechanism. (Source: Catanzariti et al. 2007.)

Effectors can be divided into diverse groups based on their localization in the host cell; such as apoplastic, cytoplasmic and nuclear or nucleolar localized effectors (Chaudhari et al. 2014). Apoplastic effectors are localized to the extracellular spaces and primarily inhibits host proteases, hydrolases, glucanases and other lytic enzymes (Giraldo and Valent 2013). Cytoplasmic effectors are dealing with host cytoplasm to target defence signaling and in some cases it may use cytoplasm as passage to reach cellular organelles as their final destination.

P. infestans, the causal agent of late blight disease in potato and tomato belongs to filamentous oomycetes which deploys a large group of effectors targeting multiple subcellular compartments in host due to their huge diversity in structure, sequence and mechanisms for the trafficking to the cytoplasm. AVR1 is an effector protein molecule of RXLR effectors family secreted by *P. infestans* and delivered inside into the host cell (potato and tomato). The virulence activity of AVR1 is to suppress cell death. However, if the plant genotype contains the resistance protein R1, then Avr1 is recognized, ETI is launched and results in HR. Sometimes specific sequence is required for translocating effector proteins within subcellular compartments. For R1-Avr1 interaction, host targeting is governed by N-terminal translocation domains followed by a general secretory signal peptide containing a conserved RXLR motif (Jiang et al. 2008). Sometimes, such as N-terminal domain of ARR3a effector from *P. infestans* is required for translocation in potato cells (Whisson et al. 2007). Whisson and collegues (2007) also showed that RXLR domain functions as a leader sequence and mediates the cellular targeting.

HaRxL17, a HaRxL effector of *Ha* is associated with the tonoplast in the uninfected cells, but in infected cells it localizes to the extra-haustorial membrane. Some RXLR effectors (HaRxLL3b, HaAtr13 Emoy2 and HaRxL44) localizes to the plant nucleolus. Nuclear localized effectors potentially reprogram transcription in host cells for their own benefit. For example, 33% of putative effectors from *Ha* are strictly localized to the nucleus and other 33% are nucleo-cytoplasmic (Caillaud et al. 2012a). CRN effectors from *P. capsici* localize to the nucleus, but only CRN1_719 effectors accumulate in the nucleolus which specifies the involvement of subnuclear domains. On the other hand CRN79_188 accumulate around the nucleolus and unknown nuclear bodies (Stam et al. 2013).

1.12.1 Subcellular localization of *M. larici-populina* effectors

To better understand the molecular background of leaf rust caused by *M. larici-populina*, it is important to study the subcellular accumulation of *M. larici-populina*

effectors in the host cell (Petre et al. 2015b). Using *M. larici-populina* effectoromics pipeline and transient expression in *N. benthamiana*, twenty candidate effectors subcellular localization have recently been studied (Petre et al. 2015b). Out of twenty, fifteen candidate effectors localized to nucleus, the five others localized to the nucleolus, the choloroplasts and cytosolic bodies (Table 1.7, Fig. 1.16) (Petre et al. 2015b). It should be noted that some of the effectors listed in Table 1.7 were also studied in our laboratory, in fact, *Mlp124478* is the gene investigated in this thesis.

Table 1.7. Subcellular accumulation of candidate effectors of *M. larici-populina.*

Protein ID	Family	CPG or class	Accumulation pattern
Mlp102036	SSP42 (5)	CPG2528	Nucleus and cytosol
Mlp105684	SSP72 (3)	CPG1133	Nucleus and cytosol
MLP106985	SSP79 (3)	CPG335	Nucleus and cytosol
Mlp107772	-	-	Chloroplasts, mitochondria
Mlp109567	-	-	Nucleus
Mlp123227	SSP102 (2)	CPG1059	Nucleus and cytosol
Mlp123524	SSP142 (2)	CPG3994	No accumulation
Mlp123532	SSP146 (2)	CPG4557	Nucleus and cytosol
Mlp123731	SSP8 (12)	CPG423	No accumulation
Mlp124017	-	-	Nucleus and cytosol
Mlp124111	SSP15 (8)	Class III	Chloroplasts, cytosolic aggregates
Mlp124266	SSP6 (13)	CPG5464	Nucleus and cytosol
Mlp124353	SSP12 (10)	CPG4890	Nucleus and cytosol
Mlp124371	SSP57 (4)	CPG3477	Nucleus and cytosol
Mlp124478	SSP14 (9)	CPG2811	Nucleolus
Mlp124497	SSP3 (32)	CPGH1	Nucleus and cytosol
Mlp124499	SSP3 (32)	CPGH1	Nucleus and cytosol
Mlp124530	-	-	Nuclear and cytosolic bodies
Mlp124543	SSP4 (17)	CPG510	Nucleus and cytosol

Protein ID	Family	CPG or class	Accumulation pattern
Mlp124561	SSP64 (4)	CPGH4	Nucleus and cytosol
Mlp37347	-	-	Cytosolic bodies
Mlp64894	-	-	No accumulation
Mlp67606	SSP54 (4)	CPG1252	Nucleus and cytosol
Mlp91075	SSP7 (12)	CPG332-333	No accumulation

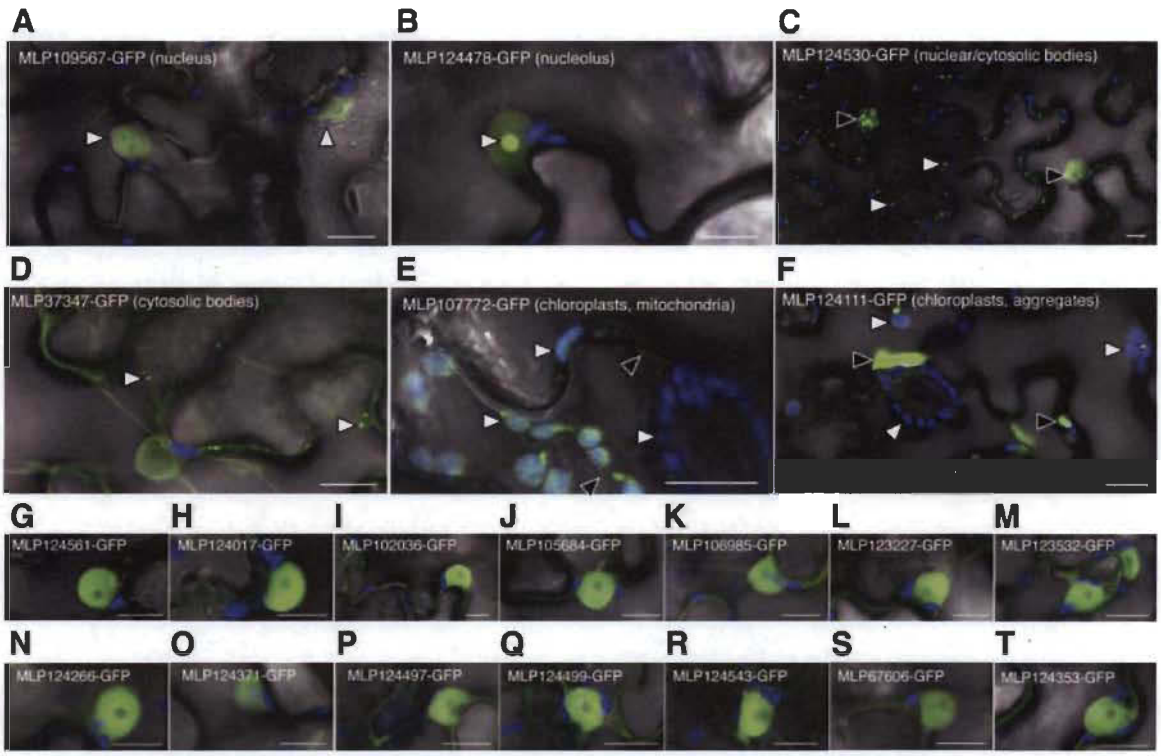


Fig. 1.16 Transient expression of tweenty candidate effectors of *M. larici-populina*. A to F represents subcellular accumulation of six candidate effectors of *M. larici-populina* in diverse subcellular compartments indicated in parentheses. G to T shows nuclear and cytosolic accumulation of 14 candidate effectors corresponding to their fusion proteins. White arrowheads mark nuclear bodies (A), nucleolus (B), cytosolic bodies (C,D) and chloroplasts (E, F). Black arrowheads indicate nuclear bodies (C), mitochondria (E) and large cytosolic aggregates (F). (Source: Petre et al. 2015b.)

Recently our laboratory also used transient expression and stable transgenic to show that *M. larici-populina* effector Mlp124202 accumulated with membrane and cytoplasm of plant cells (Gaouar et al. 2016).

Virulence effect of *M. larici-populina* in host plants depends on the cumulative functional effects of its diverse effectors including their interaction with host proteins or how they manipulate host subcellular programs. Therefore, it is certainly important to uncover the subcellular accumulation of diverse effector proteins.

1.12.2 Nuclear localized effectors

Subcellular localization of effector proteins can be predicted using numerous bioinformatic softwares (PSORT II, WoLF, NOD, NLStradamus), however, not all of them have been confirmed experimentally (Horton et al. 2007, Nair and Rost 2005). Translocation of effector proteins containing Nuclear Localization Signal (NLS) into nucleus is an active cellular process (Nigg, Baeuerle and Lührmann 1991). NLS is not stringent to any domain (N/C terminus) of the effector protein sequence, but most NLS motif are rich in lysine (K) and arginine (R) either in monopartite or bipartite (K-R/K-X-R/K) (Chelsky, Ralph and Jonak 1989, Dingwall and Laskey 1991). NLSs can be positioned anywhere within the protein sequence in single or multiple copies, although multiple copies might have additive effect (Garcia-Bustos, Heitman and Hall 1991). Some transcription factors of viral pathogens bear NLS and enter into the nucleus to modulate gene expression (V Citovsky and Zambryski 1993).

A number of secreted effector proteins have been documented as nuclear localized proteins. *Xanthomona* sp secretes large number of avirulence (*avr*)/pathogenicity (*pth*) genes which possess NLSs. Monopartite NLS motifs has been identified at the C-terminus amino acid sequences of PthA, Avrb6, AvrBs3 and AvrXa10, and induces canker synmptoms on citrus (Yang and Gabriel 1995). One class of *H. arabidopsidis* effectors (HaRxLs) localize to the nucleus (Caillaud et al. 2012a). *C. graminicola* Effector Protein 1 (CgEP1) is being synthesized at the early stage of infection; it possesses an NLS which enhances the anthracnose development in maize (Vargas et al. 2016). Several CRNs effectors possess C-terminus NLS and *in planta* transient assay in *N. benthamiana* confirmed the requirement of NLS for CRN8 subcellular accumulation to nucleus to induce plant cell death (Schornack et al. 2010).

The nucleolus is inside the nucleus of a cell and is only present in eukaryotic cells. In the nucleolus, DNA is transcribed into rRNA, and these rRNAs are combined with proteins to assemble the large and small ribosomal subunits. These subunits are then exported from the nucleolus to nucleus and through the endoplasmic reticulum to the cytosol in order to carry out protein synthesis (Venema and Tollervey 1999). Generally one or more specific stretches of basic amino acids are needed for nuclear/nucleolar localization signals (NLSs/NoLSs). Now it is understood that NoLS is embedded within NLS (Savada and Bonham-Smith 2013). Recent study on *Arabidopsis* showed the effect of mutation within NLS/NoLS on subcellular protein accumulation (Savada and Bonham-Smith 2013). Savada and collaborators mutated all eight NLS/NoLSs from RPL23aA individually and also in groups and showed transient expression in tobacco cells. They observed that nucleolar accumulation of RPL23aA was disrupted by mutation of several combinations of five or more NLSs/NoLSs. When all eight pNLSs/NoLSs are mutated, in total basic charge of RPL23aA has been reduced 50%, resulting in the complete disruption of nucleolar localization, but the protein can still localize to the nucleus. Since no individual or specific combination of NoLSs was absolutely required for nucleolar localization, they suggested that nucleolar localization or retention of RPL23aA is dependent on the overall basic charge. On the other hand, in *Arabidopsis* RPS8A and RPL15A, mutation of just two and three N-terminal NLSs disrupted both nuclear and nucleolar localization of these two proteins. The latter result indicated the requirements of differential signal for nuclear and nucleolar localization of *Arabidopsis* RPL23aA, RPL15A and RPS8A proteins (Savada and Bonham-Smith 2013).

1.13 Effectors suppression of PTI

To avoid plants basal resistance barrier, pathogen often suppress PTI by delivering effectors inside the host cells. T3SS of *P. syringae* translocate effectors into the host to modulate host cellular responses. *P. syringae* effector AvrPto is being secreted via T3SS, binds to FLS2 and EFR in *Arabidopsis* and LeFLS2 in tomato and promotes infection in susceptible plants (Xiang et al. 2008). HopAI1, broadly conserved bacterial

pathogenic effector from *P. syringae* in both animals and plant, inactivates PAMPs by eliminating the phosphate group from phosphothreonine through phosphothreonine lyase activity, which is necessary for HopAI1 function (Zhang et al. 2007). A cell based genetic screening of effectors of *P. syringae* revealed that AvrPto and AvrPtoB arrest multiple PAMP mediated signalling at the upstream of MAPKs. Both AvrPto and AvrPtoB showed suppression of flg22 activation of *FRK1-LUC*, a PAMP reporter gene (He et al. 2006). Several assays revealed that bacterial effectors suppress PTI, the early defence of plants, such as HopPtoD2, HopPtoE, HopPtoK, AvrPto (DC) and AvrPtoB from *P. syrinage* DC3000. They suppress several host activities related to defence (Abramovitch et al. 2006, Espinosa et al. 2003, Hauck, Thilmony and He 2003, He et al. 2006).

1.13.1 Effectors and host cellular reprogramming

Once pathogenic effectors are within host cell, emerging evidence confirmed that it may perturb normal cellular processes resulting in visual changes on host (Fevre et al. 2015). *Xanthomonas* effectors (AvrBs3/PthA) or transcription activator-like (TAL) family proteins functions as transcription activators, target the host nucleus and interfere with transcription to alter plant immune responses. It changes host gene expression levels (Boch et al. 2009, Yang and Gabriel 1995, Gu et al. 2005, Zhang, Yin and White 2015). PITG_03192, a RXLR effector from *P. infestans* localize into host endoplasmic reticulum and interact with NTP1 and NTP2 (NAC transcription factors), and following to the suppression of P/MAMP, it prevents translocation of the effector into nucleus upholding disease progression at the end (McLellan et al. 2013). *H. arabidopsidis*, a filamentous obligate biotrophic pathogen, has effectors that target the nucleus. One of them, HaRxL44, goes to the nucleus and interacts with the Mediator complex MED19a, inducing its proteasome-mediated degradation. This, in turn, leads to transcriptional changes resembling jasmonic acid and ethylene induction with repressed salicylic acid signaling enhancing susceptibility to biotrophs (Caillaud et al. 2013).

In the case of fungus, spores germinate and form haustoria. Recent findings with *C. bigginsianum-A. thaliana* pathosystem revealed that upon haustoria formation, fungal pathogens acquire nutrients via haustoria and release effectors from its emergent hyphae which may influence host cellular processes (Kleemann et al. 2012). Transcriptome profiling of *M. larici-populina* revealed that SSPs expressed in resting spores (uredeniospores) (Duplessis et al. 2011b), are afterward secreted into the host cell and accumulate in diverse subcellular compartments (Petre et al. 2015a, Petre et al. 2015b) to modulate host processes. Until recently, not much was known on the how *M. larici-populina* effectors reprogram host subcellular processes.

Several WRKY transcription factors exert vital roles in transcriptional reprogramming of defence response genes (Eulgem and Somssich 2007, Pandey and Somssich 2009), hence effector may target WRKY to alter plant immunity such as is demonstrated by Pop2. C-terminus WRKY domain containing TIR-NBS-LRR type R protein RRS1-R/WRKY52 interacts with PopP2 effector of *Ralstonia solanacearum* in the nucleus (Deslandes et al. 2003, Deslandes et al. 2002, Tasset et al. 2010, Lahaye 2004). RRS1-R/WRKY52 is identical to *SENSITIVE TO LOW HUMIDITY* 1 (*SLH1*) and an inactive form of *SLH*1 acts as transcriptional repressor of several downstream defence responsive genes, such as *PR1, PR2, PR5, EDS1* and *PAD4* (Noutoshi et al. 2005). Effectors DNA binding domain can provoke transcriptional reprogramming of defence genes. For example, PopP2 effector is recognized by RRS1-R/WRKY52 and induces downstream resistance signaling either through suppressing negative function of its WRKY DNA binding domain or by activating other positive transcription regulators (Lahaye 2004). In this case the WRKY domain of RRS1 serves as a decoy for Pop2, the interaction leads to the immune activation of RRS1.

CgEP1 effector of *C. graminicola* bears NLS and DNA-binding domain. It localizes into nucleus and specifically expressed in primary hyphae during biotrophic stage. Pathogenicity assay using CgEP1 knockout strain, Ko14 and Ko20 revealed that the effector CgEP1 is necessary for the development of anthracnose disease in leaves, stalks and roots. Since CgEP1 has DNA-binding domain, Chromatin

Immunoprecipitation Assay (ChIP) followed by cloning and BLAST database of DNA sequences at the 2000 bases upstream of translational start site (TSS) of maize gene models confirmed that by binding to DNA it enhances the anthracnose development in maize at the early stage of development (Vargas et al. 2016). Dynamic interplay is underway to unravel the effectors role in rewiring of cellular processes which will enrich our understanding of molecular interaction of plant and pathogens.

1.14 Specific problematic

For deeper understanding of the Mlp-host pathosystems, it is important to unravel the molecular functions of each effector. So we thought few questions are needed to be answered, which are as follows:

- How does Mlp124478 accumulate in plant?
- Does it manipulate plant defense?
- If Mlp124478 manipulate host defence, then how does it occur?

1.15 Objectives

To address the questions stated in the section 1.14, we have selected objectives.

Objective I: Study the effect of the effector Mlp124478 on plant morphology.

- Selection of candidate effector from Mlp CSEPs
- *In silico* analysis of the selected effector

Objective II: Study of the subcellular localization and role in virulence.

- Subcellular accumulation of Mlp124478 using two different model plants
- Assess the role of effector in virulence

Objecive III: Study the role of Mlp124478 in host manipulation.

- Alteration of plant cellular processes in response to Mlp124478

In this study, we focused on the functional studies of Mlp124478, a poplar leaf rust effector. We used *in planta* pathogen assays, genotyping, live-cell imaging, comparative transcriptomics, protein-nucleic acid interaction and yeast two hybrid assay to infer the functional nature of Mlp124478. Live-cell imaging in *A. thaliana* and *N. benthamiana* revealed that Mlp124478 proteins accumulate in the nucleus and nucleolus, while transcriptomics suggests that, by binding to DNA, Mlp124478 reprograms plant transcription, thereby altering host susceptibility. Taken together, we proposed that Mlp124478 manipulates plants by remodeling transcription via direct DNA-binding, suppress normal transcriptional responses to pathogens, and mislead the host into up-regulating the expression of genes unrelated to defence.

CHAPTER II

MATERIAL AND METHODS

2.1 Plant Materials and growth conditions

A. thaliana and *N. benthamiana* plants were soil-grown (Fafard's Agromix soil) in a growth chamber (BioChambers) under a 14 h/10 h light/dark cycle with temperature set at 22°C and relative humidity of 60%. *A. thaliana* accession Col-0 was used as wild type in all experiments. GFP was transformed in Col-0 background (i.e. Col-0-GFP) and used as GFP control in the subcellular localization experiment. Seeds were sterilized using seed sterilization solution (1-5% bleach and 0.1% Tween 20), kept at 4°C for 48 h before placing on petri dishes or soil. The transgenic plants were germinated in Petri dishes for the selection of single-insertion homozygous transgenic *Mlp124478* with ½ Murashige and Skoog medium containing 0.6% agar and 15 mg/ml Basta. Salk T-DNA insertion line (SALK_017461) of AT4G14830.1/heat shock protein 20-like protein 1 was obtained from Arabidopsis Biological Resource Center (ABRC), Columbus OH, USA.

2.2 Growth of *P. syringae* pv. *tomato* and infection assay

Pseudomonas syringae pv. *tomato* (*Pst*) strain DC3000ΔCEL (*Pst*DC3000ΔCEL) (Alfano et al. 2000) containing or not *Mlp124478* was grown overnight and infiltrated in leaves of 4-weeks-old Col-0 and transgenic *Mlp124478* plants at optical density of 0.001 at 600 nm (OD600). Prior to inoculation, bacterial growth was assessed at different times by OD_{600} measurements. *Pst* infections were performed by syringe infiltration using bacterial suspension containing or not *Mlp124478* in 10 mM $MgCl_2$ in 4-weeks-old *Arabidopsis* plant leaves (Katagiri, Thilmony and He 2002). At day 0 and day 3, three samples (6 mm size of four leaf discs/sample) were harvested from infiltrated leaves for each genotype, ground in 0.5 mL $MgCl_2$, serial dilutions were plated on

LB medium with appropriate antibiotics. Bacterial colony forming units (cfu) were counted at 36 h after incubation at 28°C. Experiments were repeated three times.

2.3 *H. arabidopsidis* Noco2 infection

H. arabidopsidis strain Noco2 infection assay was performed as described previously (Jing et al. 2011). *H. arabidopsidis* Noco2 isolates were maintained in a growth chamber under a 10 h/14 h light/dark cycle at 16°C and relative humidity of 60%. Two-weeks-old Col-0, transgenic *Mlp124478* and *eds1* were spray inoculated with freshly isolated spores at a concentration of 4×10^3 spores/mL on the adaxial side of leaves. At 7 days-post inoculation (dpi), spores were counted in triplicates (spores/gFW [$\times10^3$]) using a hemocytometer.

2.4 Plasmid construction

For effector delivery via the type three secretion system (T3SS), stable transgenic *Arabidopsis* plants and transient expression in *N. benthamiana*-constructs were developed via Gateway cloning (Invitrogen, Life Technologies). *Mlp124478* coding sequence without its signal peptide (thereafter *Mlp124478ΔSP*) was ordered from GenScript in a lyophilized form (in pUC57) and primer pairs (Supplementary Table 2.1, Primers no 1-3) with a recombination sequence were used to amplify the open reading frame (ORF) of *Mlp124478*. The PCR amplicons were then cloned into the pDONR^{TM}221 vector (Supplementary Fig. 2.1) using Gateway BP recombination followed by recombination using Gateway LR reaction either into pVSP*Ps*Spdes vector for *Pst* infection assay (effector delivery) or pB7FWG2.0 (Supplementary Fig. 2.2) to generate stable transgenic *Arabidopsis* plants expressing C-terminal GFP tagged *Mlp124478* and agroinfiltration into *N. benthamiana*. pVSP*Ps*Spdes harbors the AvrRpm1 secretion signal (Rentel et al. 2008). Either empty vector pVSP*Ps*Spdes (Supplementary Fig. 2.3) or carrying *AvrRpm1-Mlp124478*ΔSP-HA constructs were transformed into *P. syringae* pv. *Tomato* DC3000ΔCEL.

2.5 Cloning of genes

Genes were cloned using Gateway cloning Technology (Karimi, Inzé and Depicker 2002) which completes in two recombination reactions: (I) BP recombination and (II) LR recombination (Fig. 2.1). In brief, (I) first ORF of *Mlp124478* was amplified from pUC57 using gene specific primer pairs comprising the recombination sites (*att*B1 to forward primer and *att*B2 to reverse primer). Equal amount (150 ng) of PCR amplicon and donor vector (pDONR^{TM}221) are added to the BP reaction mix (5X BP clonase reaction buffer, TE buffer pH8.0) and incubated for 3 h at room temperature (RT). Following to the addition of proteinase K solution, incubated for 10 min at 37°C. Then BP reaction mixture was transformed into *E. coli* DH10β, platted on Luria-Bertani (LB) agar with 50 µg/mL Kanamycin and incubated overnight at 37°C. (II) Miniprepped positive entry clone (gene recombined in pDONR^{TM}221) were added to the LR recombination reaction mixture (destination vectors: pB7FWG2.0/pK7WGR2.0, LR clonase II and TE buffer pH8.0). Similar to the BP reaction, LR recombination mix were incubated for 3 h at RT, followed by adding proteinase K solution, incubation for 10 min at 37°C and plated on LB agar with specific antibiotics. After overnight incubation positive clones were confirmed by colony PCR.

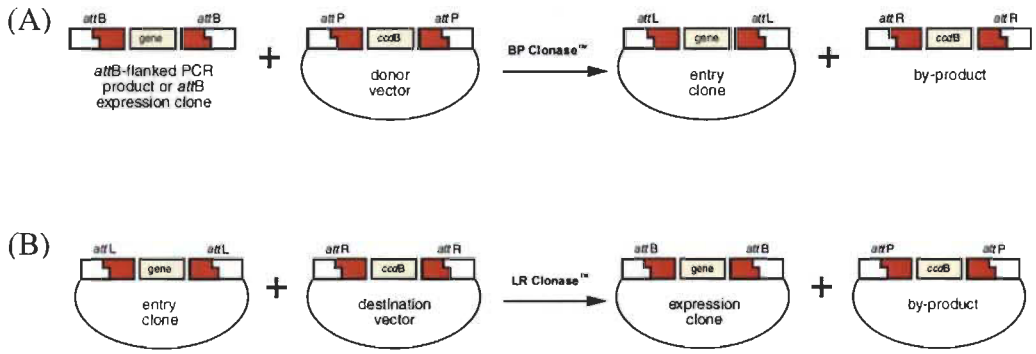


Fig. 2.1 Gateway recombination reactions.
(A) Recombination of an *att*B PCR products with an *att*P sites in donor vector to generate entry clone containing *att*L sites. (B) Recombination of entry clone comprising *att*L sites with destination vector consisting *att*R sites to generate expression clone containing gene of interest flanked by *att*B sites. (Source: Karimi, Inzé and Depicker 2002.)

2.6 Colony PCR

To confirm the genes cloned either in donor vector or in destination vector colony PCR was performed. For this purpose, single colonies were selected, picked from the agar plate using sterile pipette tip and the tip was rubbed at the bottom of a PCR tube. Then PCR master mix (5X Phusion HF buffer, 10 μM forward and reverse primers, 10 μM DNTPs, Phusion polymerase and nuclease free H2O) was added to the PCR tube having bacterial colony. PCR conditions were as follows 98°C for 3 min, 34 cycles of 98°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec; and finally 72°C for 5 min in a T100 thermal cycler machine (Bio-Rad, Canada). Reactions were electrophoresed at 100 volt for 45 min and finally visualized and image captured by ChemiDOcTM Touch Imaging Machine (Bio-Rad, Canada).

2.7 Generation of stable transgenic lines

Stable transgenic *A. thaliana* lines expressing the effector *Mlp124478* were developed by using *Agrobacterium tumefaciens* mediated floral dipping method reported previously (Mireault, Paris and Germain 2014). Briefly, destination vector containing C-terminal GFP tagged effector were transformed in *A. tumefaciens* strain C58C1 competent cells and grown overnight at 28°C in yeast extract peptone (YEP) medium supplemented with spectinomycin (50 mg/L). The cells were precipitated by centrifugation at 5000 rpm for 10 min and resuspended in 300 mL of 5% sucrose and 0.05% OFX-0309 (Norac Concepts Inc., Guelph, Ontario, Canada) in water and adjusted to OD_{600} of 0.8. Then the flower buds of 5 weeks old soil grown Col-0 plants were dipped in the solution of *A. tumefaciens* carrying effector. Plants were covered for 48 h and seeds were harvested 3 weeks later.

2.8 Transient expression

Solutions of *A. tumefaciens*-carrying recombinant plasmids were infiltrated into abaxial leaf pavement cells of 6-week-old *N. benthamiana* plants (Sparkes et al. 2006).

Briefly, *A. tumefaciens* C58C1-competent cells were transformed with pB7FWG2-containing *Mlp124478* and grown overnight in yeast extract peptone medium supplemented with spectinomycin (50 mg/L). The cells were precipitated by centrifugation at 5000 rpm and adjusted to OD_{600} of 0.5 in infiltration buffer (10 mM $MgCl_2$ and 150 μM acetosyringone). After 1 h incubation, the agro-suspension was infiltrated into the abaxial side of leaves, and the plants were returned to the growth chamber. At 4 dpi, water-mounted slides of epidermal peels from agro-infected leaves were visualized by confocal microscopy.

2.9 Microscopy

Cells were viewed by Leica TCS SP8 confocal laser scanning microscopy (Leica Microsystems). Images were obtained with HC PL APO CS2 40X/1.40 oil immersion objective, acquired sequentially to exclude excitation and emission crosstalk (when required). Leaf peels were immersed in water containing 0.2 μg/ml DAPI for 15 min for nuclei staining at room temperature. The samples were then observed at excitation/emission wavelength of 405/444-477 nm, 488/503-521 nm and 502/552-638 for DAPI, eGFP and eRFP, respectively. Images were annotated with LAS AF Lite software (Leica Microsystems).

2.10 Chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) assay

ChIP-PCR assays were conducted, as described previously, with minor modifications (Yamaguchi et al. 2014). ChIP-PCR was performed in six different steps (Fig. 2.2): briefly (I) crosslinking of protein and DNA by collecting 300 mg of 2-week-old *A. thaliana Mlp124478* stable transgenics and Col-0 in tubes containing 10 mL of phosphate-buffered saline (PBS). Then PBS were replaced by 10 mL of 1% formaldehyde to cross-link protein and DNA under vacuum infiltration. To quench the cross-linker, 0.125 M glycine was added after removal of formaldehyde, followed by vacuuming, incubation for 5 min, and tissue-rinsing with 10 mL cold PBS; (II) second

step was to isolate nuclei and shear chromatin, which has been performed by drying up cross-linked tissues on paper towel followed by adding nuclei extraction buffer (100 mM MOPS pH 7.6, 10 mM MgCl2, 0.25 M Sucrose, 5% Dextran T-40, 2.5% Ficoll 400) and freshly prepared protease inhibitors (1X) and β-mercaptoethanol (40 mM). Samples were grinded using Qiagen TissueLyser II (28 cycle/sec, 1 min), nuclei extract was collected after passing through Miracloth followed by spinning at 4°C for 5 min at 10,000 g. Then sheared chromatin was mixed with ChIP dilution buffer (16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.2 mM EDTA and 0.01% filter-sterilized SDS) without Triton. (III) The third step was chromatin fragmentation. Sheared chromatin was fragmented by using sonicator (5 times for 10 second each) and ChIP dilution buffer with Triton (16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100 and 0.01% filter-sterilized SDS) was added and fragmented chromatin were collected after centrifugation at 10,000 rpm for 5 min at 4°C. (IV) In the fourth step, sheared chromatin was immunoprecipitated with 50 µL/mL anti-GFP microbeads (MACS, Miltenyi Biotec Inc.) and incubated for 2 h at 4°C. The beads were placed in the µ-column, in the magnetic field of a µMACS separator, and washed twice. (V) the fifth step was to recover crosslinked DNA. To this end, reverse crosslinking was performed using nuclei lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0 and freshly prepared 1% SDS) and 5 M NaCl. After reverse crosslinking of DNA-protein, ChIP samples underwent DNA purification using QIAquick spin columns according to a previously-described method (Yamaguchi et al. 2014). Finally, purified ChIP sample proceeded for PCR amplification with specific primer pairs listed in Supplementary Table 2.1 (Primer Nos. 4-38).

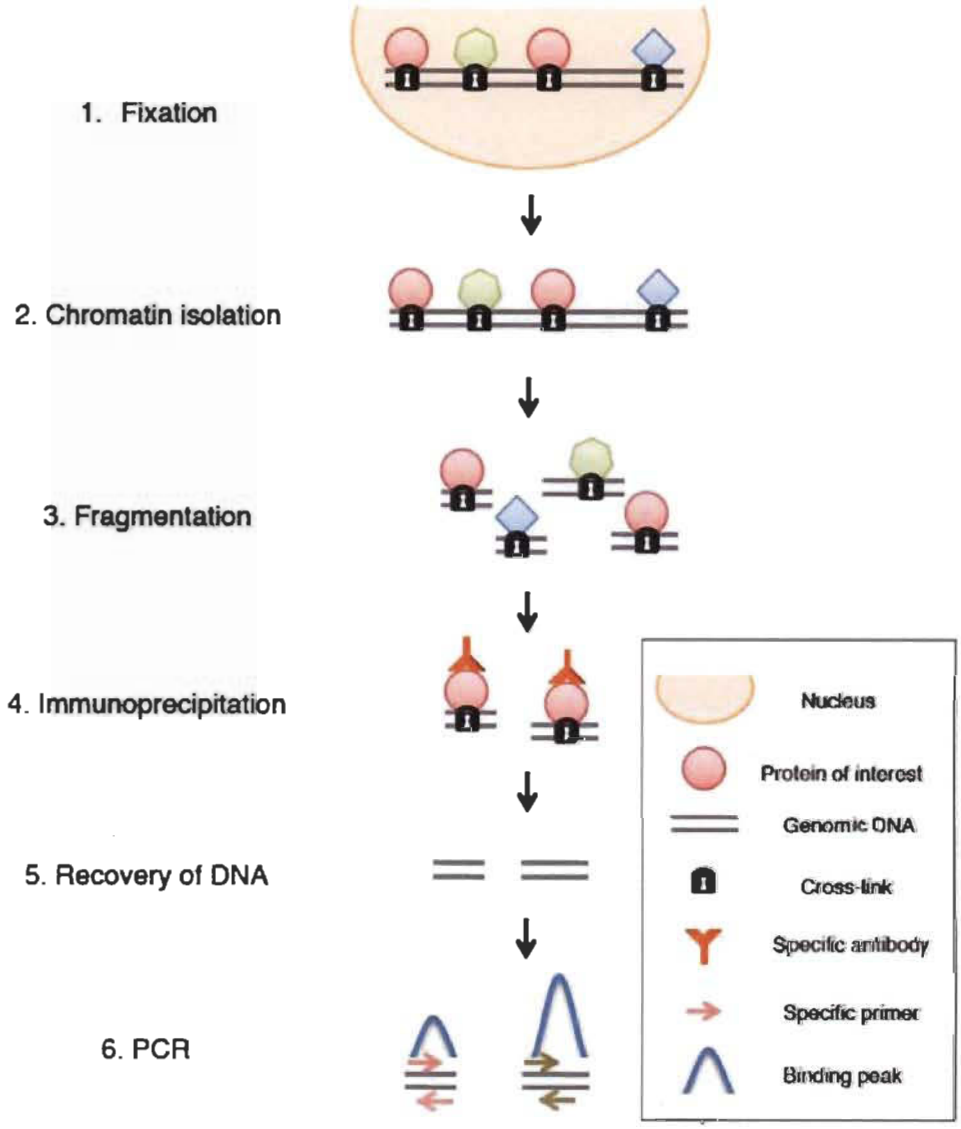


Fig. 2.2 Sketch of ChIP-PCR.
Protein and DNA were crosslinked with formaldehyde. Cells were lysed, chromatin from nuclei was isolated. Sheared chromatin were fragmented using sonication and immunoprecipitated with antibodies. Crosslinked chromatin was recovered, purified and DNA bound to the protein of interest was amplified by PCR. (Source: Yamaguchi et al. 2007.)

2.11 Electrophoretic mobility shift assay (EMSA)

EMSA was undertaken, as described earlier (Kass, Artero and Baylies 2000), with minor modifications. Unlabeled and digoxigenin (DIG)-labeled forward TGA1a

oligonucleotides were ordered from Integrated DNA Technologies. Double-stranded (DS) oligonucleotides were annealed by heating 1 nmol of each oligonucleotide at 95°C for 10 min, then slowly cooled down to 20°C. DS oligonucleotides were diluted in TEN buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8, 100 mM NaCl) to a final concentration of 50 pmol/μL. Dot blotting was carried out by serial dilutions and spotted on positively-charged nylon membranes to test efficiency of the DIG-labeled probe. 3 pmol of probe was found to be efficient for detection with anti-DIG primary antibody. Gel shift reaction was performed with 3 pmol of DS oligonucleotides and 100 ng of synthetic peptide in binding buffer (100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% Tween 20 and 150 mM KCl). After binding reaction at 25°C for 15 min, the samples were placed on ice for 15 min, and the mixtures were electrophoresed immediately through 0.25X TBE 20% polyacrylamide gel at 12.5 volts/cm. Bio-Rad semi-dry transfer cells were electroblotted on positively-charged nylon membranes at 25 volts for 10 min. DNA was then cross-linked to the membrane by baking at 80°C for 40 min. For DIG detection, the membranes were blocked in TBS [(50 mM Tris and 150 mM NaCl) + 1% BSA], followed by 2 washes with TBS for 10 min and 1 wash with TBST (TBS and 1% Tween 20), then incubated overnight at 4°C with anti-DIG monoclonal antibody diluted 1:1,000 in TBS + 1% BSA. The membranes were washed 4 times in TBS for 5 min and once in TBST. Finally, they were incubated with HRP-conjugated secondary antibody diluted 1:3,000 in TBST + 5% milk at room temperature for 45 min. The membranes were washed 4 times in TBS and once in TBST for 5 min. Bio-Rad's Clarity Western ECL blotting substrate was then applied for detection. EMSA was performed at least 3 times with independent dilution of synthetic peptides and freshly-hybridized DIG probe.

2.12 RNA extraction and transcriptome analysis

Total RNA was extracted from 4-day-old whole plant of *A. thaliana Mlp124478* stable transgenics and Col-0 with RNeasy Plant Mini Kit (Qiagen, Inc.) according to the manufacturer's specifications. Control and transgenic plants were extracted in triplicate. Eluted total RNA was quantified, sent to the Genome Analysis Platform at IBIS (Institut

de Biologie Intégrative et des Systèmes), Université Laval (Quebec, QC, Canada) for library construction, and sequenced with Ion Torrent Technology. Differential expression was analyzed with green line workflow of the DNA subway in the iPlant collaborative pipeline (Fig. 2.3) (Oliver et al. 2013) (Cold Spring Harbor Laboratory), including *A. thaliana*-Ensembl TAIR 10 as reference genome. Green line workflow uses three different steps. In step one, 108 million RNA-Seq reads were uploaded to iPlant and reference genome was selected. Then RNA-Seq reads were aligned to *A. thaliana*-Ensembl TAIR 10 as reference genome using TopHat, a fast splice junction mapper for RNA-Seq reads and then mapping results were analyzed for the identification of splice junctions between exons. In the second part, Cufflinks RNA-Seq alignments were assembled into a set of transcripts and relative abundances were calculated based on the number of reads support. The third part includes Cuffdiff which uses the output of cufflinks set of transcripts. Transcripts were assembled, compared and levels of expression were compared in multiple conditions to test for significant differences.

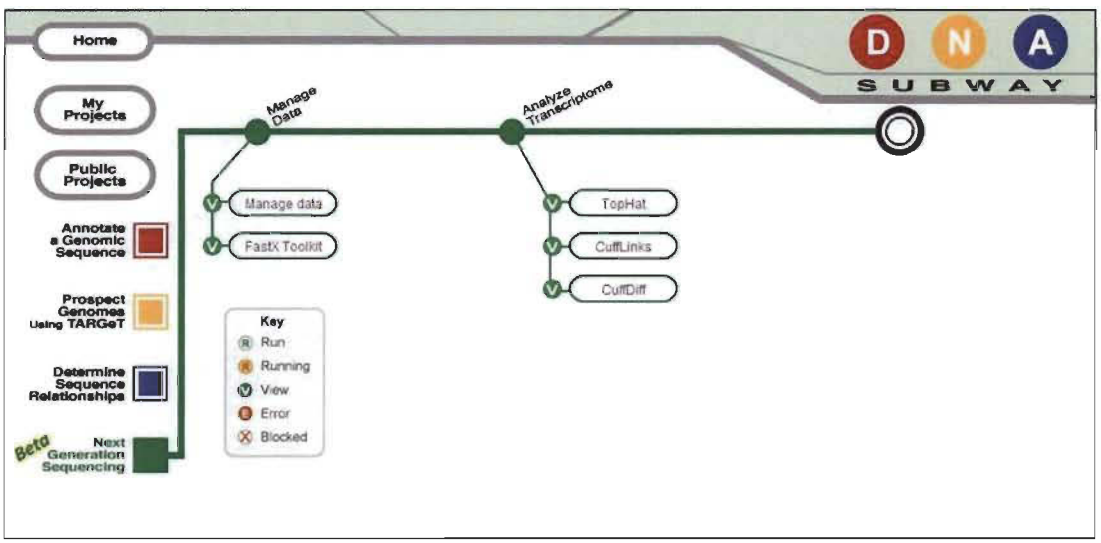


Fig. 2.3 Screenshot of green line workflow.
Upon uploading RNA-Seq reads, green line workflow analyses in three different steps such as TopHat, CuffLinks and CuffDiff. (Source: www.iPlant collaborative.org.)

2.13 Yeast Two-Hybrid Assay

Yeast two-hybrid (Y2H) screens was performed with the Matchmaker GAL4 Two-Hybrid System 3 (BD Biosciences Clontech). An *A. thaliana* cDNA library was prepared from leaves of 4-weeks-old plants (Col-0) grown at 22°C, relative humidity of 60% and under 14 h/10 h light/dark cycle. Tissue samples were grinded using Qiagen TissueLyser II (28 Hz, 1 min). RNAs were extracted using the Qiagen RNeasy Plant Mini Kit and reverse transcribed using M-MLV Reverse Transcriptase to produce cDNA (Invitrogen, Thermo Fisher Scientific Corporation). Then resulting cDNA libraries were amplified according to Clontech's method, cloned in the pGADT7 vector and transferred into Y187 yeast cells. *Mlp124478* sequence was cloned in the pGBKT7 vector and the resulting bait plasmid was transferred into AH109 yeast cells. In total, 3.2×10^5 interactions were screened. Diploid yeast cells (carrying pGBKT7 and pGADT7) were first grown on medium stringency SD/-His/-Leu/-Trp growth medium. Later on all positive colonies were replicated onto high stringency SD/-Ade/-His/-Leu/-Trp/X-α-gal growth medium. Plasmids were extracted from all blue colonies and retransformed into *E. coli*. Then plasmids were isolates by miniprep and verified by sequencing.

2.14 Bioinformatics analyses

Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) aligned sequences of the 9-member CPG2811 effector family and annotated them later manually. Phylogenetic trees were generated by COBALT (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi). SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) predicted signal peptides. PSORT (http://www.psort.org/) predicted the subcellular localization of protein and NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/) predict nuclear-localizing signals. Transcription factor-binding sites (TFBS) were identified and analyzed with the AthaMap (http://www.athamap.de/search_gene.php) (Steffens et al. 2005), Pscan (http://www.beaconlab.it/pscan) (Zambelli, Pesole and Pavesi 2009) and PlantPan (http://plantpan2.itps.ncku.edu.tw/index.html) (Chang et al. 2008) databases.

Consensus TFBS sequences were retrieved from the Pscan database. Promoter sequences were obtained individually with TAIR's SeqViewer (http://tairvm09.tacc.utexas.edu/servlets/sv), and TFBS-specific primers (Supplementary Table 2.1, Primer Nos. 4-38) were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Genevestigator (http://genevestigator.com/gv/doc/intro_plant.jsp) provided gene expression data under different biological conditions. Expression values were copied from Genevestigator, and a heatmap was created in Excel. Protein DNA-binding sites were predicted by MetaDBSite (http://projects.biotec.tu-dresden.de/metadbsite/) (Si et al. 2011). ChIP-PCR-positive genes on the up-regulated gene list from the Genevestigator expression dataset were searched with the National Center for Biotechnology Information (NCBI) protein blast search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to identify similar genes in poplars. PLEXdb (http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Poplar) (Dash et al. 2012) visualized gene expression profiles in poplars.

CHAPTER III

RESULTS

3.1 The candidate effector *Mlp124478* was selected for functional characterization

Mlp124478, the effector investigated in this study belongs to the CPG2811 gene family of *M. larici-populina* gene families. CPG2811 has 9 members in the genome of *M. larici-populina* isolate 98AG31. None of them bears any protein sequence similarity outside of *M. larici-populina* and the Pucciniales order or no homolog found in *M. lini* (Duplessis et al. 2011b, Hacquard et al. 2012). *Mlp124478* expression is strongly enhanced during infection and reaches 50-fold induction at 96 h after infection. Given the kinetics of *M. larici-populina* infection, this corresponds to the biotrophic growth stage in mesophyll cells (Duplessis et al. 2011b). In addition, the CPG2811 family presents a rapid evolution signature, a feature of pathogen effector families (Hacquard et al. 2012). Each family member (Mlp124478, Mlp124479, Mlp124480, Mlp124481, Mlp124482, Mlp124483, Mlp124484, Mlp124485, and Mlp124486) is composed of 2 exons encoding short peptides (75-96 amino acids) with 6 conserved cysteine residues and a signal peptide (SP) (Fig. 3.1A). Eight effectors (*Mlp124478, Mlp124479, Mlp124480, Mlp124481, Mlp124482, Mlp124483, Mlp124485,* and *Mlp124486*) of CPG2811 family comprises a SP of 26 amino acids long, except Mlp124484 which has a SP of 27 amino acids (Fig. 3.1A). Overall, amino acid conservation is low in the family. Amino acid identity ranges from 28% to 60% between Mlp124478 and the other family members (Fig. 3.1B). The maximum amino acids identity of 60% is found between Mlp124478 and Mlp124483. Amino acids identity of 52%, 45%, 45%, 42%, 40%, 34% and 28% were observed between Mlp124478 and Mlp124479, Mlp124480, Mlp124482, Mlp124485, Mlp124481, Mlp124484 and Mlp124486, respectively. *Mlp124478* is the only member of the CPG2811 family that

has a putative nuclear localization signal (NLS) (29-38 amino acids) and a putative DNA-binding domain (amino acids 58 to 80) (Fig. 3.2).

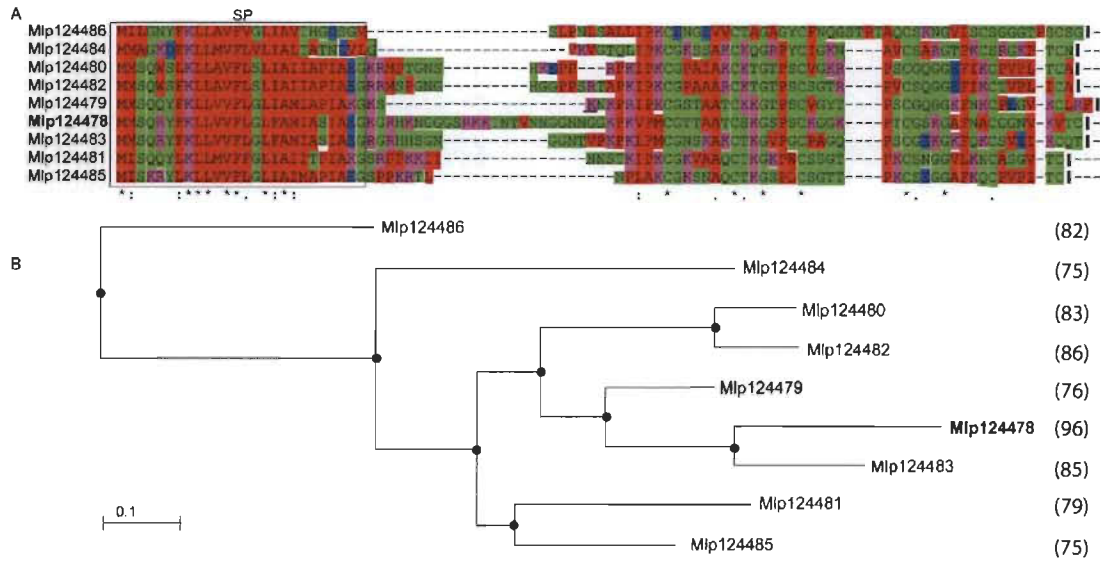


Fig. 3.1 Sequence alignment and phylogenetic tree.

(A) Multiple sequence alignment of 9 members of CPG2811 showing sequence similarity. Signal peptide is marked by a black box. Identical/highly conserved residues (*); semi conserved residues (:) and designate conserved residues (.) Color represents the conservativeness of amino acids according to their features and distribution within the column. (B) Phylogenetic tree of 9 effector members of the CPG2811 family obtained with COBALT using Kimura distance value and neighbor joining tree method. Number within parenthesis at the right site represents the length of amino acids.

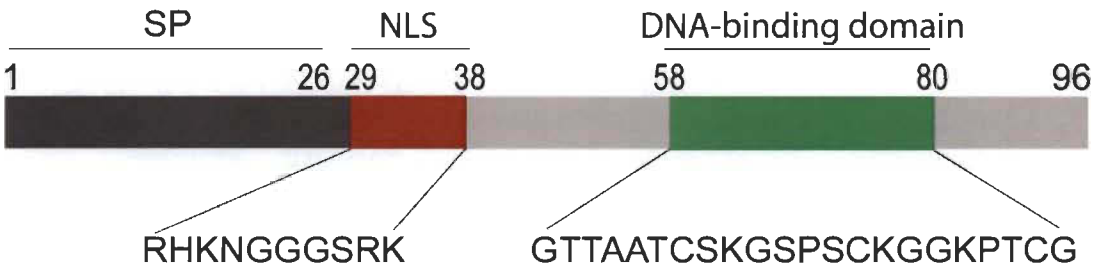


Fig. 3.2 Signal peptide and nuclear localization sequences within the peptide sequence of Mlp124478.

Both SP and NLS resides to the N-terminus region and stretch of NLS amino acids are very close to the SP. DNA-binding domain of 22 amino acids reside to the C-terminus. SP: Signal peptide; NLS: Nuclear localization sequence.

Since *Mlp124478* is expressed during infection and appears to harbor specific features, making it unique within this family, we decided to investigate if Mlp124478 plays a role *in planta* during pathogen growth. We anticipated to characterize Mlp124478 using functional genomic assays (Fig. 3.3). Reading frame of mature Mlp124478 (Fig. 3.1A) were cloned using Gateway cloning technology (Fig. 3.3B) in *P. syringae* pv. *tomato* DC3000ΔCEL (Fig. 3.3D) and *A. tumefaciens* for use in pathogenic infection assay, development of transgenic plant and subcellular localization (Fig. 3.3E). Moreover, the transcriptome of plants overexpreesing *Mlp124478* was analyzed to study the role of Mlp124478 in host cellular processes (Fig. 3.3F).

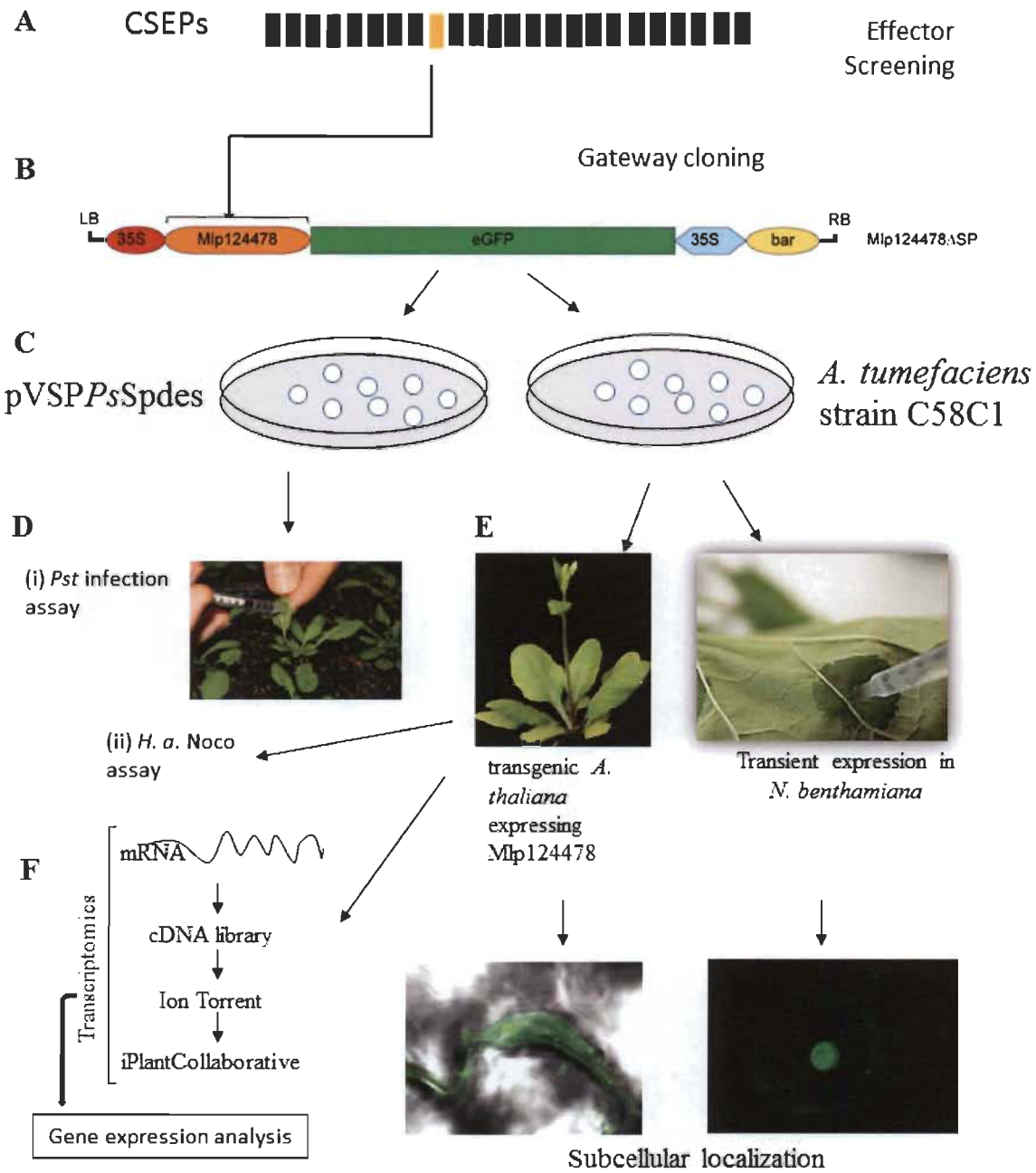


Fig. 3.3 Overview of functional studies of Mlp124478.

(A) Mlp124478 effector was mined from the set of candidate effectors of *M. larici-populina*. (B) Mature form of Mlp124478 (without SP, *Mlp124478*ΔSP) was cloned using Gateway Cloning Technology. (C) *Mlp124478*ΔSP was further recombined into pVSPPsSpdes vector for *Pst* infection assay and pB7FWG2.0 for subcellular localization. *Mlp124478* recombined into pB7FWG2.0 was then transformed into *A. tumefaciens* strain C58C1. (D) Pathogenicity assay has been carried out using bacterial pathosystem, *Pst* infection assay using pVSPPsSpdes vector for effector

delivery in Col-0 plants (i), and *Pst*DC3000ΔCEL on Col-0 and stable transgenic Mlp124478 plants (ii). (E) Stable transgenic *A. thaliana* plants expressing *Mlp124478* was developed via *A. tumefaciens* mediated genetic transformation method using floral dipping technique, and those stable transgenic Mlp124478 plants were used for subcellular accumulation of Mlp124478 effector. Transient expression of Mlp124478 was carried out in *N. benthamiana.* (F) Stable transgenic *A. thaliana* expressing Mlp124478 were used for transcriptomics and gene expression analysis.

3.2 Mlp124478 affects the shape of *A. thaliana* leaves

To evaluate the biological consequences of *Mlp124478*'s presence in plant cells, we generated a stable transgenic *A. thaliana* line expressing the mature form of Mlp124478 (i.e., without the signal peptide) fused to GFP under the control of a 35S promoter (pro35S::*Mlp124478-GFP*) in the Col-0 background (Fig. 3.4A & B). We transformed *Mlp124478* and T1 plants were screened using the Basta herbicide (active agent glufosinate). T2 (the 2nd generation) and T3 (the 3rd generation) were selected on Basta plate to select for single insertion homozygous lines using Mendeleian segregation of the dominant resistant marker. T3 seeds obtained from the T2 plants which survived Basta treatment, were used for further characterization. We then compared the phenotype of transgenic lines with wild type plants Col-0. The transgenic lines manifested altered leaf morphology, characterized by waviness of leaf margins, while no curvature in the margins was evident in Col-0 plants (Fig. 3.4A) or control GFP plants (data not shown). Anti-GFP immunoblotting for proteins extracted from Mlp124478-GFP and Col-0 lines revealed a band at the expected size of 37 kDa only in the transgenic line (Fig. 3.4B), indicating that constitutive *in planta* expression of Mlp124478-GFP fusion alters plant morphology.

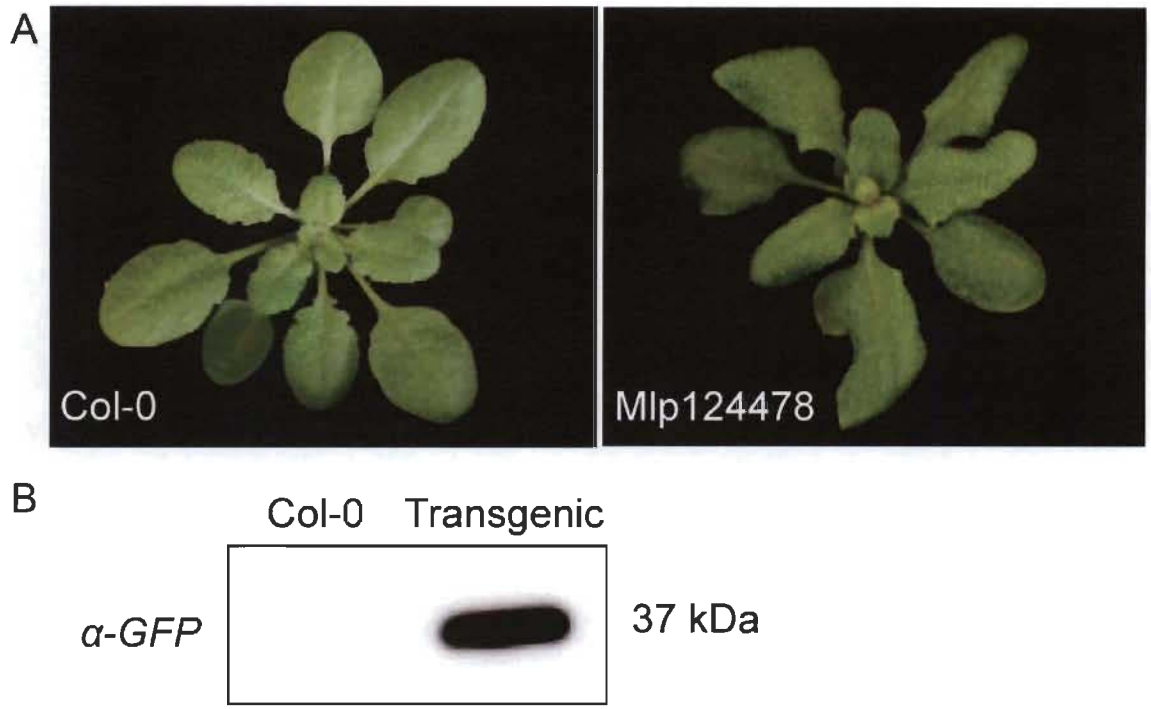


Fig. 3.4 Phenotype of Mlp124478 in *A. thaliana* transgenic.
(A) Morphology of 4-weeks old soil grown Col-0 and stable transgenic *Mlp124478* plant grown at 22°C under 14 h/10 h photoperiod in growth chamber. (B) Immunodetection of GFP protein in Col-0 and stable transgenic seedlings. Only stable transgenic line revealed a band of 37 kDa.

3.3 Mlp124478 accumulates in the nuclear area in *A. thaliana* leaves

To ascertain subcellular localization of Mlp124478, we undertook confocal laser scanning microscopy of leaves from 4-day-old *A. thaliana* seedlings expressing Mlp124478-GFP fusion (Fig. 3.5). We detected the GFP signal in the nucleolus, with a weaker signal in the nucleoplasm and cytosol of epithelial cells (Fig. 3.5, top panel). In contrast, in control plants expressing GFP, the fluorescent signal accumulated only in the nucleoplasm and cytosol, with no signal in the nucleolus (Fig. 3.5, bottom panel). The absence of GFP fluorescent signal in the control plant compared to the one observed in the nucleolus of plant expressing Mlp124478-GFP confirmed that Mlp124478-GFP specifically accumulates in the nucleolus of leaf cells, with weaker accumulation in the nucleoplasm and cytosol (Fig. 3.5).

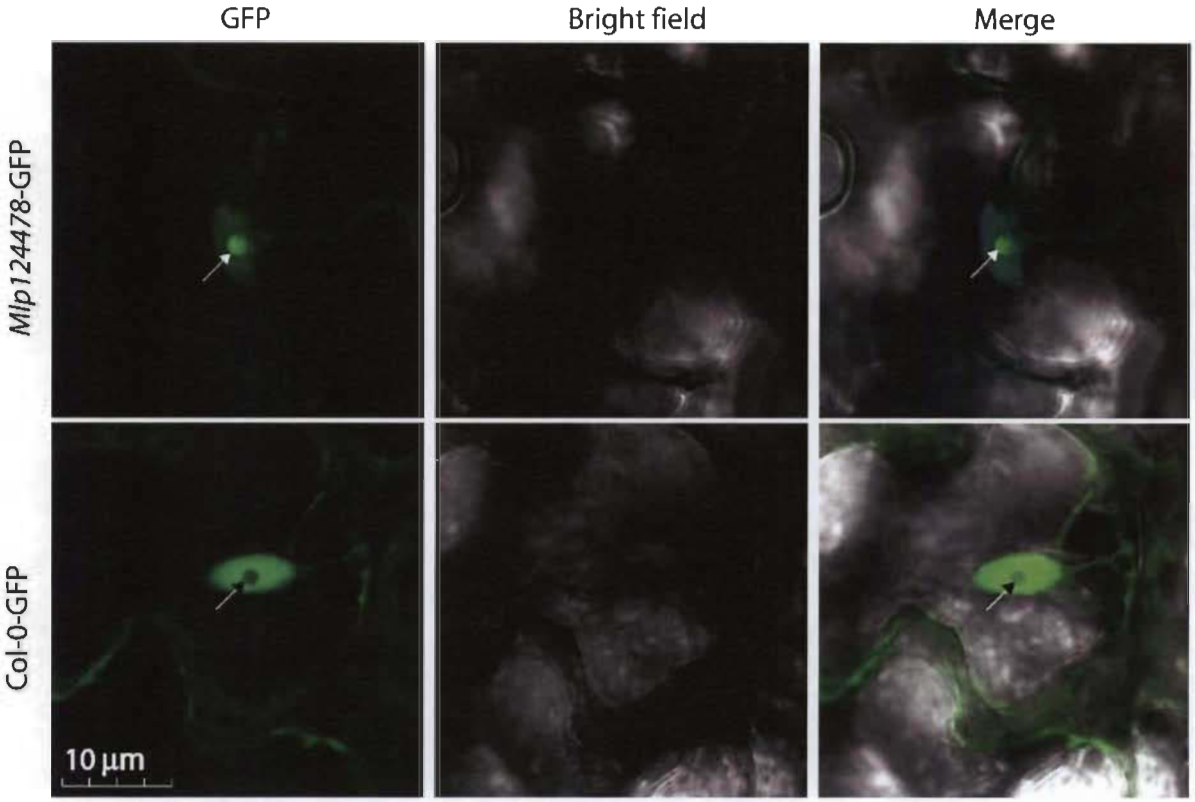


Fig. 3.5 Subcellular accumulation of Mlp124478 in *A. thaliana* leaves.
Live cell imaging using confocal microscope of epidermal cells of 4-days old stable transgenic *Mlp124478* plantlets with C-terminal GFP under the control of 35S promoter. GFP in the Col-0 background was used as control. Fluorescence in the green channel (left panel), bright filed (middle panel) and merge of all channels (right panel) are shown. Single focal plane showed GFP localizes to the nucleus and cytoplasm. In contrary Mlp124478-GFP localizes to both nucleus and nucleolus (white arrow heads) which is absent in GFP (black arrow heads). The similar pattern of distribution for Mlp124478-GFP and control GFP were observed in at least five independent lines.

3.4 Mlp124478 carries a Nuclear Localization Sequence

Mlp124478 carries a predicted Nuclear Localization Sequence (NLS) consisting of 10 amino acids within the N-terminal part of the mature form ($Mlp124478_{29\text{-}38}$::RHKNGGGSRK) (Fig. 3.2). To assess whether the predicted NLS was required for nuclear localization, we used a GFP tagged construct with the mature form of Mlp124478 (i.e., without the signal peptide) fused to GFP under the control of a

35S promoter (pro35S::*Mlp124478-GFP*), and designed another GFP tagged construct lacking the predicted NLS, hereafter named Mlp124478$_{\Delta 29\text{-}38}$-GFP. Both Mlp124478-GFP and Mlp124478$_{\Delta 29\text{-}38}$-GFP were expressed transiently in *N. benthamiana* leaf cells by agro-infiltration (Fig. 3.6A). Consistent with our *A. thaliana* observation, three-dimensional (3D) reconstructed image of Mlp124478-GFP subcellular accumulation (supplementary Fig. 3.1) confirmed that Mlp124478-GFP fusion accumulated in both the nucleus and nucleolus of *N. benthamiana* epithelial cells (Fig. 3.6B). However, Mlp124478$_{\Delta 29\text{-}38}$-GFP accumulated solely in the nucleus, and its signal was almost completely excluded from the nucleolus (Fig. 3.6B, lower panel).

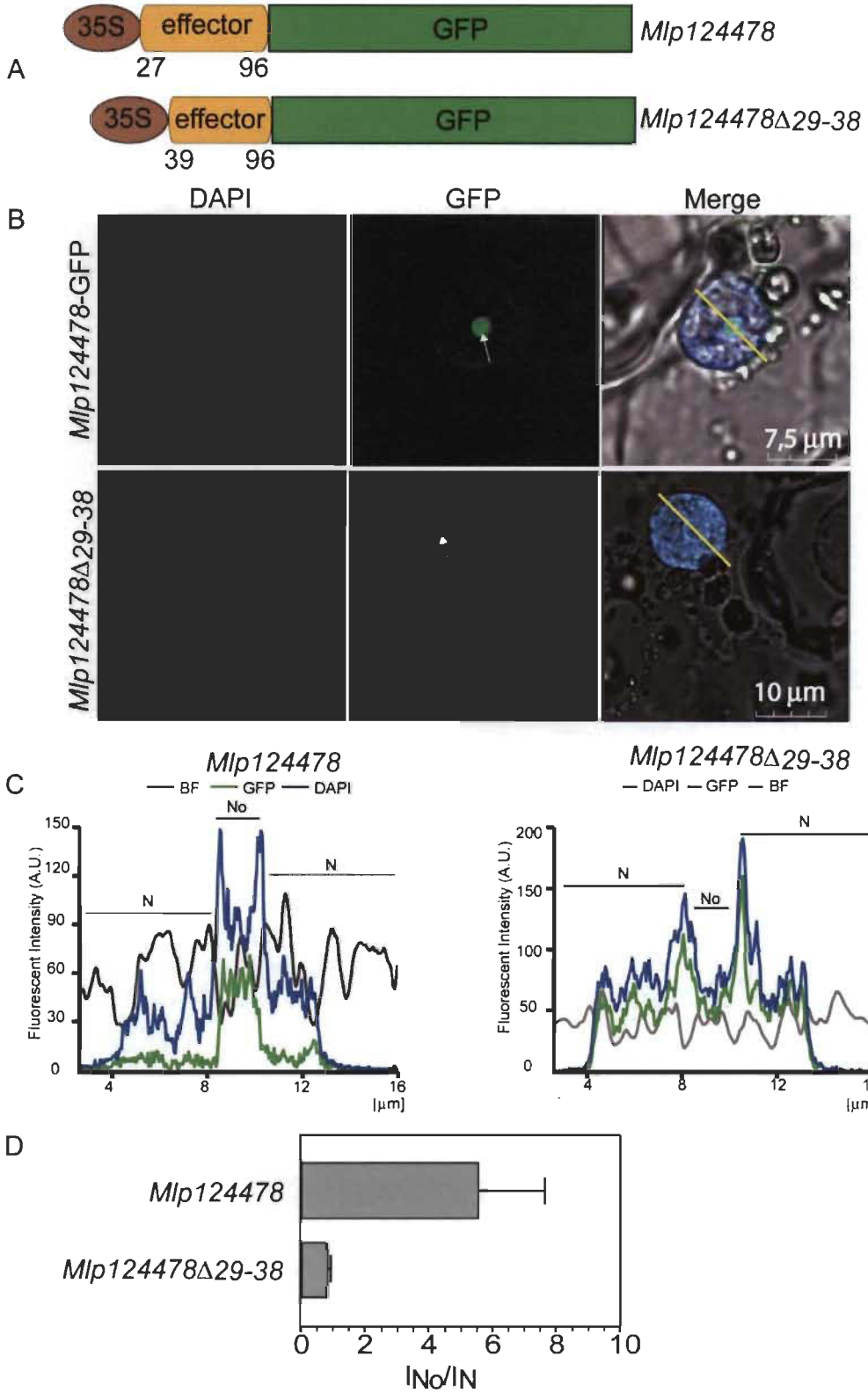


Fig. 3.6 Transient expression of *Mlp124478* showed NLS acts as NoLS.
(A) Schematic representation of the constructs (full length and truncated) used for transient expression in *N. benthaminan*. NLS (29-38 AA) has been

removed from the N-terminus of the truncated construct (*Mlp124478*$\Delta_{29\text{-}38}$). For both constructs GFP is tagged to the C-terminus, but lacks the signal peptide at the N-terminus region. Expression was controlled by the 35S promoter. (B) Subcellular accumulation of Mlp124478-GFP and Mlp124478$\Delta_{29\text{-}38}$ in *N. benthamiana* epidermal cells. At 4-days post infiltration, the nucleus was stained by DAPI staining dye and epidermal cells were observed under the blue channel (left panel), green channel (middle panel) and merge of all channels (right panel). Arrowheads point the nucleolar localization of Mlp124478, which is absent for Mlp124478$\Delta_{29\text{-}38}$. A solid yellow line is transected in the merge channel over the nuclear and nucleolar region to draw the intensity plot in C. (C) Intensity plot represents intensities strongly differ between nucleus (N) and nucleolus (No). (D) Nuclear-nucleolar distribution of the fluorescent fusion proteins according to the fluorescent intensity ratios: nucleolar intensity (I_{No}) divided by nuclear intensity (I_N). Average fluorescence intensity ratios (± SD) were determined from the fluorescent intensities on the nucleus and nucleolus in confocal images with the Leica LAS X software.

To quantify the changes in subcellular distribution, we generated intensity plots of the fluorescent signals, which clearly showed decreased fluorescence in the nucleolus between the two Mlp124478 constructs (Fig. 3.6C). Moreover, we noted average distribution ratios by comparing fluorescence intensities in the nucleus and nucleolus from confocal images acquired under identical settings which helped to get an assessment of the reliability of subcellular distribution. The higher values for I_{No}/I_N represent a nucleolar prevalent accumulation. Mlp124478-GFP had a significantly higher nucleolar/nuclear ratio of 5.55 compared to a Mlp124478$_{\Delta 29\text{-}38}$ protein ratio of 0.8 (Fig. 3.6D). Taken together, these results suggest that the predicted NLS acts as nucleolar localization signal (NoLS) and resides at the N-terminus region of mature Mlp124478.

3.5 Mlp124478 augment oomycete pathogen growth

To better understand the molecular effect of Mlp124478 in pathogenic situations, we conducted pathogen assays. We used two different model pathosystem (*P. syringae* pv. *tomato* and *H. arabidopsidis* Noco2) to test whether Mlp124478 alters pathogen virulence in *A. thaliana* (Fig. 3.7). *P. syrinage* pv. *tomato* is a bacterial pathogen that has a type three secretion system (T3SS). Since no rust fungi infect *A. thaliana*, we used the

obligate biotrophic oomycete pathogen *H. arabidopsidis* as a proxy for filamentous pathogen.

We inoculated transgenic *Mlp124478-GFP*, Col-0 WT (negative control) and *enhanced disease susceptibility 1-1 (eds1-1)* plants (positive controls hypersensitive to *H. arabidopsidis*). At 7 days after inoculation, we quantified the number of spores and observed 10,000, 25,000 and 85,000 spores, respectively, on average, for each genotype. We noted a significant increase of susceptibility in *Mlp124478* transgenic plants compared to Col-0 ($P<0.0001$), although not as strong as that encountered in *eds1-1* plants (Fig. 3.7A). This finding demonstrates that *Mlp124478* can enhance plant susceptibility to obligate biotrophic filamentous pathogens.

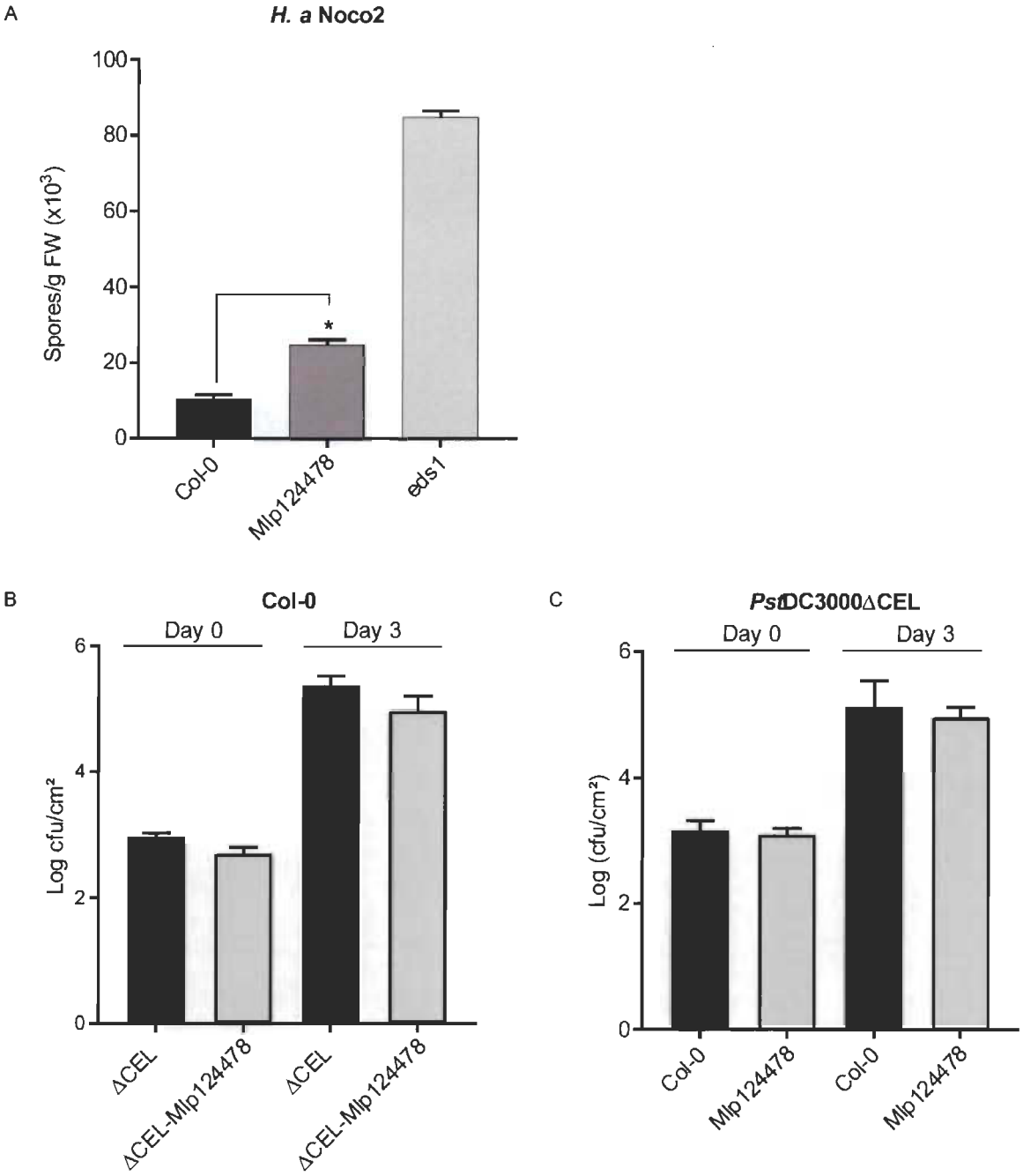


Fig. 3.7 Defence response of *Mlp124478* against bacterial and oomycete pathosystem.

(A) Four-weeks-old soil grown Col-0, stable transgenic Mlp124478 and *eds1-1* plants were spray inoculated with *Hyaloperonospora arabidopsidis* Noco2 (50,000 conidiospores/mL) and number of conidiospores were quantified at 7 days after inoculation. (B) Quantification of the growth of *Pst* strain DC3000ΔCEL carrying or not *Mlp124478* in Col-0 WT. In this system effector is expressed in bacteria and delivered *in planta* via the T3SS. Bacterial poulations were averaged immediate after inoculation (0 dpi) and 3 dpi. Student's *t*-test *P*-value 0.066. (C) Quantification of *Pst*DC3000ΔCEL

growth in Col-0 and stable transgenic *A. thaliana* expressing *Mlp124478*. The effector was expressed *in planta*. Growth of bacteria was measured at 0 and 3 dpi. *P*-value 0.4368. Statistical significance was evaluated using student's *t* test. For B and C, four weeks old plants were syringe infiltrated with bacteria at OD_{600}=0.001. The experiments were repeated three times with similar results. dpi: days postinfiltration, cfu: colony forming unit/mL.

To investigate whether Mlp124478 could enhance susceptibility to bacterial pathogens, we infiltrated 4-weeks-old Col-0 leaves with *Pst*DC3000ΔCEL bacteria carrying *Mlp124478* or not (Fig. 3.7B). Prior to pathogenic assay, bacterial growth was assessed at different times by OD_{600} measurements (supplementary Fig. 3.2). In this system, the effector is expressed in bacteria and delivered *in planta* via the T3SS. No significant difference was evident between bacterial strains carrying *Mlp124478* and the empty vector strain (Student's *t*-test, *P*-value 0.066) (Fig. 3.7B). We also undertook infection assays with *Pst*DC3000ΔCEL in transgenic plants overexpressing *Mlp124478* and Col-0. The effector was expressed *in planta* in this system. Bacterial growth was not significantly different between Mlp124478 and control plants (*P*-value 0.4368) (Fig. 3.7C). From these experiments, we conclude that Mlp124478 enhances the growth of *H. arabidopsidis* but not *P. syringae* in *A. thaliana*.

3.6 Mlp124478 interacts with several proteins in yeast two hybrid

Since Mlp124478 accumulate in the nuclear compartment, we sought to know whether Mlp124478 has possible targets within host cells. Therefore, we used Yeast Two Hybrid (Y2H) screening system in which Mlp124478 served as the bait and a 4-weeks-old *Arabidopsis* library served as the preys. Screening of *Arabidopsis* cDNA library by using Mlp124478 as a bait revealed 11 candidate interactors (Table 3.1). Members of superfamilies were excluded as their large functional redundancy precludes them as specific targets of effector. Since Mlp124478 accumulate in the nucleolus, we analyzed the presence of NoLS within the protein sequence of 11 interactor partners of Mlp124478, by using Nucleolar Localization Sequence Detector (NoD) software. Only Heat shock protein 20 like protein 1 (HSP1) identified as NoLS positive with

a stretch of 21 amino acids ($HSP1_{1-21}$:TPKKKRPMKIHPLPRNENNNN) at the N-terminal part (Supplementary Fig. 3.3).

Table 3.1. Potential interactors of Mlp124478 identified through Y2H.

SL No.	Protein name	E-value	NoLS
1	Post-illumination chlorophyll fluorescence increase protein	e-107	No
2	Heat shock protein 20 like protein 1	e-100	Yes
3	Hypothetical protein	e-152	No
4	RNA-binding KH domain-containing protein	0	No
5	Mlp-like protein 28	e-58	No
6	AT4G20360 Elongation factor Tu	e-56	No
7	Uncharacterized protein	e-52	No
8	Ferredoxin--nitrite reductase	e-158	No
9	Thiol protease aleurain cysteine proteinase AALP	0	No
10	F-box protein PP2-B1	e-100	No
11	Ubiquitin activating enzyme	e-28	No

Since Mlp124478 comprises a NoLS, and subcellular localization in *A. thaliana* and transient expression in *N. benthamiana* confirmed its accumulation into the nucleolus, we considered HSP1 as a potential putative interactor partner for further studies. We generated a construct with HSP1 fused to a C-terminal Red Fluorescent Protein (RFP) under the control of 35S promoter (pro35S::*RFP-HSP1*). To assess colocalization, Mlp124478-GFP and RFP-HSP1 were co-infiltrated at the abaxial side of *N. benthamiana* leaves to transiently expressed in the epithelial cells. Consistent with the previous observation of transient expression of Mlp124478-GFP in nucleus and nucleolus (Fig. 3.6B), Mlp124478-GFP and RFP-HSP1 also accumulated in both the nucleus and nucleolus of *N. benthamiana* epithelial cells (Fig. 3.8). Merged image captured with the DAPI (Fig. 3.8A), bright field (Fig. 3.8B), GFP (Fig. 3.8C) and RFP

(Fig. 3.8D) channel showed that both Mlp124478-GFP and RFP-HSP1 accumulates in the nucleus and nucleolus (Fig. 3.8E). Taken together with the Y2H and NoLS prediction, we concluded that Mlp124478 and HSP1 are both present in the nuclear compartment.

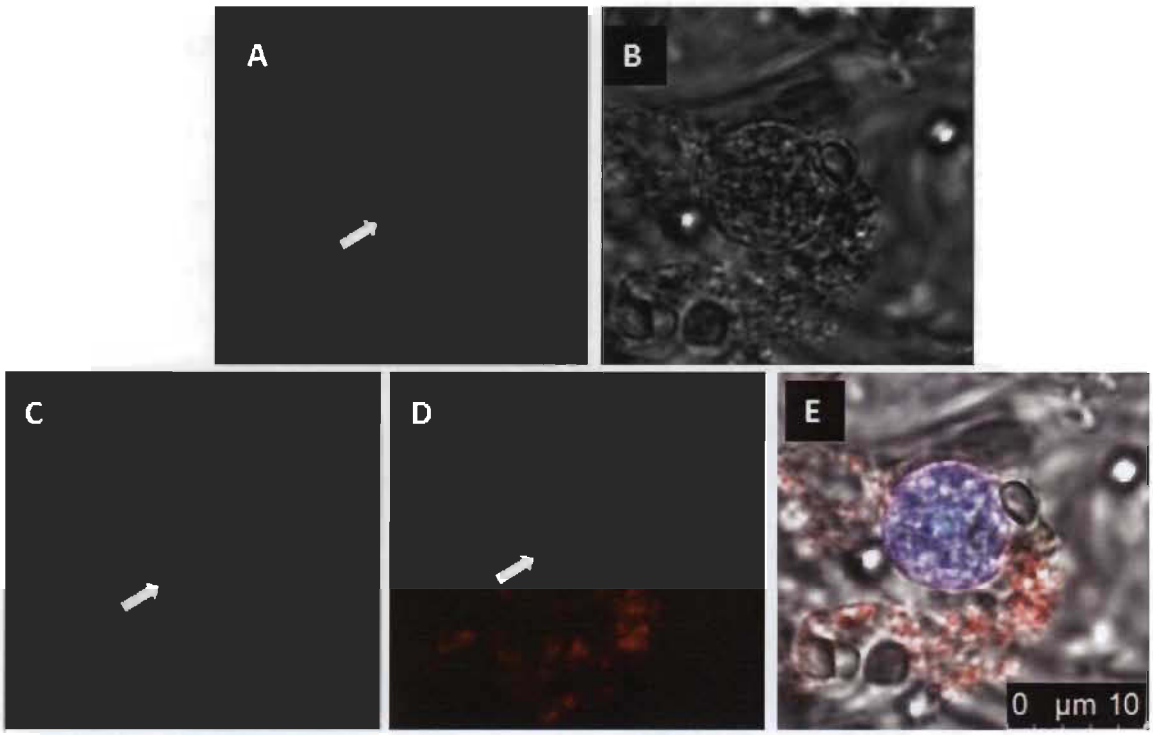


Fig. 3.8 Colocalization of Mlp124478-GFP and RFP-HSP1.
Subcellular accumulation of Mlp124478-GFP and RFP-HSP1 in *N. benthamiana* epidermal cells. *A. tumefaciens*-carrying recombinant plasmids were infiltrated into abaxial leaf pavement cells of 6-week-old *N. benthamiana* plants. At 4-day post infiltration, leaf peels are emerged in water mounted slide and stained by DAPI staining dye. Epidermal cells were observed under the blue channel (A), bright field (B), green channel for GFP (C), red channel for RFP-HSP1 (D) and merge of all channels (E). Arrowheads point the nucleolar localization of Mlp124478 and HSP1.

To assess biological relevance of Y2H screen and test the hypothesis that HSP1 would be involved in plant defence (as an effector target could be) as an interacting partner of Mlp124478, we ordered T-DNA insertion line for HSP1 (SALK_017461) from Arabidopsis Biological Resource Center (ABRC) at Ohio State University (USA). Homozygous line was selected by T-DNA genotyping. We assessed pathogenic growth

to quantify whether HSP1 enhance or suppress pathogen growth. To this end, we infiltrated 4-weeks-old leaves of HSP1, Col-0 (negative control) and SNC1 (suppressor of npr1, constitutive 1), a mutant containing a gain-of-function mutation in TIR-NBS-LRR mediated resistance which leads constitutive resistance to pathogens (positive control) with *Pst*DC3000ΔCEL. We observed that bacterial growth was not significantly different between HSP1 and Col-0 (control plants) (*P*-value 0.8879) (Fig. 3.9). This finding demonstrates that HSP1 does not enhance nor suppressed the growth of *P. syringae* in *A. thaliana*.

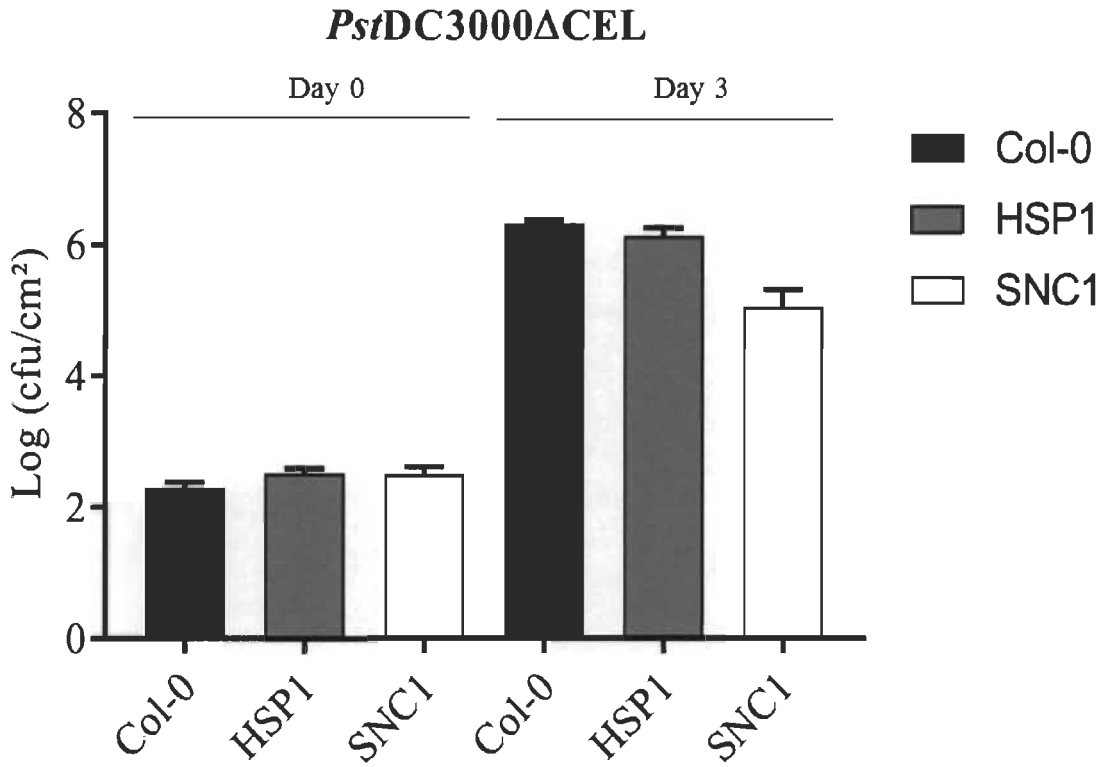


Fig. 3.9 Pathogenic growth response of HSP1 using bacterial pathosystem. Quantification of *Pst*DC3000ΔCEL growth in Col-0, HSP1 and SNC1. Four weeks old plants were syringe infiltrated with bacteria at OD_{600}=0.001. Growth of bacteria was measured at 0 and 3 dpi. *P*-value 0.4368. Statistical significance was evaluated using student's *t* test. The experiments were repeated three times with similar results. dpi: days postinfiltration, cfu: colony forming unit/mL.

We also crossed *hsp1* with snc1 to assess whether HSP1 is affecting plant resistance. F_2 were selected, genotyped and phenotypes were scored (snc1 has stunted morphology). No exception to simple Mendelian inheritance were identified in the F_2 generation. We concluded that hsp1 did not suppress the phenotypic effect of snc1, suggesting that hsp1 has no role in disease resistance.

3.7 Mlp124478 effect is revealed by gene expression network

To better understand how Mlp124478 functions in plant cells, and since it localizes to the nucleus and nucleolus, we investigated whether Mlp124478 can alter gene expression patterns in *A. thaliana* transgenics. We performed transcriptome profiling of the 4-days-old *A. thaliana* Mlp124478 stable transgenic line and Col-0 (Fig. 3.10). Our cDNA library followed by Ion Torrent based sequencing revealed a total of 108 million reads (80 M corresponding to control plants and 28 M to Mlp124478 plants, triplicate were done for both the transgenic and the control). Raw reads were processed using DNA subway of iPlantCollaborative (now CYVERSE) transcriptome pipeline from Cold Spring Harbor Laboratory, New York, USA, which revealed a total of 14,327 genes (6036 up-regulated and 8291 down-regulated genes). After applying filters for modulated genes with fold-change greater than 2.0 and Q-values of $P \leq 0.05$, we obtained 98 and 294 up- and down-regulated genes, respectively (Fig. 3.10, Supplementary Tables 3.1 & 3.2).

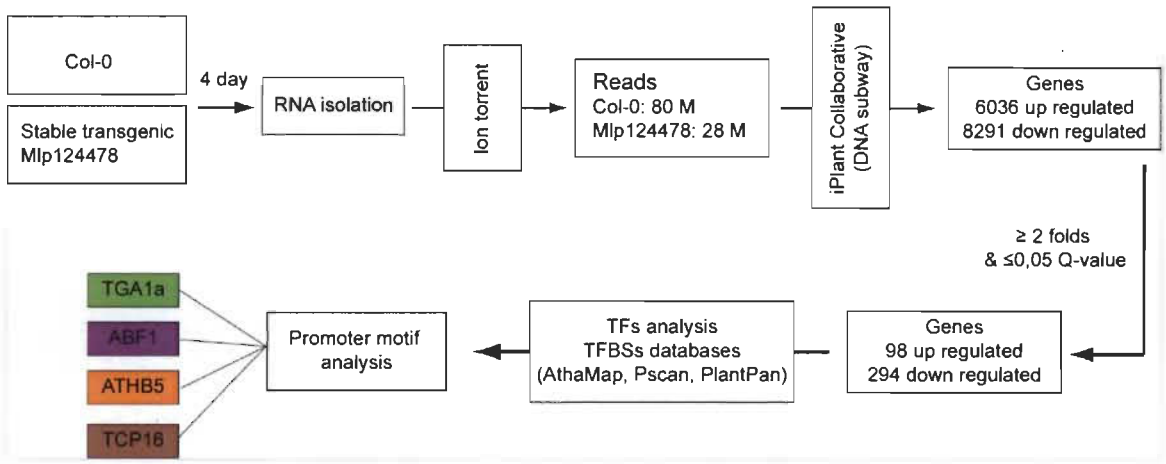


Fig. 3.10 Work flow of transcriptomics.

RNA was isolated from 4-days-old Col-0 plants and stable transgenic *A. thaliana* expressing Mlp124478. Eluted RNA was quantified, library was constructed and sequenced using ion torrent. Transcripts were analyzed using CYVERSE DNA subway and deregulated genes were considered for further gene ontology (GO) analysis. Three different Transcription Factor Binding Sites (TFBSs) databases were implemented to analyse Transcription Factors (TFs) within the promoter region of deregulated genes.

Deregulated genes (98 up and 294 down-regulated genes) were considered for gene ontology (GO) study to know their involvement in biological processes. GO term among these up- and down-regulated genes revealed 7 functional groups (GO groups 0-6) of 15 GO terms significantly enriched (i.e. response to virus, response to bacterium, response to brassinosteroid, indole-containing compound biosynthetic process, cell wall organization, response to red or far red light signaling and negative regulation of ethylene-activated signaling pathway) (Fig. 3.11). Few GO terms were enriched for up-regulated genes in comparison to down-regulated genes in the *A. thaliana* Mlp124478 transgenic lines. We noticed that some of the genes are related to defence. Among up-regulated genes, 3 expansin genes involved in cell wall organization, which are CRK21 (Cysteine-rich receptor-like protein kinase 21), ESM1 (Epithiospecifier modifier 1) and LOS2 (transcriptional activator) are involved in defence response. Only two up-regulated genes are enriched in response to ethylene and negative regulation of signal transduction (Table 3.2, Fig. 3.11). We also noticed that out of 15 GO terms, only 7 GO terms were enriched among up-regulated genes. This analysis indicates that all of the 15 GO terms of 7 functional groups enriched among down-

regulated genes. Among the 294 down-regulated genes (Supplementary Table 3.2), 42 belongs to cell wall organization, 37 belong to the xyloglucan transglycolase XTH, XRT and EXT families. The defence-related transcription factors WRKY18, WRKY27, WRKY33, MYB51, defence-related proteins NHL3, RPP8, YLS9, AZI1, CRK11, and the jasmonate pathway and regulation genes JAZ1, ASA1, ASB1 were identified in the down-regulated gene list. However, some genes involved in diverse mechanisms are also down-regulated, such as the chitinase *CHI*, the brassinostreoid-related genes *BAS1*, *BES1*, PAR1, *BEE1*, the salicylic acid-related genes *NPR3*, the ethylene-related response genes *ARGOS* and *ARGOS-like* (*ARL*), *EBF2*, *ERF6*, *ETR2*, *RTE1*, the carbon metabolism-related genes *EXO* and red/far red light signalization-related genes *FAR1*, *GA2OX2*, *PAR1*, *PIF3*, *PKS4*, *RR5*. This results indicate that the alterations in Mlp124478 transgenic plant might occur due to the down-regulation of expression of genes involved in diverse functions, which are related to the regulation of defence response.

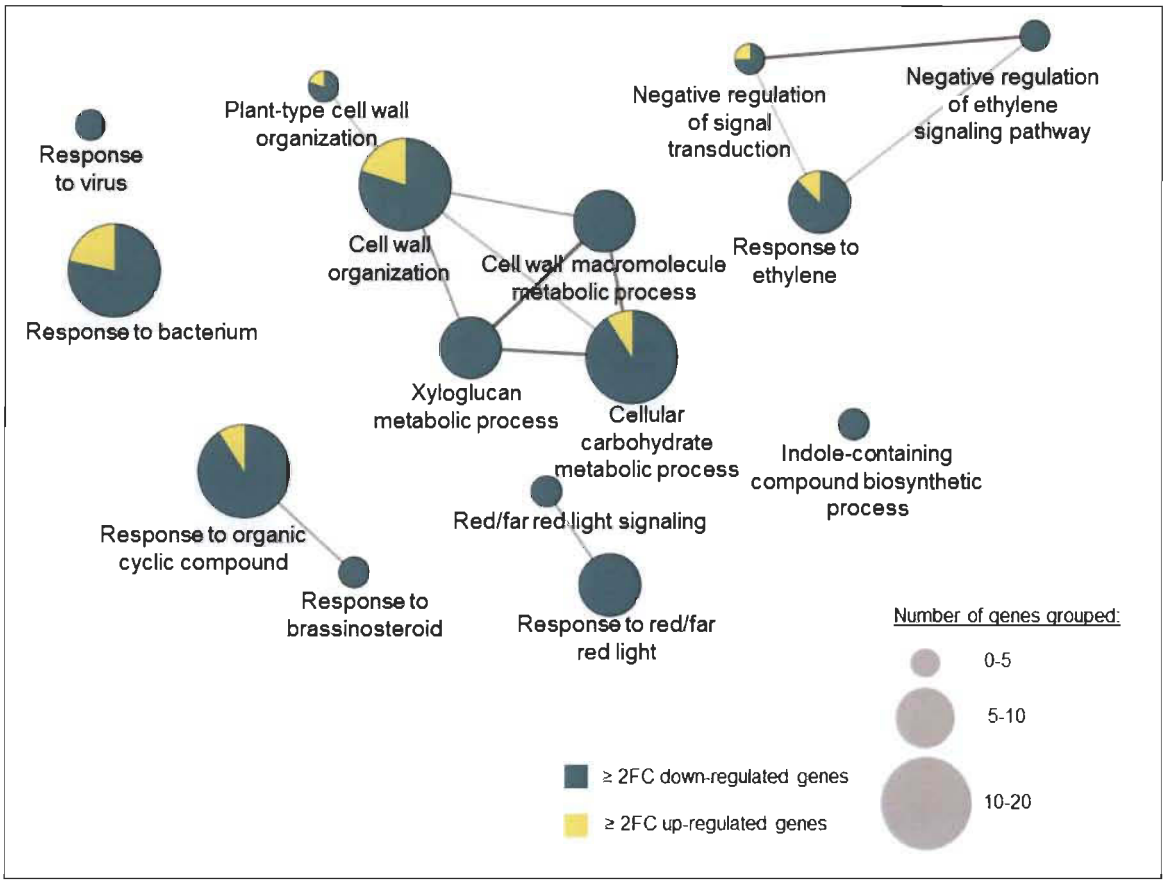


Fig. 3.11 Gene Ontology (GO) term enrichment network analysis using Cytoscape. Go term enrichment was performed with deregulated genes filtered with Q-value ≤0.05 and fold-change ≥2 using the Cytoscape software (version 3.1.1). Cytoscape was performed with the plug-in ClueGO and CluePedia to visualize functions enriched in the deregulated genes. The GO terms presented are significantly enriched in up-regulated and down-regulated genes with FDR≤0.05 (Benjamini-Hochberg p-value correction) and revealed 15 GO terms belongs to 7 functional groups. All the down-regulated genes represented in all functional groups and GO terms, but only 7 GO terms corresponds to 4 functional groups represents among up-regulated genes.

Table 3.2. GO enrichment of down-regulated (cluster 1) and up-regulated genes of Mlp124478 versus Col-0 lines.

GO Term	GO Group	Nr. Gene	Genes Down-regulated (cluster 1)	Genes up-regulated (cluster 2)	% Genes (cluster 1)	% Genes (cluster 2
Response to virus	0	4	[CHI, NHL3, RPP8, YLS9]	[]	100,00	0,00
Response to bacterium	1	14	[AZI1, BAS1, BES1, CRK11, JAZ1, MYB51, NHL3, NPR3, WRKY18, WRKY27, WRKY33]	[CRK21, ESM1, LOX2]	78,57	21,43
Response to organic cyclic compound	2	11	[ARL, BAS1, BES1, CHI, EXO, LTL1, MYB51, PAR1, RPP8, WRKY18]	[MYB4]	90,91	9,09
Response to brassinosteroid	2	5	[ARL, BAS1, BES1, EXO, PAR1]	[]	100,00	0,00
Indole-containing compound biosynthetic process	3	4	[ASA1, ASB1, MYB51, WRKY33]	[]	100,00	0,00
Cellular carbohydrate metabolic process	4	11	[ICL, MLS, XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[MIPS1]	90,91	9,09
Cell wall organization	4	15	[EXT3, EXT4, MYB51, PRP2, XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[AT1G60590, EXPA10, EXPB3]	80,00	20,00
Cell wall macromolecule metabolic process	4	9	[CHI, XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[]	100,00	0,00
Plant-type cell wall organization	4	5	[EXT3, EXT4, PRP2, XTH33]	[EXPA10]	80,00	20,00

GO Term	GO Group	Nr. Gene	Genes Down-regulated (cluster 1)	Genes up-regulated (cluster 2)	% Genes (cluster 1)	% Genes (cluster 2
Xyloglucan metabolic process	4	8	[XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[]	100,00	0,00
Response to red or far red light	5	8	[BEE1, FAR1, GA2OX2, PAR1, PIF3, PKS4, RR5]	[]	93,50	0,00
Red or far-red light signaling pathway	5	4	[FAR1, PIF3, PKS4]	[]	94,64	0,00
Response to ethylene	6	8	[ARGOS, EBF2, ERF6, ETR2, MYB51, PRP3, RTE1]	[SNZ]	87,50	12,50
Negative regulation of signal transduction	6	4	[EBF2, ETR2, RTE1]	[KEG]	75,00	25,00
Negative regulation of ethylene-activated signaling pathway	6	3	[EBF2, ETR2, RTE1]	[]	100,00	0,00

3.8 Mlp124478 alter the pattern of gene expression

Mlp124478 is predicted to possess a DNA-binding domain. We inferred that it might interfere with transcription through direct interaction with DNA. Thus, we screened for Transcriptional Factor Binding Sites (TFBS) within the promoter sequences of all up- and down-regulated genes. We identified 4 different TFBS (Fig. 3.12) which were very abundant among the up- (43 genes out of 98) (Table 3.3) and down-regulated genes (30 genes out of 294) (Tables 3.4). TFBS abundant in the up-regulated gene set included ABF1 and TGA1a, bound by the basic region/leucine zipper motif (bZIP) transcription factor (TF) family, and TCP16, recognized by the TCP (TEOSINTE BRANCHED 1, CYCLOIDEA AND PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR 1) TF family. The TFBS ATHB5 and TCP16 were abundant among the down-regulated genes, bound by Homeodomains associated with the Leucine Zipper (HD-ZIP) and TCP TF families.

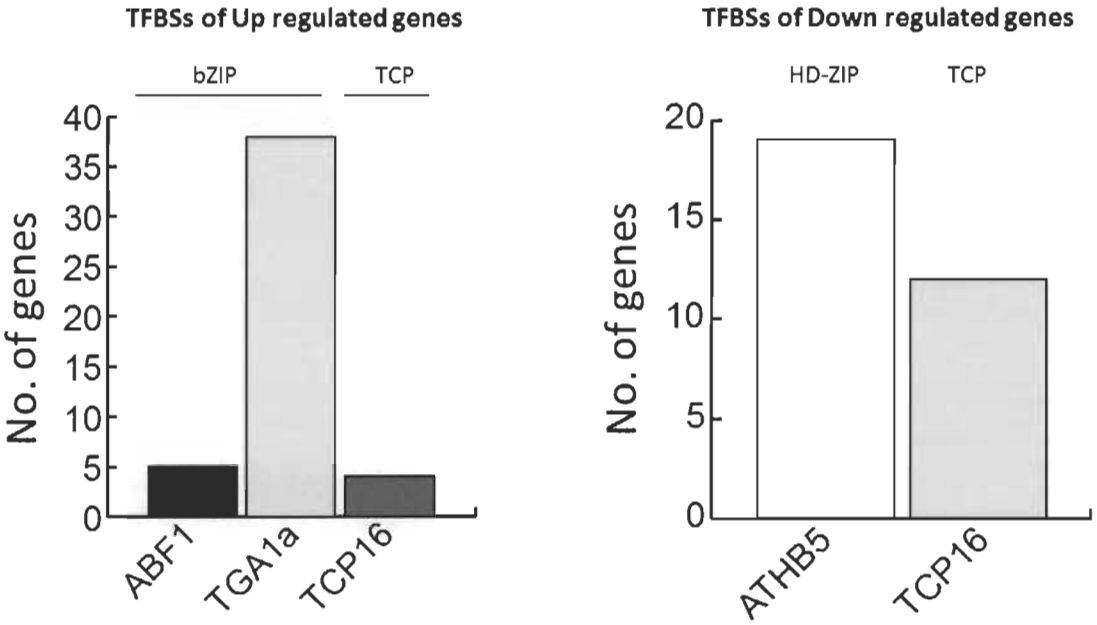


Fig. 3.12 Abundance of TFBSs among up- and down-regulated genes.
Deregulated genes were considered for Transcriptional Factor Binding Sites (TFBS) within the promoter sequences usinf TFBSs databases. Three different TFBSs (ABF1, TGA1a and TCP16) were found abundant among up-regulated genes. On the other hand, two TFBSs (ATHB5 and TCP16) were abundant among down-regulated genes.

Table 3.3. List of up-regulated genes abundant with TFBSs.

Gene ID	Fold Change	Q-Value	Description
At1g24580	9,53	0,015	RING/U-box domain-containing protein
At2g15020	8,54	0,0016	Uncharacterized protein
At2g37770	7,06	0,0016	NAD(P)-linked oxidoreductase-like protein
At3g58990	3,85	0,0162	Isopropylmalate isomerase 1
At2g34450	3,78	0,0299	High mobility group (HMG1/2) domain-containing protein
At1g04770	3,07	0,0016	Male sterility MS5 family protein
At1g18773	3,02	0,0478	Uncharacterized protein
At5g41410	2,87	0,0052	Homeobox protein BEL1-like protein
At3g54600	2,78	0,0029	Class I glutamine amidotransferase domain-containing protein
At5g42760	2,73	0,0052	Leucine carboxyl methyltransferase
At5g49480	2,68	0,0016	Ca2+-binding protein 1
At1g62050	2,61	0,0016	Ankyrin repeat-containing protein
At3g51660	2,48	0,0202	Macrophage migration inhibitory factor family protein
At3g04140	2,47	0,0041	Ankyrin repeat-containing protein
At3g21670	2,44	0,0016	Major facilitator protein
At2g39800	2,4	0,0016	Gamma-glutamyl phosphate reductase
At4g08870	2,34	0,0016	Putative arginase
At1g33170	2,3	0,0097	Putative methyltransferase PMT18
At5g17300	2,27	0,0097	Myb family transcription factor
At5g24470	2,27	0,0018	Two-component response regulator-like APRR5
At3g48310	2,24	0,0018	Cytochrome P450 71A22
At2g47750	2,24	0,0082	Putative indole-3-acetic acid-amido synthetase GH3,9
At1g13650	2,23	0,0016	Uncharacterized protein

Gene ID	Fold Change	Q-Value	Description
At3g14440	2,19	0,0341	9-cis-epoxycarotenoid dioxygenase NCED3
At2g41870	2,17	0,0052	Glutathione peroxidase 7
At5g17550	2,16	0,0131	Peroxin 19-2
At5g53280	2,16	0,0223	Plastid division protein 1
At5g58770	2,13	0,0088	Dehydrodolichyl diphosphate synthase 2
At5g62130	2,13	0,0016	Per1-like family protein
At4g03400	2,12	0,0016	Auxin-responsive GH3 family protein
At2g40480	2,11	0,0509	Uncharacterized protein
At1g26770	2,09	0,026	Expansin A10
At1g52400	2,09	0,0097	Beta glucosidase 18
At3g63160	2,08	0,0016	OEP6, OUTER ENVELOPE PROTEIN 6
At2g39250	2,08	0,0052	AP2-like ethylene-responsive transcription factor SNZ
At2g32990	2,07	0,0431	Endoglucanase 11
At1g52590	2,06	0,0029	Putative thiol-disulfide oxidoreductase DCC
At1g22590	2,05	0,0062	Protein AGAMOUS-like 87
At1g75030	2,05	0,0243	ATLP-3, THAUMATIN-LIKE PROTEIN 3, TLP-3
At1g18360	2,04	0,0016	Alpha/beta-hydrolase domain-containing protein
At4g10120	2,04	0,0018	Sucrose-phosphate synthase
At3g22104	2,03	0,009	Phototropic-responsive NPH3 family protein
At4g39800	2,02	0,0016	Inositol-3-phosphate synthase isozyme 1

Table 3.4. List of down-regulated genes abundant with TFBSs.

Gene ID	Fold Change	Q-Value	Description
At1g71900	2	0,0398	Uncharacterized protein
At5g57887	2,01	0,0069	Uncharacterized protein
At1g27670	2,02	0,0358	Uncharacterized protein
At5g64620	2,03	0,0016	Cell wall / vacuolar inhibitor of fructosidase 2
At2g37130	2,05	0,0016	Peroxidase
At4g37800	2,07	0,0018	Xyloglucan:xyloglucosyl transferase
At4g01410	2,11	0,0041	Late embryogenesis abundant hydroxyproline-rich glycoprotein
At3g25600	2,15	0,0018	Putative calcium-binding protein CML16
At5g20650	2,16	0,0016	Copper transporter 5
At5g65200	2,19	0,0314	U-box domain-containing protein 38
At5g13190	2,25	0,0018	Uncharacterized protein
At2g43150	2,3	0,0018	Proline-rich extensin-like family protein
At1g78890	2,43	0,0018	Uncharacterized protein
At1g64640	2,46	0,0018	Early nodulin-like protein 8
At5g57910	2,49	0,0314	Uncharacterized protein
At1g78460	2,62	0,0018	SOUL heme-binding protein
At1g67910	2,76	0,0018	Uncharacterized protein
At1g29090	2,92	0,0079	Cysteine proteinase-like protein
At3g12710	3,02	0,0018	DNA-3-methyladenine glycosylase I
At2g39200	3,06	0,0018	MILDEW RESISTANCE LOCUS O 12, MLO12
At1g50740	3,1	0,0018	Transmembrane proteins 14C
At1g29465	3,12	0,0079	Uncharacterized protein
At4g40010	3,15	0,0486	Serine/threonine-protein kinase SRK2F
At4g09890	3,17	0,0018	Uncharacterized protein

Gene ID	Fold Change	Q-Value	Description
At3g02550	3,32	0,0018	LOB domain-containing protein 41
At2g27080	3,75	0,0018	Late embryogenesis abundant hydroxyproline-rich glycoprotein
At2g20835	4,29	0,0357	Uncharacterized protein
At5g39610	4,5	0,0057	NAC domain containing protein 6
At4g16515	5,31	0,0016	CLE-LIKE 6
At2g35980	5,95	0,0156	ARABIDOPSIS NDR1/HIN1-LIKE 10, ATNHL10

Next, we analyzed the gene expression profiles of up- and down-regulated genes by transcriptomics during different biotic perturbations. We accessed Genevestigator (http://www.genevestigator.com) towards this end. Expression levels in 5 different biotic conditions (*Golovinomyces orontii*, *Phytophtora infestans*, *H. arabidopsidis*, *G. cichoracerum*, *Plectosphaerella cucumerina*) were retrieved for all up- and down-regulated genes within the *Mlp124478*-expressing line (Fig. 3.13A & B). Most genes up-regulated in the *A. thaliana* transgenic line overexpressing *Mlp124478* were down-regulated in response to these pathogens. Only 1 gene (*At3g51660*) seemed to be up-regulated (maximum fold change of 2.4) in most conditions analyzed (Fig. 3.13A) and also up-regulated in the transgenic line expressing *Mlp124478*. The same conditions were imposed to analyze the expression pattern of down-regulated genes from our transcriptome (Fig. 3.13B). Of the 30 down-regulated genes, 8 were up-regulated in almost all conditions considered (*At2g37130, At3g25600, At5g13190, At5g57910, At2g39200, At1g50740, At5g39610, At2g35980*). We further analyzed the identity of these genes. *At2g37130* encoded a peroxidase which was strongly up-regulated in response to fungal infection. *At5g13190* encoded a plasma membrane protein regulating cell death. *At2g39200* encoded *MILDEW RESISTANCE LOCUS O 12* (*AtMLO12*) whereas the product of the At2g35980 gene was very similar to *Arabidopsis NONRACE-SPECIFIC DISEASE RESISTANCE 1* (*NDR1*), a central integrator of defence responses downstream of the coiled-coil-nucleotide-binding leucine-rich repeat (CC-NBS-LRR). Taken together, we conclude that Mlp124478 rewires host transcription specifically to

induce genes not normally expressed during defence against biotrophic pathogens while concurrently down-regulating genes normally up-regulated in response to such pathogens.

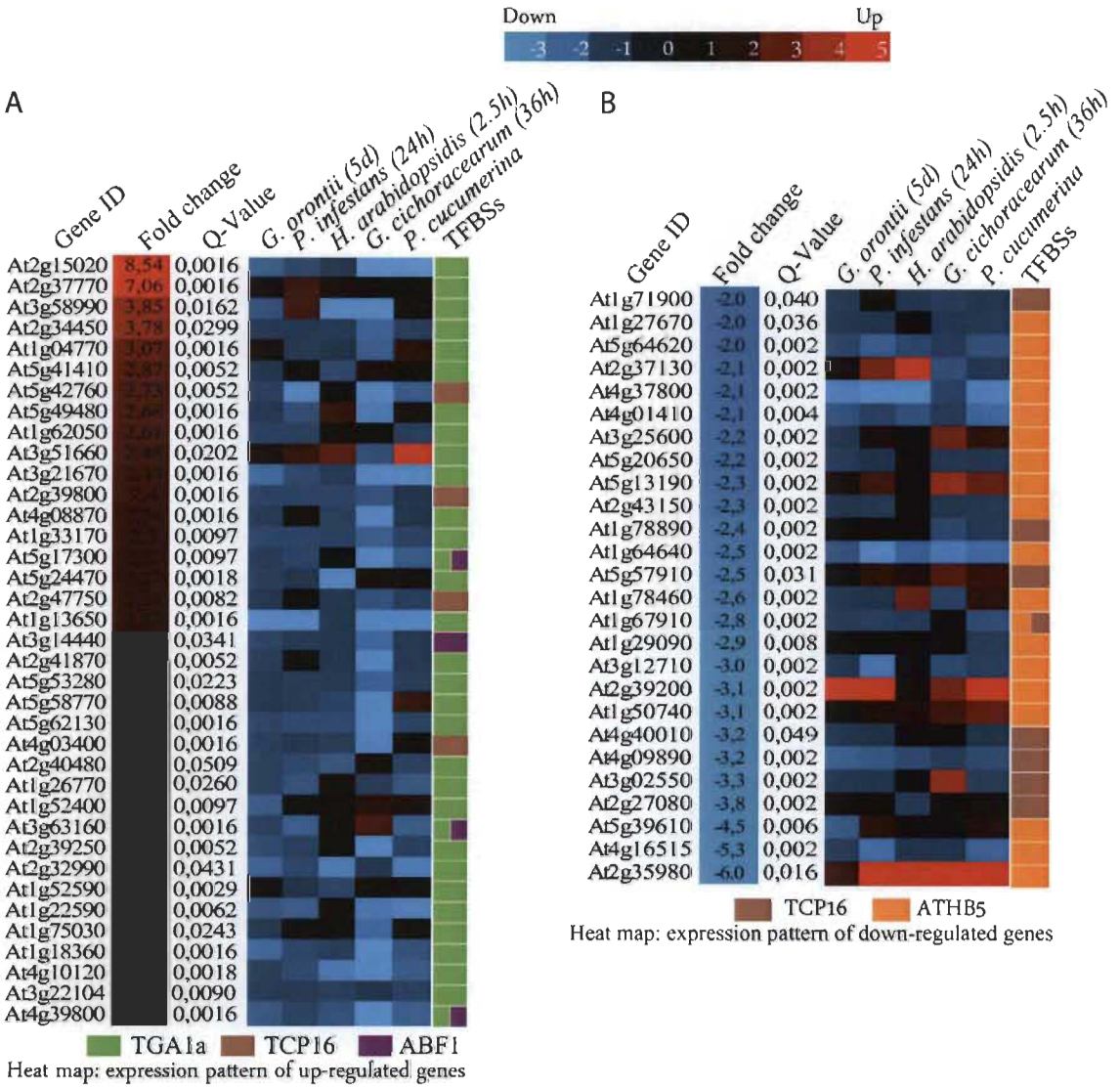


Fig. 3.13 Regulation of gene expression level.

Up and down-regulated genes were considered for analysing gene expression patter against five diffrrent biotrophic pathogens in Genevestigator microarray database. Fold change of genes in response to different biotrophic conditions were copied in Microsoft excel to generate Heat Map. (A) Heat Map of up-regulated genes and (B) Heat Map of down-regulated genes against different biotrophic pathogens. Gene IDs are listed according to their fold change obtained from transcriptome and intensity of color represents the level of expression.

3.9 Mlp124478 reprogram host processes by binding to DNA

The nuclear localization of Mlp124478, the presence of a DNA-binding motif and alterations at the transcriptional, morphological and defence levels prompted us to investigate whether Mlp124478 associates with DNA molecules. For this purpose, we performed a ChIP-PCR experiment. More precisely, we cross-linked proteins and DNA using formaldehyde, and then immunoprecipitated (IP) GFP with anti-GFP beads to pulldown DNA bound to GFP-tagged proteins. We designed 32 primer pairs that could amplify the promoter regions of deregulated genes containing either TCP, ATHB5, TGA1a and ABF1. We also tested Col-0 genomic DNA as PCR-positive control and subjected Col-0 to the same ChIP procedure for negative control. Only 1 of the primer sets resulted in specific amplification, revealing interaction of Mlp124478 with the promoter of a HMG-box (high mobility group) DNA-binding family gene (AT2G34450) containing a TGA1a-binding site among the most strongly up-regulated genes in Mlp124478 expressing plants. We did not observe any band in the IP with Col-0 DNA, which served as negative control, but a band was produced with *A. thaliana* genomic DNA as positive control (Fig. 3.14). AT2G34450 was up-regulated in the presence of Mlp124478 and showed down-regulation against biotrophic pathogens (Fig. 3.13A).

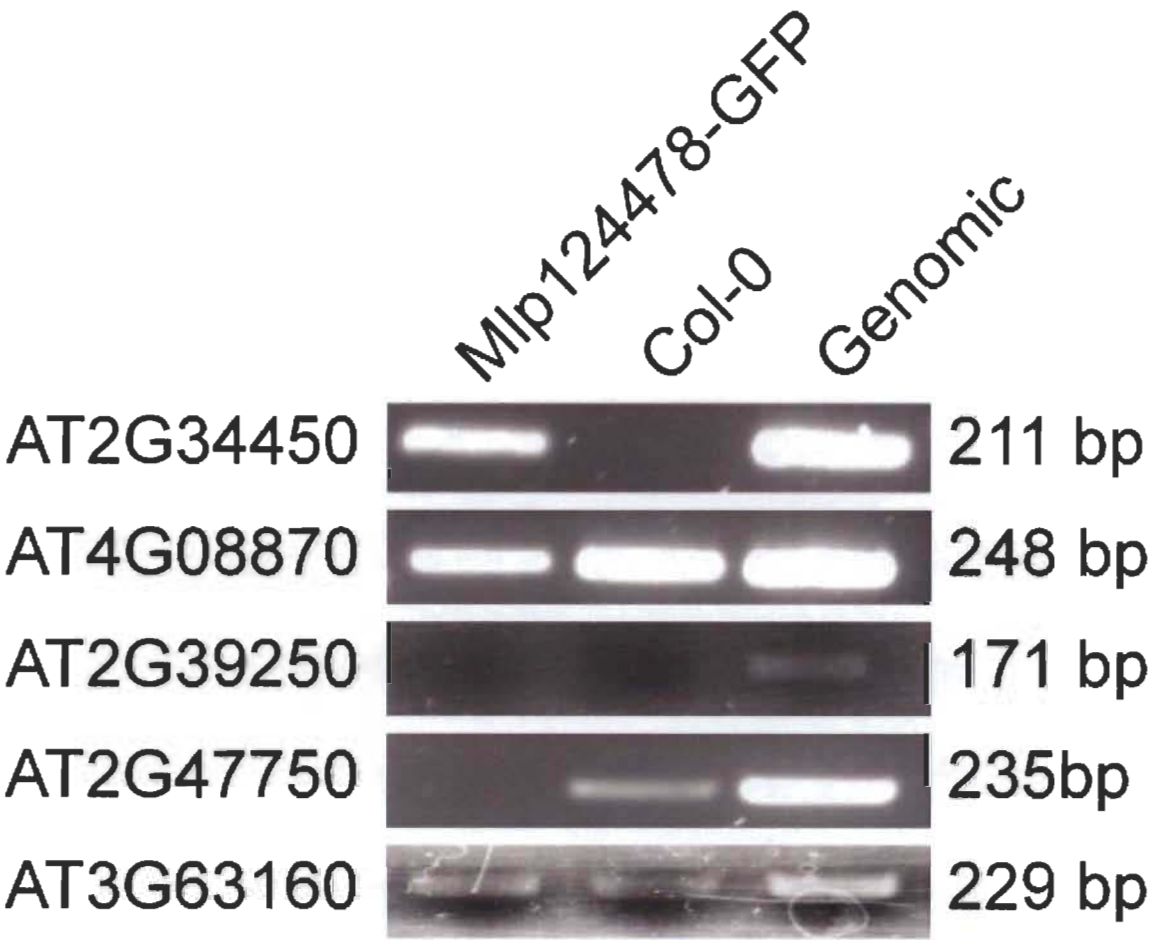


Fig. 3.14 ChIP-PCR assay of TFBSs determining DNA-binding ability.
Two weeks old plants tissues of Col-0, stable transgenic *Mlp124478* and *A. thaliana* genomic DNA were used for chromatin preparation using ChIP assay with antibody against GFP as described in the material and methods section. TGA1a associated site was PCR amplified with TGA1a specific primer pair. Expected bands (211 bp) was obtained from transgenic and *Arabidopsis* genomic DNA for TGA1a at the promoter region of AT2G34450 gene. AT4G08870 and AT3G63160 showed band of 248 and 229 bp with all three types of DNA (Mlp124478, Col-0 and genomic DNA); whereas AT2G39250 showed amplification with only genomic DNA, and AT2G47750 showed amplification with Col-0 and genomic DNA. Col-0 DNA: negative control; *A. thaliana* genomic DNA: positive control.

We aimed at performing electrophoretic mobility shift assay (EMSA) to evaluate the ability of the predicted DNA binding domain of Mlp124478 to interact with a TGA1a consensus DNA sequence. Since we could not produce a recombinant Mlp124478 in *E. coli*, we used a synthetic peptide corresponding to the predicted DNA

binding domain of Mlp124478 instead and a double-stranded oligonucleotide displaying the consensus TGA1a sequence. The TGA1a sequence of AT2G34450 (our ChIP positive interactor) oligonucleotides was labeled with digoxigenin (DIG) and incubated with Mlp124478 synthetic peptide, but we did not discern any interaction. No shift was observed for TGA1a and the synthetic peptide compared with the lane of the oligo without the synthetic peptide (Fig. 3.15). EMSA result suggests that the DNA binding domain of Mlp124478 cannot adopt a configuration that enables *in vitro* interaction with the TGA1a sequence of AT2G34450.

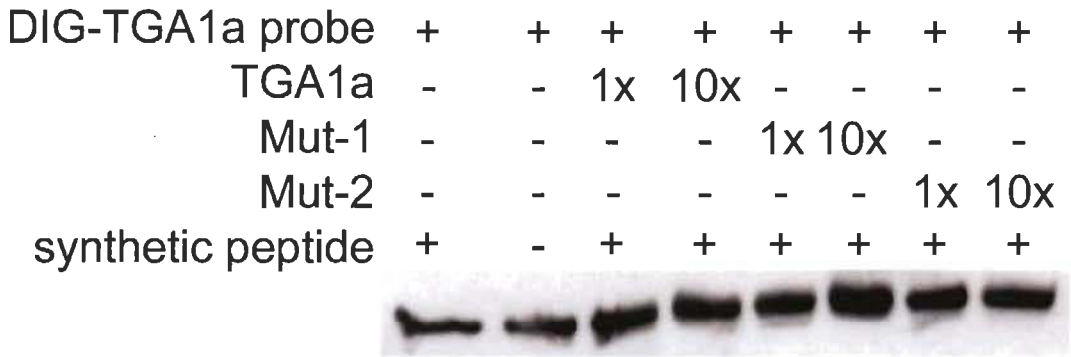


Fig. 3.15 EMSA of TGA1a and DNA-binding domain of Mlp124478 to assess binding activity.

EMSA was carried out with DNA-binding domain specific synthetic peptide and digoxigenin labelled TGA1a probe. Gel shift reaction was performed with 3 pmol of DS oligonucleotides and 100 ng of synthetic peptide in binding buffer. After binding reaction, the mixtures were electrophoresed through 0.25X TBE polyacrylamide gel. Bio-Rad semi-dry transfer cells were electroblotted on positively-charged nylon membranes. DNA was then cross-linked to the membrane by baking. Bio-Rad's Clarity Western ECL blotting substrate was then applied for detection. 10-fold excesses of oligonucleotides were added as competitors including wild type (TGA1a) and mutated (Mut-1 and Mut-2). EMSA did not discern any interaction.

3.10 Mlp124478 ChIP interactor has a poplar homolog with similar regulatory sequence

Since poplars are *M. larici-populina*'s natural host, and not *Arabidopsis*, we searched for the sequence immunoprecipitated in the ChIP experiment and present in

poplars. We anticipated from ChIP assay that Mlp124478 targets and interferes with transcription by binding to TGA1a at the upstream of gene contains TGA1a consensus sequence, such as the ChIP-PCR positive gene AT2G34450. Since this candidate effector was retrieve from the *M. larici-populina* genome, we searched if AT2G34450 had a putative homolog in poplar that contained a similar regulatory sequence. Our homology search revealed sequence similarities between AT2G34450 and a poplar genes and their regulatory sequences. We searched for promoter motifs and exonic-intronic structures of AT2G34450 (Fig. 3.16A) in TAIR database (http://www.arabidopsis.org/) and found that it belongs to the HMG-box (high mobility group) DNA-binding family protein, and functions in sequence specific DNA binding transcription factor activity. We found a poplar putative homolog corresponding to AT2G34450 identified as POPTR_0004s13630.1 (gene ID Ptp.5659.1.S1_at) in JGI Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html). Hence, poplars contained a promoter with 57% similarity to the promoter of AT2G34450 (gene model POPTR_0004s13630.1). Comparison between the two revealed that both belong to a HMG (high mobility group -box) DNA-binding family protein. Both are characterized by 6 exons and 5 introns and contains TGA1a regulatory sequences (Fig. 3.16B). Taken homology result suggests that Mlp124478 could bind TGA1a regulatory sequences in poplar as it does in *Arabidopsis* to regulate transcription.

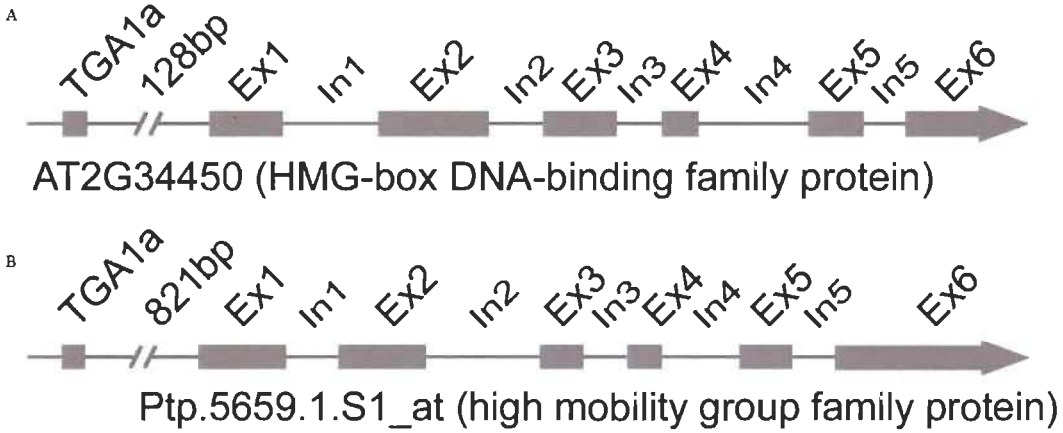


Fig. 3.16 Complete exon and intron structure with TFBSs at the upstream of gene. *A thaliana* gene *AT2G34450* showing the generalized structure of exons and introns with TGA1a at the upstream of transcription start site (TSS) (upper one). Lower image represents Ptp.5659.1S1_at, the homolog of *AT2G34450* in poplar represents similar number of exons and introns.

CHAPTER IV

DISCUSSION

Heterologous systems are used to investigate the function, localization and interaction of effectors from biotrophic pathogens (Caillaud et al. 2012a, Caillaud et al. 2012b, Du et al. 2015, Gaouar et al. 2016, Petre et al. 2015a, Petre et al. 2016, Petre et al. 2015b, Kunjeti et al. 2016). It has also been shown that many effectors target the nucleus and, in some cases, alter transcription (Canonne and Rivas 2012, McLellan et al. 2013, Rennoll-Bankert et al. 2015). Here, we undertook functional genomics to study Mlp124478, a small secreted protein from the poplar leaf rust pathogen *M. larici-populina*. We conducted *in planta* pathogen assays, live-cell imaging, comparative transcriptomics, and protein-nucleic acid interaction assay to assess Mlp124478 functions.

4.1 *Mlp124478* alters plant phenotype and response to pathogens growth

We observed that stable transgenic *Arabidopsis* line expressing *Mlp124478* showed distinct morphology. The transgenic lines manifested altered leaf morphology, characterized by waviness of leaf margins compared to Col-0 plants. Clear divergence in the leaf morphology of *Mlp124478* stable transgenic *Arabidopsis* enabled us to confirm the direct consequence of over-expression of *Mlp124478*. Previous studies showed that morphology characterized by the shape of leaf margins, such as smooth, wavy (undulate) or lobate is an imperative feature to define and classify specific phenotypes. Different genetic perturbations are involved in developing entire leaves lobate or wavy (Berger et al. 2009, Nath et al. 2003, Nikovics et al. 2006). Recent studies on quantitative measurements of phenotypes of leaf margins in *Arabidopsis* showed that leaf waviness is associated with oscillating normal curvature along the leaf margins provided by either as an outcome of induction level of gene misexpression or as an

action of time (Armon et al. 2014). Our findings with the altered leaf morphology and later on alteration in gene expression via modification of plant transcription suggest that the altered phenotype in the stable transgenic line is the outcome of the modification in the host cellular processes.

Since model pathosystem offers an advantageous method to verify whether effectors from eukaryotic pathogens can trigger or suppress plant defence mechanisms (Fabro et al. 2011), we used model plant pathosystems (the bacteria *Pseudomonas syringae* and oomycete *Hyaloperonospora arabidopsidis* Noco2) to unravel the virulence function of Mlp124478. Our pathogenicity assays used either a bacterial delivery system in which Mlp124478 was translated in *P. syringae* and translocated via the T3SS to the host cell, or constitutively expressed *in planta*. In both cases, we did not reveal significant bacterial growth alteration. Recently, another *M. larici-populina* effector, Mlp124202 has also been studied by our group using *P. syringae* pv. *tomato* DC3000ΔCEL to assess defence response (Gaouar et al. 2016). We also developed stable transgenic *A. thaliana* Ler line expressing Mlp124202 effector and noticed that Mlp124202 did not affect the growth of bacteria in Ler plants.

However, when *Arabidopsis* was exposed to a filamentous pathogen *H. arabidopsidis* Noco2 strain that possesses a lifestyle similar to rusts, we observed more pathogen growth, which indicates that this effector may target an immunity component specifically affected by pathogens with filamentous lifestyles. Previously, multiple effectors (HaRxLs) of *H. arabidopsidis* have also been assessed for their capacity to manipulate host defence (Fabro et al. 2011). They evaluated the role of host manipulation of HaRxLs candidate effectors in various *Arabidopsis* accession using T3SS of *P. syringae* pv. *tomato* and *H. arabidopsidis* Noco2. *Arabidopsis* transgenic line expressing HaRxLs effectors showed enhanced susceptibility to *P. syringae* pv. *tomato* (*Pst*-LUX), and 7 lines showed increased susceptibility of *H. arabidopsidis* Noco2 strain (Fabro et al. 2011). It was consistent with our usage of methods for assessing the pathogenic response of candidate effector by combining the use of a

heterologous system for candidate effector delivery with a consequence for pathogenic growth *in planta*.

Although it was previously reported that independently evolved effectors, arising in different kingdoms can converge onto molecular hubs and facilitate their various life cycle strategies (Mukhtar et al. 2011), our results confirm kingdom effector specificity directed towards pathogen lifestyle. As a matter of fact, a large group screening initiative (16 effectors) in our laboratory supports some degree of kingdom specificity from the effector repertoire.

4.2 Mlp124478 accumulate in plant nuclear compartment

We have observed accumulation of Mlp124478 in the nuclear compartment of plant epithelial cells. Also we observed mesophyll cells, but in all the cases it is dense of chloroplasts, which prevented to observe effector proteins accumulation. So for the technical reason, we observed in the epithelial cells. In previous studies (Petre et al. 2015b), Mlp124478 has been shown as nucleolar accumulated effector protein in *N. benthamiana*. Petre and his colleagues successfully demonstrated subcellular accumulation of 20 effectors of *M. larici-populina* in *N. benthamiana* and identified their interacting partners using coimmunoprecipitation assay followed by mass spectrometry. In this study, we confirmed subcellular accumulation of Mlp124478 both in the nucleus and nucleolus by using two different heterologous model system: stable transgenic *A. thaliana* expressing Mlp124478 and transient expression in *N. benthamiana*. Over the decades, expression of candidate effectors in *N. benthamiana* has been proven as the method of choice to assess localization, since it represents a fast and robust system for studying protein subcellular localization and the effect of ectopic expression (Caillaud et al. 2012a, Du et al. 2015, Gaouar et al. 2016, Lim et al. 2015, Petre et al. 2016, Petre et al. 2015b, Wang et al. 2016).

A *M. larici-populina* effector Mlp124202 has been shown to accumulated both in the plasma membrane and cytoplasm in *N. benthamiana* (Gaouar et al. 2016).

Using transient expression assay in *N. benthamiana* confirmed that majority of HaRxLs effectors of *H. arabidopsidis* localize to the nucleus and 11 out of 16 nuclear-localized HaRxLs effectors were observed to accumulate in the nucleolus (Caillaud et al. 2012b). Nuclear accumulating effectors have also been shown to be involved in cell death and virulence activity, such as crinkler effector, PcCRN4 of *Phythophthora capsici* localize to the nucleus and required for the cell death activity and virulence function (Mafurah et al. 2015). In some cases, both effector and corresponding R protein accumulates in the nuclear compartment, such as *P. infestans* RXLR effector AVR1 and its matching resistance protein R1 accumulate in the nucleus and cytoplasm in a close proximity, and their nuclear localization is essential for the immune activation of R1 (Du et al. 2015). Nuclear accumulation of effectors can also be associated with disease development in plants. For instance, *Colletotrichum graminicola* effector CgEP1 is synthesized at the early stage of disease development, target maize nucleus and develop anthracnose in the leaves, stems and roots (Vargas et al. 2016). Nucleolar accumulation of effector protein, interaction with protein in the host and re-localization within nuclear compartment promote plant diseases; as for example, studies in *N. benthamiana* showed that Pi04314, a RXLR type effector of *P. infestans* accumulate strongly in the nucleolus and nucleoplasm with additional cytoplasmic background. Pi04314 interacts with host protein phosphatase 1 catalytic (PP1c) (Boevink et al. 2016). Quantification of the fluorescence ratio of Pi04314 and 3 different isoforms of PP1c in *N. benthamiana* confirmed their interaction and re-localization in the nucleus. They also found that Pi04314 contains a motif of R/KVxF (KVTF), which is required for the interaction with PP1c. Moreover, it re-localizes from nucleolus to the nucleoplasm and is involved in late blight disease development (Boevink et al. 2016). Some bacterial pathogens, such as *Legionella* and *Burkholderia*, secrete SET-domain effectors that target the nucleolus to control gene expression and promote virulence, thereby promoting their intracellular survival (Bierne 2013).

Since default GFP distribution in plant cells is nucleo-cytoplasmic, localization of a GFP-tagged effector displaying nucleo-cytoplasmic distribution is considered non-informative. In the case of Mlp124478, localization in nucleoli indicates that GFP is not

masking the Mlp124478 localization sequence and that localization is driven by the effector sequence. The nucleolus has long been recognized as a hallmark of virus infection (Hiscox 2002, Hiscox 2007, Salvetti and Greco 2014), essentially to recruit nucleolar proteins and facilitate virus replication (Hiscox 2007). While viral lifestyle easily explains the need to target the nucleolus, the reasons why a rust effector would do so is not as intuitive. Given that little is known in plants about what could link the nucleolus and the biological processes of plant defences it would be highly speculative at this point to suggest a reason why we observed Mlp124478 accumulation in the nucleolus. Interestingly, however, the amino acid sequence, predicted to act as a nuclear localization sequence, in fact served as a nucleolar localization sequence. Thus, Mlp124478 localized in the nucleus and nucleolus.

4.3 Mlp124478 contains novel NoLS

We showed that the predicted NLS of Mlp124478 in fact serves as a NoLS using stable expression in *A. thaliana* and transient expression in *N. benthamiana*. This led us to further examine the NoLS of Mlp124478. The overall role of Mlp124478 effector in plant nuclear compartment could bring an important aspect for manipulating cellular processes. Our *in silico* study identified the NoLS of Mlp124478 as a novel amino acid sequence for NoLS. We verified NoLS amino acid sequence of Mlp124478 within the *Arabidopsis* nucleolar protein database (AtNoPDB) which provides information on 217 nucleolar localized proteins identified in a proteomic analysis in nucleoli isolated from *Arabidopsis* cell culture (Brown et al. 2005). We did not find any identical amino acid sequence of the NoLS of Mlp124478 to any other known NoLS in the AtNoPDB, thus defining a putative novel NoLS. The NoLS of Mlp124478 is composed of 10 amino acids. Therefore, we will be cloning this small fragment of gene from Mlp124478 which is only of 30 base pairs (bp) to perform expression and mutation studies *in planta,* expecting NoLS-GFP would entirely accumulate in the nucleolus, but not the mutated NoLS-GFP fusion.

4.4 Mlp124478 nuclear accumulation benefit the pathogen

Nucleolus is a prominent subnuclear structure whose core function in transcription of genes for ribosomal RNA (rRNA), processing and modification of precursor rRNA (pre-rRNA) and assembly of ribosomal subunit (Venema and Tollervey 1999). Moreover, some other functions are associated with nucleolus, such as biogenesis and transport of varieties of RNAs and RNPs, maturation of mRNA, control of cell cycle and stress responses (Olson, Dundr and Szebeni 2000, Rubbi and Milner 2003, Pederson 1998). Nucleolar protein content is dynamic and is altered under the stressful conditions. In mammalian cells, proteomic analysis showed the dynamics of the nucleolar proteome and the reorganization of the nuclear architecture, which can lead to either apoptosis or arrest of the cell cycle in response to stresses including viral infection and DNA damage (Andersen et al. 2005, Boulon et al. 2010). Since nucleolus is associated with translational processes (rRNA maturation), any alteration/malfunction in the nucleolus would bring modification in gene expression which eventually affect function or morphology of plant. Previous study showed that nucleolar localized HaRxLs effectors give various morphological phenotypes (Caillaud et al. 2012b). Caillaud and his colleagues developed stable transgenic *A. thaliana* plants expressing HaRxLs effectors under the control of 35S promoter and observed leave curvature, abnormal number of leaves, early flowering, formation of two apical meristems, bushy, albino leaves and twist of the organs in the aerial part. They observed that nuclear localized HaRxLL3b induced enhanced leave curvature (Caillaud et al. 2012b). Morphological alteration in leaves due to the nuclear localized effector HaRxLL3b observed by Caillaud and his colleagues is analogous to our findings, as nucleolar-localized Mlp124478 in stable expressed transgenic *A. thaliana* showed leaf curvature (Fig. 3.4A). Hence, the altered phenotype observed in the stable transgenic *A. thaliana* expressing *Mlp124478* is the consequence of the modification of the transcriptional process due to the ectopic expression of Mlp124478 in plant. We concluded that nuclear accumulation of Mlp124478 can manipulate host nuclear regulatory components for the benefits of the pathogen.

4.5 Mlp124478 may interacts with multiple proteins in the host

We chose HSP1 as a putative interactor of Mlp124478, since HSP1 contains predicted NoLS. Although transient expression in *N. benthamiana* showed colocalization of Mlp124478 and HSP1 in nucleus, our pull down assay showed no *in planta* interaction of Mlp124478 with HSP1, indicating that HSP1 may not interact *in planta* with Mlp124478. Moreover, F_1 plants of *hsp1×snc1* did not show any suppression of *snc1* autoimmune phenotype, indicating that HSP1 does not have significant role in immunity. Studies showed that heat shock proteins (HSPs) function as molecular chaperone regulating folding and accumulation of proteins, as well as localization and degradation in plants and animals (Hu, Hu and Han 2009, Panaretou and Zhai 2008, Feder and Hofmann 1999). Recent studies revealed that some HSPs are critical for defence responses in plants, such as HSP90 which plays as molecular chaperone for modulating structure and/or stability of R proteins (Elmore, Lin and Coaker 2011, Sangster and Queitsch 2005). In this case, cochaperones, such as SGT1 is required to interact with HSP90 (Azevedo et al. 2006). We cannot exclude that Mlp124478 does interact with an HSP protein *in planta*, since we found an HSP interactor by yeast two hybrid, however *in planta* interaction could be with another HSP. Taken together, we concluded that HSP1 is not an *in planta* interactor of Mlp124478. Therefore, any of 10 other putative interactor proteins of Mlp124478 found in Y2H assay could be the positive interactor protein, or HSP1 might require cochaperone and/or cofactors in the nucleus to assist Mlp124478 in plant cells, it is also possible that all 10 are false positive, yeast two hybrid is notoriously prone to false positives.

4.6 Mlp124478 tricks plant defence by suppressing immune regulators

Our stable transgenic line overexpressing the effector was subjected to phenotype analysis, pathogenicity assays, and comparative transcriptomics, insightful approaches taken more easily than adopting *N. benthamiana* as a heterologous expression system. Stable transgenic plants could provide clues with regard to putative interacting proteins, if a well-described phenotype is copied. In our case, although plants overexpressing

Mlp124478 clearly displayed wavy leaf margins, it did not enable us to speculate about a putative interacting protein because of the plethora of mutants displaying similar phenotypes (Abe et al. 2010, Graciet et al. 2009, Koyama et al. 2010, Reed 2001). However, the clear leaf morphology phenotype of *Mlp124478*-expressing plants confirmed that *Mlp124478* expression *in planta* affected plant development and prompted us to investigate the effect of *Mlp124478* expression on the plant transcriptome.

The first step in transcriptome analysis, after sorting genes through fold-induction/repression, is usually to assess whether transcript levels relative to a specific biological function or process are altered under certain conditions. As for the transgenic lines of *A. thaliana* expressing *Mlp124478*, most of the changes occur in down-regulation of defence-response associated genes. Interestingly, the genes that were found enriched down-regulated in our study corresponds to GO terms that were also reported recently (Hacquard et al. 2016a). Hacquard and collaborator have shown a transcriptomic analysis of the *A. thaliana* responses during colonization of two fungal species, *Colletotrichum tofieldiae* (a beneficial root endophyte) and *Colletotrichum incanum* (parasite). This study highlighted the same GO terms as in our case, except that genes are activated during the colonization of *C. incanum* (Hacquard et al. 2016a) and down-regulated in *Mlp124478* transgenic lines. As for example, they identified defence related transcription factor WRKY33 and ethylene responsive factor MYB51 in their list of hub genes (Hacquard et al. 2016a). However, we found WRKY33 encoding proteins in response to bacterium, and MYB51 encoding proteins in response to both bacterium and ethylene (Table 3.1). But both WRKY33 and MYB51 appeared as down-regulated in the transcriptome of *Mlp124478* transgenic. Hence, the expression of this single effector (*Mlp124478*) appears to bear broad transcriptional impact as it appears to counter the normal defence output described by Hacquard (2016) using a very similar analysis.

Transcription process is highly sophisticated and dynamic in eukaryotes (Lelli, Slattery and Mann 2012) and transcription factor binding sites (TFBSs) at the upstream

of a gene are key elements that determines transcriptional regulation (Van de Velde, Heyndrickx and Vandepoele 2014). We analyzed the promoter regions of deregulated genes in transgenic plants, and observed that TGA1a, ABF1, TCP16 and ATHB5 regulatory sequences were very abundant in the promoters of deregulated genes. Genes corresponding to those 4 TFBSs were selected to analyze the expression pattern towards biotrophs in *Arabidopsis* microarray data. We noticed that gene expression pattern has been changed in response to the candidate effector i.e. up-regulated genes were mostly down-regulated and down-regulated genes showed up-regulation against selected biotrophic pathogens. We observed that the list of up-regulated genes did not specifically contain genes involved in plant defence, but rather genes involved in defence were among the most repressed. To our knowledge this is the first attempt at using transcriptomics of transgenic *Arabidopsis* plants expressing *M. larici-populina* effector to understand the expression pattern of genes differentially expressed in presence of an effector. Since Mlp124478 accumulates in the nucleus and nucleolus, the study of transcriptome of transgenic plants over-expressing effector might boost our understanding of the regulatory pattern of gene expression in response to biotrophs, which could not as easily be performed using transient expression in *N. benthamiana.*

Cross-talk between defence signalling pathways is believed to assist plants deciding on a defence strategy depending on the type of pathogen encountered. Until now it appears that pathogens have also evolved to manipulate plants for their own interest by suppressing defence related genes or by altering defence signalling network (Pieterse and Dicke 2007). We did not find genes significantly enhancing/suppressing salicylic acid or jasmonic acid signaling pathways, but few genes correspond to negative regulation of ethylene. Since those genes were down-regulated in the transcriptome of *Mlp124478*, it gives clue that *Mlp124478* might manipulate the plant's signaling infrastructure or transcriptional processes by mimicking functions to trick the plant into activating inappropriate responses.

We observed that Mlp124478 effector altered leaf morphology and compromise plant defence to their advantage. Earlier study (Herms and Mattson 1992) showed that

plants have evolved sophisticated mechanisms in order to balance between growth and defence. Depending on different stresses (biotic or abiotic), plant face growth-defence tradeoffs, which are vital for plant production and fitness. Many of the molecular mecanisms are underlying these tradeoffs in plants, which are needed to be elucidated. So cross-talk between plant defence signaling in response to pathogen needed to achieve a balance condition (Huot et al. 2014). Our transcriptomic study showed that Mlp124478 alters plant transcription, which could be in support of growth-defence tradeoffs. Plant resource diversification has been reported to be occurred at different levels, including machinery involved in the process of transcription, translation and protein secretion (Wang, Amornsiripanitch and Dong 2006, Bilgin et al. 2010). It has been reported that transcriptional reprograming induced by the activation of defence is accompanied by the repression of signaling of growth hormone (Huot et al. 2014). Recently it has been shown that tradeoff between plant innate immunity and growth is mediated by a transcription factor, BZR1. It regulates the level of expression of defence genes which are associated by WRKY transcription factors (such as WRKY40), resulting quantitative immune suppression (Lozano-Durán et al. 2013).

Since Mlp124478 exhibits a DNA-binding domain, we investigated if Mlp124478 affect transcription by binding to plant DNA. Defence responsive proteins exhibiting DNA-binding domain have been reported to affect transcription by recognizing specific promoter motif (Ulker and Somssich 2004). Several of the genes observed in the up and down-regulated list are likely to be indirectly mis-regulated following interaction of Mlp124478 with the expression of transcriptional regulators. The presence of a DNA-binding domain in Mlp124478 and the fact that we could confirm Mlp124478 interaction with DNA in a sequence-specific manner suggest that it may alter gene expression to deceive plant immune systems. The fact that our EMSA with synthetic peptide encompassing the DNA-binding domain showed no *in vitro* binding with an oligonucleotide representing the consensus TGA1a sequence indicates that a longer peptide may be needed to adopt proper DNA-binding conformation, or DNA interaction may require host factors or co-effectors. Recently, effectors that bind DNA have started to emerge. CgEP1, a *C. graminicola* effector with DNA-binding properties has been

shown to enhance anthracnose development in maize (Vargas et al. 2016). Like Mlp124478, the oomycete effector PsCRN108 exhibits a DNA-binding domain, localizes to the nucleus and it binds with the HhH promoter motif to down-regulate the expression of defence-related genes (Song et al. 2015). Following the discovery of TGA1a, different group designed experiments to unravel their role *in planta*. For example, TGA1a TF has been reported as a regulator of DNA-binding and *trans*-activation repressor, where they showed that xenobiotic stress promptly and transiently intrudes the normal binding capacity of TGA1a to bind to the cognate activation sequence-1 (*as*-1) promoter element for the activation of transcription (Johnson, Glover and Arias 2001b). However, first use of ChIP for analyzing targets of stress responsive TFs *in vivo* showed in tobacco that under xenobiotic stresses, TGA1a selectively binds to *as-1* promoter elements and trigger transcription (Johnson et al. 2001a). Our results suggest that TGA1a found in this study might have some important role in helping *Mlp124478* in immune responses against biotrophic pathogens.

4.7 Homolog gene in *Arabidopsis* and poplar

Homology search in poplar database for the *Mlp124478* ChIP target in *A. thaliana* revealed that it belongs to the HMG-box DNA-binding family protein in poplar (Ptp.5659.1.S1_at). It also contains 6 exons, 5 introns, and a TGA1a binding site located at the upstream of its transcription start site (TSS). HMG proteins are heterologous class of proteins in higher eukaryotes and relatively abundant in nucleus. They are involved in modulating the transcription of specific genes, either by direct binding to nucleosomes or by organizing complexes of transcription factors and cofactors in response to rapid environmental changes, and sometimes stably binding to the DNA (Agresti and Bianchi 2003, Bianchi and Agresti 2005). Additional genes were shown to be affected by the expression of *Mlp124478*, several down-regulated genes in the transgenic are normally up-regulated during infection while down-regulated ones would normally be up-regulated during infection.

Collectively, our results suggest that *Mlp124478* manipulates plants by targeting nuclear compartments, and remodeling transcription via direct DNA-binding to suppress the transcriptional response to pathogens, and mislead the host into up-regulating the expression of genes unrelated to defence. Therefore, we suggest to rename Mlp124478 to *SHAM TO DECEIVE (STD)* (as use thereafter), to reflect its effect on plant immunity. While our current results are consistent with STD in the nucleus, we cannot rule out that it could also have a distinct function in the nucleolus.

4.8 Proposed model for STD

To summarize, we propose the model showed in Fig. 4.1. After being secreted from *M. larici-populina*, *STD*, localizes to the nucleolar and nuclear compartments where it binds with regulatory sequences upstream of (perhaps other transcriptional regulators) gene to alter their transcription and mislead the plants with regards to the appropriate transcriptional response coherent with a pathogenic presence. To our knowledge, this study is the first report that an *M. larici-populina* effector targets plants promoters to manipulate the transcription process showing the value of using stable expressing lines to uncover processes affected by pathogen effectors.

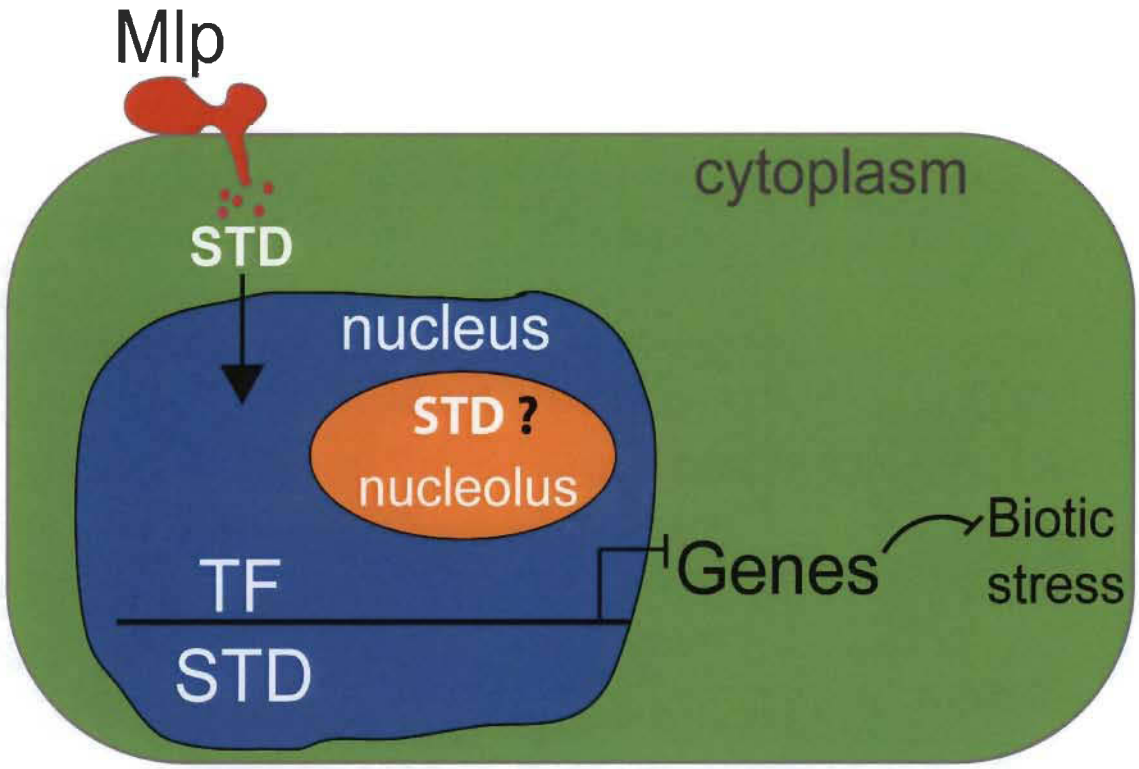


Fig. 4.1 Proposed functional model for STD.
The diagram represents how *STD* reprograms host transcription. *STD* is proposed to bind to DNA at the upstream of TSS of genes. The *STD* then alters the normal transcription process in plants like *A. thaliana* and poplar which eventually represses gene expression in response to biotic pathogens.

CHAPTER V

CONCLUSION

5.1 Conclusion

Effectors are secretory protein molecules from pathogens, which are required for colonization of the host, modification of host physiology to promote parasitic growth and eventually disease onset. The word effector has been accepted by a broad range of microbiologists and pathologists and now is being preferentially used in the field of fungi and oomycete as well (Hogenhout et al. 2009). Effectors target a variety of subcellular compartments and molecules in the host, such as proteins and DNA, and modulate their location, stability, or function to the advantage of the pathogen (Chaudhari et al. 2014, Lewis et al. 2009, Win et al. 2012). Recently, the term effector has also been used to denote secreted microbial proteins which establish symbiotic relationship with plants (Plett et al. 2011, Kloppholz et al. 2011). Plants have evolved a sophisticated resistance mechanism for the constant battle with the rapid genome evolution of diverse pathogenic components. But as a control measure, we need a defined molecular understanding of the functions of effectors. The overall objective of this thesis was to elucidate the molecular functions of *M. larici-populina* effector, *Mlp124478* in the plant. The research provides a better understanding of plant-pathogen interactions.

Genome sequencing of *M. larici-populina* provided access to DNA sequences encoding 1,184 small secreted proteins and allow functional characterization of potential candidate secretory effector proteins (CSEPs). *Mlp124478*, the effector investigated in this study, belongs to the CPG2811 gene family of *M. larici-populina*, which has 9 members in the genome of *M. larici-populina* isolate 98AG31. None of them bears any protein sequence similarity outside of the Pucciniales order. *Mlp124478* is the only member of the CPG2811 family that has a putative nuclear localization signal (NLS)

(29-38 amino acids) and a putative DNA-binding domain (amino acids 58 to 80). The transgenic lines developed in the *A. thaliana* Col-0 background manifested altered leaf morphology-characterized by waviness of leaf margins. In order to unravel how Mlp124478 modify plant cellular functions to induce this phenotype, functional characterization of this effector was conducted. First subcellular accumulation of Mlp124478 in plant cells was observed using confocal laser scanning microscopy of leaves from *A. thaliana* seedlings stably expressing *Mlp124478-GFP* fusion, as well as during transient expression in leaves of *N. benthamiana*. We detected the accumulation of Mlp124478 in the nucleolus of leaf cells, with weaker accumulation in the nucleoplasm and cytosol. The predicted NLS was subsequently validated as NoLS both in *A. thaliana* and *N. benthaliana* model system. We did not find any identical amino acid sequence of the NoLS of Mlp124478 to any other known NoLS in the AtNoPDB, suggesting it might represent a novel NoLS.

We conducted pathogenicity assays using bacterial (*P. syringae* pv. *tomato*) and oomycete (*H. arabidopsidis* Noco2) pathosystems and we observed that Mlp124478 enhances the growth of the filamentous pathogen *H. arabidopsidis* but not that of the bacterial pathogen *P. syringae* in *A. thaliana*. To better understand how Mlp124478 functions in plant cells, we investigated whether Mlp124478 can alter gene expression patterns in *A. thaliana* transgenics. We performed transcriptome profiling of 4-days-old *A. thaliana Mlp124478* stable transgenic line and Col-0 expressing GFP only. A gene ontology (GO) analysis in terms of biological function, revealed that changes in Mlp124478-GFP *A. thaliana* transgenic line transcriptomes occur mostly by a down-regulation of the expression of genes involved in diverse functions, mostly related to defence response regulation. Since Mlp124478 is predicted to possess a DNA-binding domain, it provoked us that it might interfere with transcription through direct interaction with DNA. Thus, we screened for Transcriptional Factor Binding Sites (TFBS) within the promoter sequences of all up- and down-regulated genes and identified 4 different TFBS those were very abundant among the up- and down-regulated genes. Later on, we accessed Genevestigator (http://www.genevestigator.com) to assess the expression levels of those genes found to

be up- and down-regulated in the *Mlp124478*-expressing line. In the five different biotic conditions investigated (*Golovinomyces orontii*, *Phytophthora infestans*, *H. arabidopsidis*, *Golovinomyces cichoracerum*, *Plectosphaerella cucumerina*), we observed that most genes up-regulated in the *A. thaliana* transgenic line overexpressing *Mlp124478* were down-regulated in response to these pathogens. Therefore, we concluded that *Mlp124478* rewires host transcription specifically to induce genes not normally expressed during defence against biotrophic pathogens while more importantly down-regulating genes normally up-regulated in response to such pathogens.

We also tried to identify the interaction partner of Mlp124478. Our yeast two hybrid assay revealed eleven putative interactors. Out of eleven, only one protein (HSP1) contains a NoLS and we considered HSP1 for further confirmation since Mlp124478 was found to localize to the nucleolus. Although transient expression in *N. benthamiana* confirmed the colocalization of Mlp124478 and HSP1 in the nucleolus and the nucleus, but reverse genetics using T-DNA insertion line of *HSP1* indicates that HSP1 is not be the positive interactor of Mlp124478. Therefore, we concluded that other putative interactors found in Y2H assay could be the real interactor proteins, or that HSP1 might require cochaperone and/or cofactors in the nucleus to assist Mlp124478 pathogenicity in plant cells.

The nuclear localization of Mlp124478, the presence of a DNA-binding motif and alterations at the transcriptional, morphological and defence levels prompted us to investigate whether Mlp124478 associates with DNA molecules. To this end, we performed ChIP coupled with PCR experiment and observed an interaction of Mlp124478 with the promoter of a HMG-box (high mobility group) DNA-binding family gene (AT2G34450) containing a TGA1a-binding site among the most strongly up-regulated genes in *Mlp124478*-expressing plants. However, we have found a homolog gene in the poplar which is also a HMG-box (high mobility group) DNA-binding family gene (gene model POPTR_0004s13630.1). AT2G34450 and POPTR_0004s13630.1 contains six exons and five introns and TGA1a regulatory

sequence at the upstream of the gene, which indicates that DNA interaction in *A. thaliana* could occur similar way in poplars.

This is the first attempts at using transcriptomics of transgenic *A. thaliana* plants expressing *M. larici-populina* effector to understand the expression pattern of genes differentially expressed in presence of an effector. Since Mlp124478 accumulates in the nuclear compartments, the study of transcriptome of transgenic plants over-expressing effector might boost our understanding of the regulatory pattern of gene expression in response to biotrophic pathogens, which could not as easily be performed using transient expression in *N. benthamiana*.

Considering all functional observations, we concluded that *Mlp124478* manipulates plants by targeting nuclear compartments; manipulate the plant's signaling infrastructure or transcriptional processes by mimicking functions to trick the plant into activating inappropriate responses, which provoked us to rename *Mlp124478* as *SHAM TO DECEIVE (STD)*, to reflect its function. Finally, to get a glimpse of the molecular function of STD, we propose a model. The model shows that after being secreted from *M. larici-populina*, *STD*, localizes to the nucleolar and nuclear compartments where it binds with regulatory sequences upstream of gene to alter their transcription. To our knowledge, this study is the first report that an *M. larici-populina* effector targets plants promoter to manipulate the transcription process showing the value of using stable expressing lines to uncover processes affected by pathogen effectors.

5.2 Perspectives

5.2.1 Short term perspectives

5.2.1.1 Confirmation of NoLS as a novel NoLS sequence

NLStradamus predicted nuclear localization sequence (NLS) within the amino acid sequence of Mlp124478, which is ten amino acids longer, next to signal peptide sequence. Our subcellular localization study using *A. thaliana* and *N. benthamiana*

model system confirmed that Mlp124478 strongly accumulate in the nucleolus; i.e. the predicted NLS proved as NoLS. Our *in silico* study found the NoLS as a novel amino acid sequence for NoLS within the *Arabidopsis* nucleolar protein database (AtNoPDB). Therefore, we sought to further examine the NoLS. The NoLS of Mlp124478 corresponds to a stretch of 30 bp. We aim to clone the NoLS sequence under the control of 35S promoter using a modified Gateway cloning method. We would like to express the NoLS and mutated NoLS *in planta* expecting the NoLS to uniquely accumulates in the nucleolus, in contrast with the mutated NoLS

5.2.1.2 Assessment of possible interactors of Mlp124478

Since Mlp124478 and its putative interactor HSP1 localize to the nucleolus, we sought to confirm whether HSP1 and Mlp124478 interacts within the nucleolus. Although they colocalize in the nucleolus, further genetic studies did not find HSP1 as a positive interactor. Therefore, we concluded that further studies should be carried out to find out the exact role of HSP1. Previous studies (Azevedo et al. 2006) established that HSP90, an HSP protein that functions as a molecular chaperone with the help of a cochaperone, SGT1 to modulate the structure or stability of R proteins. Therefore, we assume that it would be useful to find out possible interactors in the nucleolar compartment as cofactors of HSP1 to prove the idea that the initial complex of HSP1-cofactor assists Mlp124478 for its parasitic behavior.

5.2.2 Long term perspectives

5.2.2.1 Effect of NoLS removal on pathogenesis

We considered mature protein of Mlp124478 for *in planta* pathogenicity assay using two different model pathosystem (*P. syringae* pv. *tomato* and *H. arabidopsidis* Noco2) to check whether Mlp124478 alters pathogen virulence on *A. thaliana*. Mlp124478 enhances the growth of the filamentous pathogen *H. arabidopsidis* Noco2 but not that of the bacterial pathogen *P. syringae* in *A. thaliana*. However, we observed that Mlp124478 accumulate strongly at the nucleolus with a weak signal in the

nucleoplasm and cytoplasm. Therefore, we would like to assess whether Mlp124478 requires its NoLS to fulfill its virulence function. To this end, we will generate transgenic *A. thaliana* expressing Mlp124478 harbouring wild-type and mutated NoLS and then use the obligate biotrophic oomycete pathogen *H. arabidopsidis* as a proxy for filamentous pathogen to assess if Mlp124478 retains its virulence with a mutated NoLS. If we do not observe any alteration in the leaf morphology of the plant expressing mutated NoLS, and/or significant hypersusceptibility to *H. arabidopsidis*, then we will conclude that the nucleolar location is important for virulence.

5.2.2.2 Confirmation of possible interactors of Mlp124478

Since HSP1 has not been confirmed as a positive interactor of Mlp124478, we aim to perform additional experiments to find out possible interactors of Mlp124478. We revealed ten other interactors using yeast two hybrids which do not contain any predicted NoLS, hence, it would be beneficial to clone those possible interactors to visualize colocalization with Mlp124478, and reverse genetics using T-DNA homozygous line to confirm their role in association with Mlp124478. It is possible that Mlp124478 strongly accumulates in the nucleolus, then is later translocated in other subcellular compartments where it could interact with proteins from the nucleus or the cytoplasm. Those proteins could interact with Mlp124478 to modify the architecture of Mlp124478, and finally facilitate Mlp124478 to bind to TFBSs at the upstream of gene (s) to rewire transcriptional process in plant. Moreover, nuclear proteins could be isolated from the transgenic *A. thaliana* expressing *Mlp124478*, and proteins that accumulate in the nucleus exclusively in response to Mlp124478 could be identified using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS-MS).

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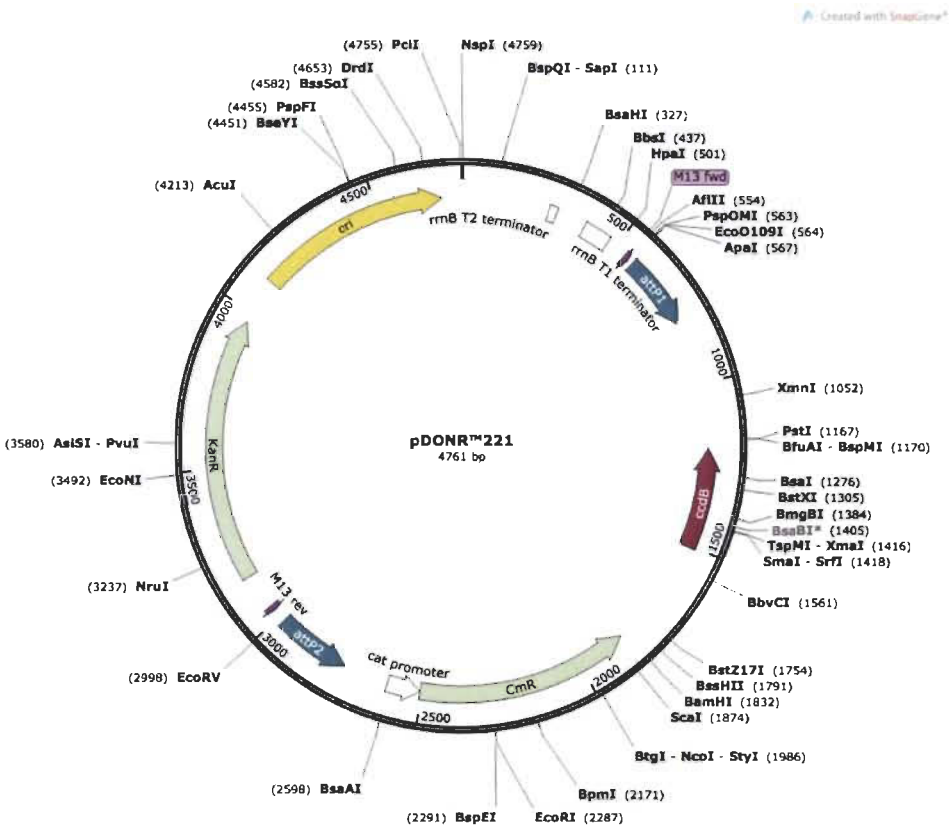
ANNEX A

SUPPLEMENTARY DATA FOR CHAPTER II AND III

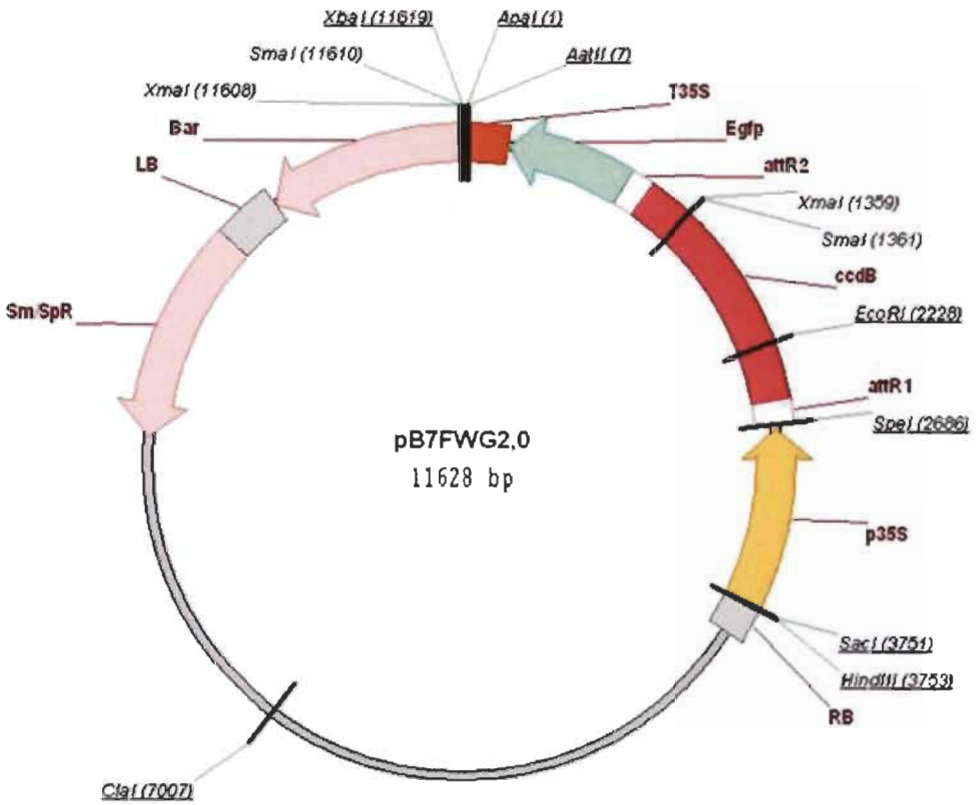


Supplementary Fig. 2.1. Gateway pDONR^{TM}221 vector.

Gateway donor vector with recmbinational sites attP1 and aatP2, and kanamycin resistance marker. pDONR^{TM}221 has a pUC origin for high plasmid yields and universal M13 sequencing sites.

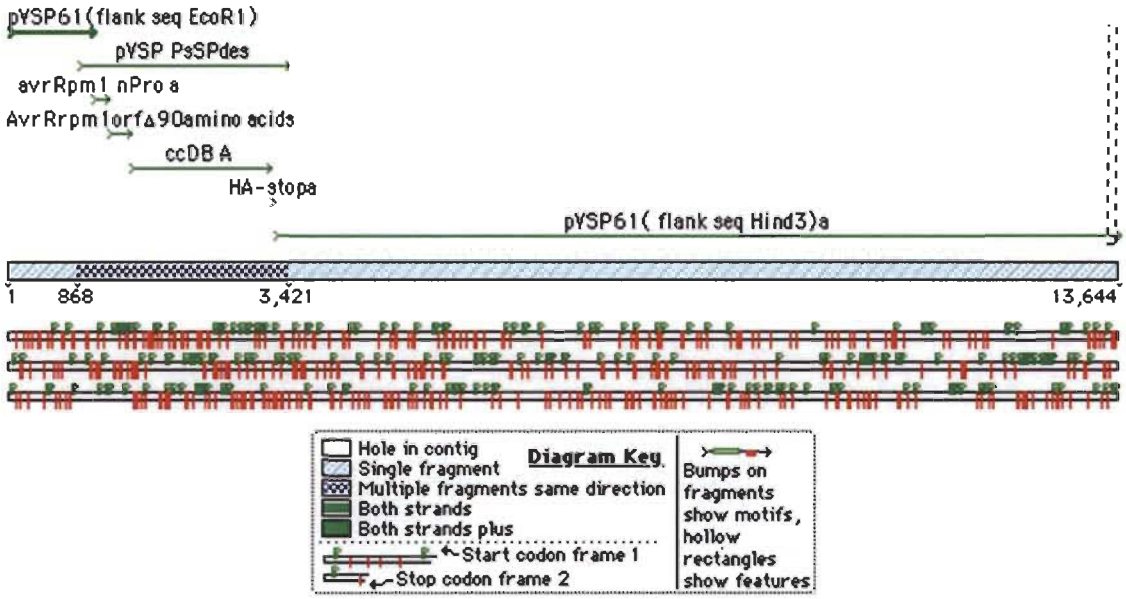
rrnB T2 transcription termination sequence (c):	268-295
rrnBT1 transcription termination sequence (c):	427-470
M13 Forward (-20) priming site:	537-552
attP1:	570-801
ccdB gene (c):	1197-1502
Chloramphenicol resistance gene (c):	1847-2506
attP2 (c):	2754-2985
M13 Reverse priming site:	3027-3043
Kanamycin resistance gene:	3156-3965
pUC origin:	4086-4759

(c)=complementary strand



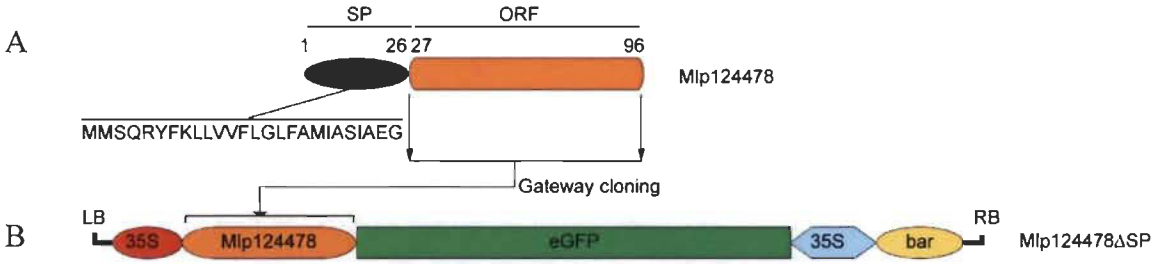
Supplementary Fig. 2.2. Gateway destination vector pB7FWG2,0.

Gateway destination vector with recmbinational sites attR1 and aatR2, and spectinomycin resistance marker. pB7FWG2,0 has a 35S promoter bar gene for selection, and Egfp at the C-terminal fusion to the protein (N-terminal fusion to fluorescence tag).

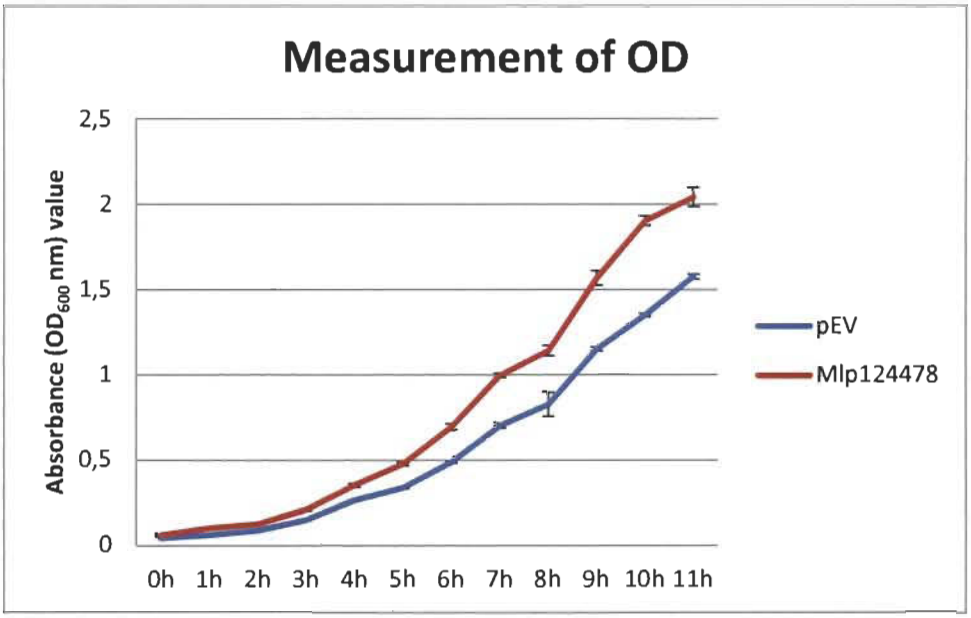


Supplementary Fig. 2.3. Map of pVSPPsSpdes.

pVSP*Ps*Spdes harbors the AvrRpm1 secretion signal, ccDBA at the N-terminus, pVSP61 (flank sequence Hind3) at the C-terminus and pVSP61 (flank sequence EcoR1) at the N-terminus.

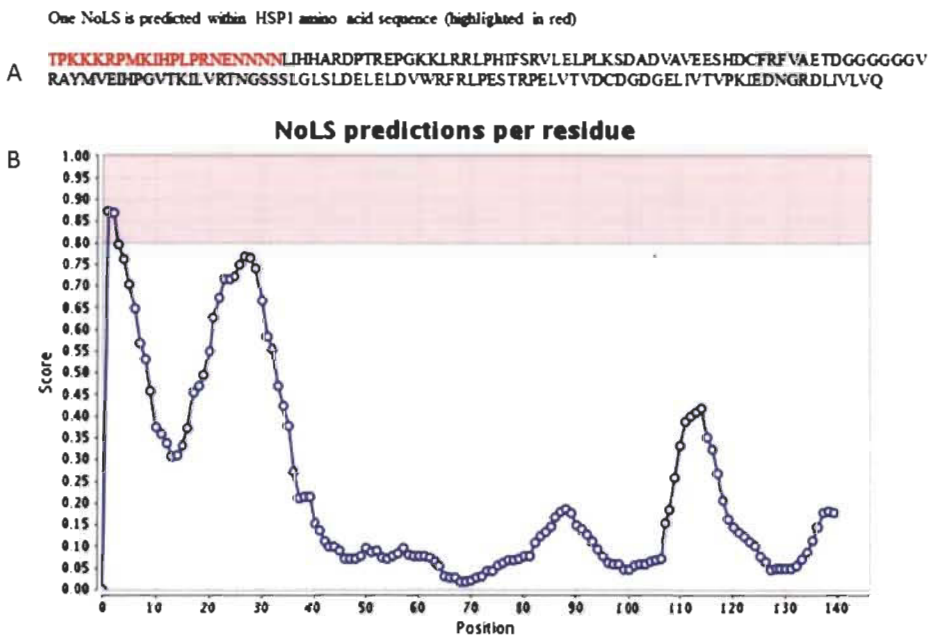


Supplementary Fig. 3.1. Schematic representation of Mlp124478 construct.
(A) Full length Mlp124478 showing SP and ORF. SP resides in first 26 amino acids. (B) ORF of Mlp124478 was cloned at the N-terminus of eGFP in pB7FWG2.0 destination vector using Gateway cloning technology. 35S was used as promoter and basta resistance gene (*bar*) as selectable marker. SP: Signal Peptide; ORG: Open Reading Frame; eGFP: Enhanced Green Fluorescent Protein; LB: Left Border; RB: Right Border.



Supplementary Fig. 3.2. Growth curve of *Pst*DC3000ΔCEL with and without *Mlp124478*.

The cell growth was determined by measuring the absorbance at OD_{600} nm of the bacterial cell culture. Experiments were performed in triplicate. The data points and error bars indicates the average values and standard errors. pEV: Empty vector (*Pst*DC3000ΔCEL); Mlp124478: *Pst*DC3000ΔCEL containing the effector Mlp124478.



Supplementary Fig. 3.3. NoLS prediction of HSP1.

Nucleolar Localization Sequence Detector (NoD) software was used to predict the presence of nucleolar localization sequence within the amino acid sequence of HSP1. (A) Twenty-one amino acid stretch of NoLS is predicted at the C-terminus of HSP1, which is highlighted in red in the full length amino acid sequence of HSP1. (B) Graphical representation of score value for the NoLS predictions per residue of HSP1 amino acids.

Supplementary Table 2.1. List of primers used.

No	Name	Sequence	Tm
1	GW-GFPc-Mlp124478-ΔSP_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAGGTCGACACAAAAATGGGGGT	60.6
2	GW-GFPc-Mlp124478$\Delta_{29\text{-}38}$_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGAGGACAGCTATGACAAACACCGTAAATAACGG	58.7
3	GW-GFPc-Mlp124478+$\Delta_{29\text{-}38}$_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCACATGTAACTTTCACGTTCCCCC	61.9

No	Name	Left primer sequence	Tm	Right primer sequence	Tm
4	Ch_U_ABF1.1	accgtgaaacgttgagtcttc	59,25	agactcatctcccacaatttga	58.65
5	Ch_U_ABF1.2	tttgcgtcttatcttccttgg	59,35	aaaaacagcgatacatttcaa	55,16
6	Ch_U_ABF1.3	ccttgaaatcatcggcattt	59,9	tttggcgcgtgagatagtta	59,47
7	Ch_U_ABF1.4	ggatggtggtgaagaaaagg	59,38	aggaggcgtaagacgtgaaa	59,88
8	Ch_U_TGA1a.1	cacgcataggcttctgtgat	58,9	cactagaccaatcagaaagagga	57,62
9	Ch_U_TGA1a.2	tggttgggaaagatttgaga	58,12	gcccatttttctttgtcagtg	58,2
10	Ch_U_TGA1a.3	tcataaaacaccctctcacaca	58,13	ggagacttttgctttttcca	57,07
11	Ch_U_TGA1a.4	gatggaagtaaaggagcgaaaa	59,74	agattgtcggagacctttgc	59,29
12	Ch_U_TGA1a.5	tctcccctccacatacaaaa	57,99	ttggtagaccaaatcgggta	57,94
13	Ch_U_TGA1a.6	acgtcgtcgtttgattgatt	58,08	cgtgatgacgtggtgaatta	57,97

No	Name	Left primer sequence	Tm	Right primer sequence	Tm
14	Ch_U_TGA1a.7	atggcttgttcacagcaaaa	59,32	aattagacgcatccctggttt	59,85
15	Ch_U_TGA1a.8	tgatcacagccattttgatg	58,04	ctcatgaatcttcacgacgta	56,37
16	Ch_U_TGA1a.9	attcatttgaccgctgcac	59,67	aatggtgaagctttttaggc	56,18
17	Ch_U_TGA1a.10	caacgaaagagtccacgttt	57,86	gaagcaggaaacgaaacaga	58,08
18	Ch_U_TGA1a.11	agcacaaactccacctttga	58,34	accacaaaaacttgggcata	58
19	Ch_U_TGA1a.12	cccgagttggtaccagtgta	58,5	tgttgggaccatgaatttct	57,84
20	Ch_U_TGA1a.13	acgtacgtaccatccttttga	58,56	ccgcatataaaatgcctcac	58,14
21	Ch_U_TGA1a.14	aaattaggagacgtggacga	57,27	tttctttcctccacagatgg	57,71
22	Ch_U_TCP16.1	ttggctaaaatctgggagtg	57,82	tagcgaaacaagagccaaac	58,17
23	Ch_U_TCP16.2	aaaagaatccacacccaaca	57,89	aagcaatcacaaccatccat	57,86
24	Ch_U_TCP16.3	cagggaaagcagttgaaaga	58,08	gggaattaccgtccacaaat	58,62
25	Ch_U_TCP16.4	cgccttgtttggtaagaaaa	57,95	ccaaagtttcatgacgatcc	57,99
26	Ch_D_ATHB5.1	cgcttgttgcatgatgttagta	58,91	agatctcatgatttcgtcaaa	54,85
27	Ch_D_ATHB5.	cttacctctctcctctttgga	58,98	gcaattgtaggcttgtgatgtt	59,16
28	Ch_D_ATHB5.3	cggataaggccctttagaga	59,7	tgacccacctttgcttctt	59,71
29	Ch_D_ATHB5.4	gcttcctacccaatttccaa	59,02	cgcgtttttggagaagaagt	59,49
30	Ch_D_ATHB5.5	cgcttgtttgttaccgtcaa	59,77	cacacttaatgggccttttgt	59,01
31	Ch_D_ATHB5.6	gagcaacgaagctcctctctat	59,29	ttaaacgcgaaacctcatgt	58,29
32	Ch_D_ATHB5.7	acgtactccccaaaataagca	58,64	ccatgaccagtcaaggcata	59,52

No	Name	Left primer sequence	Tm	Right primer sequence	Tm
33	Ch_D_ATHB5.8	aattgtcttgccaactgaacc	59,09	tcgtagatccgaattggtaatg	58,97
34	Ch_D_ATHB5.9	ctttttaggccaccaccaaa	59,97	caagcgataaaccgaggtaaa	59,26
35	Ch_D_ATHB5.10	atacttgcaccgcccatatt	59,32	aatggaataccgccgtgtag	59,85
36	Ch_D_ATHB5.11	agtgcaagcacaagggaact	59,91	ccggttggaaccttgaatta	59,79
37	Ch_D_TCP16.1	ccggaaaccataaaaccaga	59,79	aatcgccacgtaagtcatcc	59,96
38	Ch_D_TCP16.2	aaaaccttgcaccagtttcc	59,08	cttgtgttccattgtcgtctg	59,2

Supplementary Table 3.1. List of up-regulated genes.

Gene	Fold Change	Description
AT1G24580	9,53	RING/U-box domain-containing protein
AT2G15020	8,54	Uncharacterized protein
AT2G37770	7,06	NAD(P)-linked oxidoreductase-like protein
AT2G19650	4,62	Cysteine/histidine-rich C1 domain-containing protein
AT5G24660	4,41	Response to low sulfur 2
AT3G47420	4,28	Phosphate starvation-induced protein
AT4G12320	4,26	Cytochrome P450
AT3G58990	3,85	Isopropylmalate isomerase 1
AT2G34450	3,78	High mobility group (HMG1/2) domain-containing protein
AT4G14020	3,49	Rapid alkalinization factor (RALF) family protein
AT2G27420	3,28	Cysteine proteinase-like protein
AT3G45140	3,12	Lipoxygenase 2
AT3G52740	3,08	Uncharacterized protein
AT1G04770	3,07	Male sterility MS5 family protein
AT5G13530	3,03	E3 ubiquitin-protein ligase KEG
AT1G18773	3,02	Uncharacterized protein
AT4G15490	2,98	UDP-glycosyltransferase-like protein
AT4G23290	2,91	Cysteine-rich receptor-like protein kinase 21
AT5G41410	2,87	Homeobox protein BEL1-like protein
AT5G46690	2,87	Transcription factor bHLH71
AT3G54600	2,78	Class I glutamine amidotransferase domain-containing protein
AT5G42760	2,73	Leucine carboxyl methyltransferase
AT5G14760	2,69	L-aspartate oxidase
AT5G49480	2,68	Ca2+-binding protein 1
AT3G14210	2,68	Epithiospecifier modifier 1

Gene	Fold Change	Description
AT4G34950	2,64	Major facilitator family protein
AT5G39710	2,62	Pentatricopeptide repeat-containing protein
AT1G62050	2,61	Ankyrin repeat-containing protein
AT4G23290	2,59	Cysteine-rich receptor-like protein kinase 22
AT2G46810	2,56	Transcription factor bHLH70
AT5G23040	2,50	CELL GROWTH DEFECT FACTOR 1
AT4G16880	2,48	Leucine-rich repeat (LRR) family protein
AT3G51660	2,48	Macrophage migration inhibitory factor family protein
AT3G04140	2,47	Ankyrin repeat-containing protein
AT3G21670	2,44	Major facilitator protein
AT1G29640	2,43	Uncharacterized protein
AT1G06180	2,41	Myb proto-oncogene protein
AT2G39800	2,40	Gamma-glutamyl phosphate reductase
AT1G18810	2,40	Protein phytochrome kinase substrate 3
AT4G08870	2,34	Putative arginase
AT4G09770	2,32	TRAF-like family protein
AT4G39510	2,31	Cytochrome P450
AT1G57610	2,31	Uncharacterized protein
AT1G33170	2,30	Putative methyltransferase PMT18
AT5G54130	2,28	Calcium-binding endonuclease/exonuclease/phosphatase family protein
AT5G17300	2,27	Myb family transcription factor
AT5G24470	2,27	Two-component response regulator-like APRR5
AT1G10920	2,26	*NB-ARC domain-containing disease resistance protein*
AT1G60590	2,26	Pectin lyase-like protein
AT2G47750	2,24	Putative indole-3-acetic acid-amido synthetase GH3,9
AT2G43920	2,24	Putative thiol methyltransferase 1

Gene	Fold Change	Description
AT3G48310	2,24	Cytochrome P450 71A22
AT3G14200	2,23	Chloroplast import apparatus 2 protein
AT1G13650	2,23	Uncharacterized protein
AT3G48320	2,23	Cytochrome P450 71A21
AT4G12830	2,22	Hydrolase
AT1G65190	2,22	Protein kinase domain-containing protein
AT4G38620	2,21	Transcription repressor MYB4
AT1G65900	2,21	Uncharacterized protein
AT3G14440	2,19	9-cis-epoxycarotenoid dioxygenase NCED3
AT3G05830	2,18	Uncharacterized protein
AT4G31870	2,17	Glutathione peroxidase 7
AT4G26860	2,17	Putative pyridoxal phosphate-dependent enzyme
AT5G17550	2,16	Peroxin 19-2
AT5G53280	2,16	Plastid division protein 1
AT5G62130	2,13	Per1-like family protein
AT5G13170	2,13	Senescence-associated protein 29
AT5G58770	2,13	Dehydrodolichyl diphosphate synthase 2
AT4G03400	2,12	Auxin-responsive GH3 family protein
AT1G62630	2,12	CC-NBS-LRR class disease resistance protein
AT2G01290	2,12	Ribose 5-phosphate isomerase A
AT5G50950	2,11	Fumarate hydratase 2
AT2G40480	2,11	Uncharacterized protein
AT5G25120	2,10	Cytochrome P450 71B11
AT5G16980	2,09	2-alkenal reductase
AT1G26770	2,09	Expansin A10
AT1G52400	2,09	Beta glucosidase 18
AT3G26310	2,09	Cytochrome P450 71B35

Gene	Fold Change	Description
AT1G74640	2,09	Putative alpha/beta-hydrolase-like protein
AT2G39250	2,08	AP2-like ethylene-responsive transcription factor SNZ
AT4G28250	2,08	Expansin B3
AT3G63160	2,08	Uncharacterized protein
AT3G11090	2,08	LOB domain-containing protein 21
AT5G25130	2,07	Cytochrome P450 71B12
AT2G32990	2,07	Endoglucanase 11
AT1G52590	2,06	Putative thiol-disulfide oxidoreductase DCC
AT1G75900	2,05	GDSL esterase/lipase EXL3
AT1G22590	2,05	Protein AGAMOUS-like 87
AT1G75030	2,05	Thaumatin-like protein 3
AT1G18360	2,04	Alpha/beta-hydrolase domain-containing protein
AT4G10120	2,04	Sucrose-phosphate synthase
AT3G22104	2,03	Phototropic-responsive NPH3 family protein
AT1G64500	2,02	Glutaredoxin-like protein
AT4G39800	2,02	Inositol-3-phosphate synthase isozyme 1
AT1G02010	2,01	Protein transport sec1a
AT2G41870	2,01	Remorin-like protein
AT1G73870	2,01	Zinc finger protein CONSTANS-LIKE 7
AT3G09440	2,00	Protein heat shock protein 70-3

Supplementary Table 3.2. List of down-regulated genes.

Gene	Fold Change	Description
AT5G25250	-38,92	Flotillin-like protein 1
AT4G22470	-16,61	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT3G49620	-13,50	2-oxoglutarate-Fe(II)-dependent oxygenase domain-containing protein
AT3G59900	-12,02	ARGOS protein
AT2G47780	-9,41	Rubber elongation factor protein (REF)
AT1G12290	-8,68	CC-NBS-LRR class disease resistance protein
AT3G54590	-6,04	Hydroxyproline-rich glycoprotein
AT4G12480	-5,97	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT2G35980	-5,95	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT2G36690	-5,88	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein
AT4G12470	-5,68	Azelaic acid induced 1
AT2G43000	-5,55	NAC domain-containing protein 42
AT4G25200	-5,51	Small heat shock protein 23,6
AT5G67060	-5,48	Transcription factor HEC1
AT5G15120	-5,35	Uncharacterized protein
AT5G02760	-5,33	Putative protein phosphatase 2C 67
AT4G16515	-5,31	Uncharacterized protein
AT3G62680	-5,00	Proline-rich protein 3
AT4G16515	-5,00	Uncharacterized protein
AT5G53250	-4,98	Arabinogalactan protein 22
AT1G18400	-4,86	Transcription factor BEE 1
AT3G54580	-4,74	Proline-rich extensin-like family protein

Gene	Fold Change	Description
AT5G02540	-4,69	Rossmann-fold NAD(P)-binding domain-containing protein
AT1G10550	-4,56	Xyloglucan:xyloglucosyl transferase
AT3G29370	-4,55	Uncharacterized protein
AT5G39610	-4,50	NAC domain containing protein 6
AT5G25440	-4,39	Protein kinase family protein
AT5G57240	-4,38	OSBP(oxysterol binding protein)-related protein 4C
AT2G20835	-4,29	Uncharacterized protein
AT3G23150	-4,23	Putative ethylene receptor
AT4G02270	-4,12	Protein root hair specific 13
AT2G32190	-4,11	Uncharacterized protein
AT1G27020	-4,06	Uncharacterized protein
AT4G30280	-4,05	Xyloglucan:xyloglucosyl transferase
AT1G06350	-4,01	Delta-9 desaturase-like 4 protein
AT1G05135	-4,00	Pseudogene
AT2G22860	-3,88	Phytosulfokine-beta
AT5G14920	-3,82	Gibberellin-regulated protein
AT3G20395	-3,81	RING-finger domain-containing protein
AT2G38530	-3,77	Non-specific lipid-transfer protein 2
AT2G27080	-3,75	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT5G22500	-3,72	Fatty acyl-CoA reductase 1
AT4G24275	-3,68	Uncharacterized protein
AT4G15090	-3,54	Protein FAR-RED IMPAIRED RESPONSE 1
AT1G69490	-3,52	NAC domain-containing protein 29
AT5G05500	-3,51	Pollen_Ole_e_I-domain containing protein
AT1G14120	-3,47	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein
AT2G41990	-3,46	Uncharacterized protein

Gene	Fold Change	Description
AT5G13860	-3,44	Protein ELC-like protein
AT3G05150	-3,44	Sugar transporter ERD6-like 8
AT4G09030	-3,43	Arabinogalactan protein 10
AT5G65390	-3,42	Arabinogalactan protein 7
AT5G43270	-3,40	Squamosa promoter-binding-like protein 2
AT4G13400	-3,35	Dioxygenase domain-containing protein
AT3G02550	-3,32	LOB domain-containing protein 41
AT5G60460	-3,31	Protein transport protein SEC61 subunit beta
AT5G26622	-3,28	Beta-galactosidase related protein
AT2G44080	-3,24	ARGOS-like protein
AT5G03150	-3,24	Zinc finger protein JACKDAW
AT3G18200	-3,23	EamA domain-containing protein
AT5G40780	-3,18	Lysine histidine transporter 1
AT5G15230	-3,17	Gibberellin-regulated protein 4
AT4G09890	-3,17	Uncharacterized protein
AT4G40010	-3,15	Serine/threonine-protein kinase SRK2F
AT3G46280	-3,14	Protein kinase-like protein
AT1G29465	-3,12	Uncharacterized protein
AT5G04960	-3,11	Putative pectinesterase/pectinesterase inhibitor 46
AT4G08040	-3,10	1-aminocyclopropane-1-carboxylate synthase 11
AT1G50740	-3,10	Transmembrane proteins 14C
AT2G39200	-3,06	MLO-like protein 12
AT1G73330	-3,05	drought-repressed 4 protein
AT3G12710	-3,02	DNA-3-methyladenine glycosylase I
AT1G32920	-3,00	Uncharacterized protein
AT1G18570	-2,99	Myb domain protein 51
AT1G11210	-2,98	Uncharacterized protein

Gene	Fold Change	Description
AT2G19620	-2,94	N-MYC down-regulated-like 3 protein
AT1G60060	-2,93	Serine/threonine-protein kinase WNK (With No lysine)-related protein
AT1G29090	-2,92	Cysteine proteinase-like protein
AT5G03860	-2,92	Malate synthase
AT5G22310	-2,90	Uncharacterized protein
AT5G64410	-2,86	Oligopeptide transporter 4
AT2G15292	-2,86	Unknown gene
AT1G17620	-2,83	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT1G73830	-2,83	Transcription factor BEE 3
AT5G24210	-2,81	Lipase class 3 family protein
AT3G19680	-2,81	Uncharacterized protein
AT2G46330	-2,78	Arabinogalactan protein 16
AT3G11550	-2,78	Uncharacterized protein
AT1G67910	-2,76	Uncharacterized protein
AT1G19350	-2,74	Protein brassinazole-resistant 2
AT1G19180	-2,74	Protein TIFY 10A
AT5G67520	-2,73	Adenylylsulfate kinase
AT5G37660	-2,73	Cysteine-rich repeat secretory protein 60
AT2G34930	-2,71	Disease resistance-like protein/LRR domain-containing protein
AT3G15540	-2,70	Auxin-responsive protein IAA19
AT2G42870	-2,70	Phy rapidly regulated 1
AT2G41100	-2,68	Calmodulin-like protein 12
AT4G14560	-2,67	Auxin-responsive protein IAA1
AT4G01950	-2,66	Glycerol-3-phosphate acyltransferase
AT4G29140	-2,63	Delta-9 acyl-lipid desaturase 1

Gene	Fold Change	Description
AT1G76930	-2,62	Extensin 4
AT1G78460	-2,62	SOUL heme-binding protein
AT5G25930	-2,61	Protein kinase family protein with leucine-rich repeat domain
AT4G19810	-2,60	Glycosyl hydrolase family protein with chitinase insertion domain
AT5G52830	-2,60	WRKY DNA-binding protein 27
AT1G63860	-2,60	TIR-NBS-LRR class disease resistance protein
AT5G64310	-2,59	Arabinogalactan protein 1
AT2G23810	-2,59	Tetraspanin8
AT1G19610	-2,58	Defensin-like protein 19
AT5G06930	-2,57	Uncharacterized protein
AT5G05380	-2,56	PRA1 family protein B3
AT5G25350	-2,55	EIN3-binding F-box protein 2
AT4G38400	-2,55	Expansin-like A2
AT4G17260	-2,55	L-lactate dehydrogenase
AT1G57990	-2,55	Purine permease 18
AT1G65310	-2,55	Xyloglucan:xyloglucosyl transferase
AT3G46700	-2,54	Glucuronosyl transferase-like protein
AT2G27260	-2,54	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT5G06860	-2,52	Polygalacturonase inhibitor 1
AT1G17020	-2,52	Protein SRG1
AT1G21310	-2,51	Extensin 3
AT2G17880	-2,50	DNAJ heat shock N-terminal domain-containing protein
AT5G62920	-2,50	Two-component response regulator ARR6
AT2G25735	-2,50	Uncharacterized protein
AT5G57910	-2,49	Uncharacterized protein
AT1G50040	-2,49	Uncharacterized protein

Gene	Fold Change	Description
AT1G30750	-2,49	Uncharacterized protein
AT3G45730	-2,48	Uncharacterized protein
AT4G25810	-2,48	Xyloglucan:xyloglucosyl transferase
AT2G41010	-2,47	Calmodulin binding protein 25
AT1G30040	-2,47	Gibberellin 2-beta-dioxygenase 2
AT1G64640	-2,46	Early nodulin-like protein 8
AT5G13890	-2,45	Uncharacterized protein
AT4G31800	-2,45	WRKY transcription factor 18
AT2G26070	-2,45	Uncharacterized protein
AT1G19540	-2,44	NmrA-like negative transcriptional regulator-like protein
AT4G36500	-2,44	Uncharacterized protein
AT3G45970	-2,43	Expansin-like A1
AT1G03870	-2,43	Fasciclin-like arabinogalactan protein 9
AT4G35840	-2,43	NEP1-interacting protein 1
AT5G04190	-2,43	Protein PHYTOCHROME KINASE SUBSTRATE 4
AT1G78890	-2,43	Uncharacterized protein
AT3G05890	-2,42	Hydrophobic protein RCI2B
AT4G12720	-2,42	Nudix hydrolase 7
AT3G11550	-2,42	Uncharacterized protein
AT4G08950	-2,41	Phosphate-responsive 1 family protein
AT5G13890	-2,40	Uncharacterized protein
AT3G27220	-2,39	Kelch repeat-containing protein
AT1G74340	-2,38	Dolichyl-phosphate mannosyltransferase polypeptide 2
AT3G04290	-2,37	GDSL esterase/lipase LTL1
AT2G26710	-2,37	PHYB activation tagged suppressor 1 protein
AT1G48320	-2,37	Thioesterase-like protein
AT4G17500	-2,36	Ethylene-responsive transcription factor 1A

Gene	Fold Change	Description
AT3G63380	-2,36	Ca2+-transporting ATPase
AT4G23190	-2,35	Cysteine-rich receptor-like protein kinase 11
AT1G25220	-2,35	Anthranilate synthase beta subunit 1
AT2G38470	-2,34	Putative WRKY transcription factor 33
AT5G25940	-2,34	Early nodulin-related protein
AT4G32460	-2,33	Uncharacterized protein
AT2G43290	-2,32	Calmodulin-like protein 5
AT1G25230	-2,32	Purple acid phosphatase 4
AT5G08150	-2,32	Suppressor of phytochrome b 5
AT2G38870	-2,32	Serine protease inhibitor
AT1G14870	-2,31	Cadmium resistance protein 2
AT4G33050	-2,31	Calmodulin-binding protein
AT2G43150	-2,30	Proline-rich extensin-like family protein
AT3G13435	-2,30	Uncharacterized protein
AT5G15830	-2,30	Basic leucine-zipper 3
AT3G26760	-2,30	Rossmann-fold NAD(P)-binding domain-containing protein
AT2G47930	-2,29	Arabinogalactan protein 26
AT1G52190	-2,29	Putative peptide transporter
AT1G65845	-2,29	Uncharacterized protein
AT4G02800	-2,29	Uncharacterized protein
AT5G44260	-2,28	Zinc finger CCCH domain-containing protein 61
AT5G05730	-2,27	Anthranilate synthase component I-1
AT2G44500	-2,27	Axi 1 protein-like protein
AT4G22300	-2,27	Carboxylesterase
AT4G27280	-2,27	EF-hand
AT1G48930	-2,27	Endoglucanase 5
AT4G18760	-2,27	Receptor like protein 51

Gene	Fold Change	Description
AT1G71970	-2,27	Uncharacterized protein
AT2G05510	-2,27	Glycine-rich protein
AT4G34250	-2,26	3-ketoacyl-CoA synthase 16
AT2G14890	-2,25	Arabinogalactan protein 9
AT4G37450	-2,25	Lysine-rich arabinogalactan protein 18
AT4G16330	-2,25	Oxidoreductase
AT2G42840	-2,25	Protodermal factor 1
AT4G01720	-2,25	Putative WRKY transcription factor 47
AT5G13190	-2,25	Uncharacterized protein
AT1G11545	-2,24	Xyloglucan:xyloglucosyl transferase
AT4G14130	-2,24	Xyloglucan:xyloglucosyl transferase
AT2G34380	-2,24	Putative adipose-regulatory protein (Seipin)
AT5G43190	-2,23	F-box/kelch-repeat protein
AT2G21140	-2,23	Proline-rich protein 2
AT1G09530	-2,23	Transcription factor PIF3
AT4G21570	-2,21	Uncharacterized protein
AT4G26220	-2,21	Putative caffeoyl-CoA O-methyltransferase
AT4G17490	-2,20	Ethylene-responsive transcription factor 6
AT4G33960	-2,20	Uncharacterized protein
AT1G23080	-2,19	Auxin efflux carrier component 7
AT4G18340	-2,19	Glycosyl hydrolase family 17 protein
AT5G65200	-2,19	U-box domain-containing protein 38
AT1G12080	-2,19	Vacuolar calcium-binding protein-like protein
AT2G01180	-2,18	Lipid phosphate phosphatase 1
AT1G69690	-2,18	Transcription factor TCP15
AT3G62800	-2,18	Double-stranded-RNA-binding protein 4
AT2G19970	-2,17	Putative pathogenesis-related protein

Gene	Fold Change	Description
AT1G78260	-2,17	RNA recognition motif-containing protein
AT3G62570	-2,17	Tetratricopeptide repeat-containing protein
AT1G51430	-2,17	Uncharacterized protein
AT1G06850	-2,16	Basic leucine-zipper 52
AT5G24280	-2,16	Gamma-irradiation and mitomycin c induced 1
AT3G20820	-2,16	Leucine-rich repeat-containing protein
AT5G20650	-2,16	Copper transporter 5
AT5G43470	-2,16	Disease resistance protein RPP8
AT1G27770	-2,15	Autoinhibited Ca2+-ATPase 1
AT3G25600	-2,15	Putative calcium-binding protein CML16
AT3G50340	-2,15	Uncharacterized protein
AT4G07841	-2,15	Zinc ion binding protein
AT5G62280	-2,14	Uncharacterized protein
AT5G27610	-2,14	Protein ALWAYS EARLY 1
AT3G57930	-2,14	Uncharacterized protein
AT5G01950	-2,13	Leucine-rich repeat protein kinase-like protein
AT2G47760	-2,12	Alpha-1
AT4G29240	-2,12	Leucine-rich repeat-containing protein
AT3G49220	-2,12	Pectinesterase
AT3G16660	-2,12	Pollen Ole e 1 allergen and extensin family protein
AT4G35220	-2,12	Cyclase family protein
AT1G14440	-2,12	Homeobox protein 31
AT5G06320	-2,11	NDR1/HIN1-Like protein 3
AT4G01410	-2,11	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT2G42840	-2,11	Protein phosphatase 2A subunit A2
AT5G51730	-2,11	RNA-binding (RRM/RBD/RNP motifs) family protein

Gene	Fold Change	Description
AT1G26570	-2,11	UDP-glucose dehydrogenase 1
AT3G22800	-2,11	Leucine-rich repeat extensin-like protein 6
AT2G17230	-2,10	Protein exordium like 5
AT5G02020	-2,10	Uncharacterized protein
AT1G04240	-2,09	Auxin-responsive protein IAA3
AT5G13270	-2,09	Pentatricopeptide repeat-containing protein
AT1G80820	-2,09	Cinnamoyl-CoA reductase
AT3G57450	-2,08	Uncharacterized protein
AT3G55850	-2,08	Amidohydrolase family protein
AT4G16765	-2,08	Oxidoreductase
AT5G64260	-2,08	Protein EXORDIUM like 2
AT5G03360	-2,07	DC1 domain-containing protein
AT1G33590	-2,07	Leucine-rich repeat-containing protein
AT2G06850	-2,07	Xyloglucan endotransglucosylase/hydrolase protein 4
AT4G37800	-2,07	Xyloglucan:xyloglucosyl transferase
AT3G21720	-2,06	Isocitrate lyase
AT4G36410	-2,06	Putative ubiquitin-conjugating enzyme E2 17
AT2G23170	-2,06	Indole-3-acetic acid-amido synthetase GH3,3
AT1G07570	-2,06	Protein kinase APK1A
AT1G09560	-2,06	Germin-like protein subfamily 2 member 1
AT1G03850	-2,06	Monothiol glutaredoxin-S13
AT2G37130	-2,05	Peroxidase
AT5G45110	-2,05	NPR1-like protein 3
AT5G49700	-2,04	Predicted AT-hook DNA-binding family protein
AT2G45050	-2,04	GATA transcription factor 2
AT3G48100	-2,04	Two-component response regulator ARR5
AT1G15430	-2,04	Uncharacterized protein

Gene	Fold Change	Description
AT4G12520	-2,04	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT3G05880	-2,04	Hydrophobic protein RCI2A
AT3G05320	-2,04	O-fucosyltransferase family protein
AT3G60530	-2,03	GATA transcription factor 4
AT5G04720	-2,03	ADR1-like 2 protein
AT5G64620	-2,03	Cell wall / vacuolar inhibitor of fructosidase 2
AT1G69840	-2,03	Hypersensitive-induced response protein 2
AT2G45180	-2,02	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT5G62200	-2,02	Embryo-specific protein 3
AT1G27670	-2,02	Uncharacterized protein
AT1G02660	-2,02	Alpha/beta-hydrolase domain-containing protein
AT3G02640	-2,02	Uncharacterized protein
AT2G22680	-2,01	C3HC4-type RING finger domain-containing protein
AT2G14900	-2,01	Gibberellin-regulated protein
AT2G24150	-2,01	Heptahelical protein 3
AT1G72200	-2,01	RING-H2 finger protein ATL11
AT1G22330	-2,01	RNA recognition motif-containing protein
AT1G66160	-2,01	U-box domain-containing protein 20
AT3G06070	-2,01	Uncharacterized protein
AT2G43570	-2,01	Chitinase class 4-like protein
AT1G80080	-2,01	Protein TOO MANY MOUTHS
AT1G15670	-2,01	Putative F-box/kelch-repeat protein
AT5G57887	-2,01	Uncharacterized protein
AT5G44680	-2,00	DNA-3-methyladenine glycosylase I
AT5G09440	-2,00	Protein exordium like 4
AT4G19030	-2,00	Aquaporin NIP1-1

Gene	Fold Change	Description
AT1G49050	-2,00	Aspartyl protease
AT1G22500	-2,00	E3 ubiquitin-protein ligase ATL15
AT3G28200	-2,00	Peroxidase 31
AT5G13330	-2,00	Ethylene-responsive transcription factor ERF113
AT3G06750	-2,00	Hydroxyproline-rich glycoprotein family protein
AT5G02290	-2,00	Putative serine/threonine-protein kinase NAK
AT3G07800	-2,00	Thymidine kinase
AT1G71900	-2,00	Uncharacterized protein

ANNEX B

EFFECTOR BIOLOGY DURING BIOTROPHIC INVASION OF PLANT CELLS

Prateek Chaudhari, **BULBUL AHMED**, David Joly, and Hugo Germain

Annex B contains a published review article, containing extended findings on biotrophic effectors, which demonstrates the birds eye view on the molecular relationship between the functions and subcellular accumulation of effectors in the host cells.

I have equally participated for writing the review with the firth author. I have written the part entitled effectors type, localization and function, and nuclear localized effectors.

REVIEW

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Effector biology during biotrophic invasion of plant cells

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Several obligate biotrophic phytopathogens, namely oomycetes and fungi, invade and feed on living plant cells through specialized structures known as haustoria. Deploying an arsenal of secreted proteins called effectors, these pathogens balance their parasitic propagation by subverting plant immunity without sacrificing host cells. Such secreted proteins, which are thought to be delivered by haustoria, conceivably reprogram host cells and instigate structural modifications, in addition to the modulation of various cellular processes. As effectors represent tools to assist disease resistance breeding, this short review provides a bird's eye view on the relationship between the virulence function of effectors and their subcellular localization in host cells.

Introduction

Being sessile organisms, plants are constantly challenged by their environment, and their situation is compounded by biotic stresses. A number of plant pathogens, such as fungi, oomycetes, bacteria, viruses, nematodes, etc., pose serious threats to the plant well-being. Nonetheless, over the course of evolution, plants have acquired a refined, two-layered immune system to respond to pathogen attack.[1] The first line of plant immunity, thought to be the most ancient, relies on the recognition of evolutionarily-conserved pathogen molecules known as PAMPs (pathogen-associated molecular patterns), and is therefore referred to as PAMP-triggered immunity (PTI).[2-4] Pattern recognition receptors (PRRs) are plant components responsible for the detection of PAMPs[5] and for activating the immune machinery of plants. One of the best characterized PRRs in plants is FLAGELLIN SENSITIVE 2 (FLS2), a receptor kinase that activates PTI upon perception of flagellin, a conserved protein found in bacterial flagellum.[6,7]

To gain greater access to plant resources for subsequent colonization, plant pathogens, just like their animal equivalents, deploy an arsenal of highly-sophisticated molecules known as effectors. These molecules greatly augment the pathogen's capacity to propagate on its host by interfering with various cellular processes, including PTI. Fortunately, plants monitor the presence of some effectors through their resistance (R)-proteins, which constitutes the second line of defense, also known as effector-triggered immunity (ETI).[1] ETI typically results in a strong hypersensitive response, characterized by cell death, which shows some mechanistical similarities with apoptosis in animals.[8] It is regulated by direct physical interaction between a R-protein and its corresponding effector (ligand-receptor model) or between a R-protein and a host-protein modified by an effector (guard model). Resistance thus depends on the presence of both the R-protein and its corresponding effector, a situation depicted by Flor's gene-for-gene model.[9,10]

For pathogens to succeed, proper delivery of these effectors is as crucial as the molecule itself. The bacterial type three secretion system (T3SS), one of many secretion systems deployed by *Pseudomonas syringae*, is well-characterized and has been studied in great detail. The syringe-like T3SS provides bacteria with a robust mechanical structure which enables it to inject key molecules involved in pathogenicity directly into host cells.[11] Obligate biotrophic, filamentous pathogens, such as many fungi and oomycetes, are devoid of such secretion systems. Instead, they invaginate within host cells to form particular infection structures called haustoria.[12,13] To accommodate haustoria, host cells are forced to greatly expand their plasma membrane, and it is plausible that pathogens drive this process for their own benefit.

Filamentous pathogens have a large suite of predicted, secreted proteins, which could act early during infection to suppress PTI as the pathogens are establishing themselves and, at later stages, to rewire host cellular activities to meet the pathogen's metabolic needs. It has been proposed that protein trafficking from haustoria allows pathogens to hijack host cells for their own purposes. However, the precise mechanism governing effector translocation from the extra-haustorial space to host cells has eluded scientists thus far.[14] For the purpose of this review, we have classified effectors into three types based on the subcellular compartment they target: apoplastic effectors, cytoplasmic effectors and nuclear effectors. Apoplastic effectors can be secreted by appressoria and/or hyphae invading the intercellular space where they remain outside the cells. This class of effectors includes proteins with inhibitory functions, interfering with plant proteases and peroxidases. For example, the Avr2 effector from the biotrophic fungal pathogen *Cladosporium fulvum* suppresses basal defense through inhibition of specific host proteases.[15-17] On the other side, cytoplasmic and nuclear effectors affect host defense mechanisms by

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targeting proteins involved in plant immune signaling cascades. Moreover, they also manipulate various plant processes, further predisposing the host cellular machinery to act in a pathogen-conducive manner.[18,19] As their names suggest, cytoplasmic effectors target cytosolic components or are redirected to other organelles, while nuclear effectors transit via the cytosol but have a different purpose than the other two effector types (described in subsequent sections). The biology of infection of obligare biotrophic pathogens is rather unique due to the establishment of haustoria. The different strategies deployed by intracellular biotrophic hyphae produced by various pathogens to secrete their effectors are beautifully illustrated by Giraldo and Valent.[13] In this mini-review, we offer a retrospective of the molecular interactions between obligate biotrophic pathogens and their hosts, speculating on this rather intimate relationship at the molecular level and focusing on cellular components representing potential effector targets.

Effector Terminology: Virulence/Avirulence Factors vs. Effectors

It is pertinent to demystify the terminological ambiguity around effectors since, until recently, their nomenclature was contingent upon host reactions. When a molecule from a particular pathogen modulates the host's defensive cover to increase the pathogen's fitness, it is called a virulence factor. However, when the same molecule is recognized by host immunoreceptors, thereby failing to augment pathogenicity and instead triggering a defense response, it is referred to as an avirulence factor. This variation in pathogenicity is a commonly-occurring phenomenon. A particular effector may be a virulence factor on one host and an avirulence factor on another, a situation observed even within a single plant species where interactions are race-specific. Because of this inconsistency, terms such as virulence and avirulence have their limitations, since they are dependent on the specific host system in which they have been observed. The above discussed terminology in plant pathology is thus rather different from that employed in the medical field. In plant immunity, the terms virulence and avirulence are mainly related to the plant's ability to resist or succumb to the pathogen, thus depending on plant genotype.[9] In the medical field, avirulence refers to the loss of a virulence component belonging to the pathogen. Consequently, an inclusive and neutral term such as "effector" is preferred,[20] as it accounts for all the molecules secreted by a pathogen during infection that alter host cell structure or function.[21]

As mentioned earlier, Flor's work was instrumental in establishing the gene-for-gene concept.[9,10] Flor was quite foresighted when he noted that, for each gene conditioning a reaction in the host, there is a corresponding gene that conditions pathogenicity in the pathogen.[9] His deduction came from studies on the inheritance of pathogenicity in flax rust (*Melampsora lini*) and on the inheritance of resistance in flax (*Linum usitatissimum*).[10] Many years later, the flax/flax rust pathosystem remains instrumental in our understanding of the molecular aspects of gene-for-gene interactions. This pathosystem enabled inroads to be made in the molecular interaction between R- and Avr-protein, mainly through studies of L and M resistance genes and their corresponding Avr loci. Flax rust *AvrL567* genes, whose products are recognized by the L5, L6, and L7 R-proteins of flax, are highly diverse and under diversifying selection pressure, with 12 sequence variants identified from six rust strains.[22] Ravensdale et al.[23] studied direct molecular interactions between L5 and L6 (two alleles of L) and their avirulence targets in detail. Site-directed mutagenesis in *AvrL567* and the construction of chimeric L-proteins revealed that the recognition specificities of L5 and L6 are conditioned by their leucine-rich repeat regions. Their study indicated that mutations in the TIR or NB-ARC domain also affect recognition, which prompted the authors to suggest that interaction with the Avr ligand directly competes with intramolecular interactions, causing R-protein activation.[23] The AvrM effector from flax rust also interacts directly with the flax R-protein M, and this interaction can also be observed in yeast two-hybrid assays. Catanzariti et al. showed that the C-terminal domain of AvrM is required for M-dependent cell-death, consistent with the fact that it interacts with M-protein in yeast.[24] Furthermore, these authors demonstrated that C-terminal 34 amino acids formed a structured domain (unlike the N-terminal part of the protein), and gel filtration revealed that AvrM-A can dimerize.[22] Recently Ve et al. resolved the structure of AvrM and AvrM-A and showed that both possess an L-shaped fold and form a dimer with an unusual nonglobular shape.[25]

The avirulence properties of AvrM and AvrL have been described, but yield no clues with regard to their targets and their potential virulence functions. Few rust effectors have been shown to be expressed during infection and translocated to host cells. One of these effectors is rust-transferred protein 1 (RTP1), which belongs to a family of effector proteins specific to the order Pucciniales.[26] RTP1 from *Uromyces fabae* was the first rust effector demonstrated to localize in host cells, and it was also observed that the transfer of the protein was dependent on the developmental stage of haustoria.[27] RTP1 translocates from the extra-haustorial matrix, where it first accumulates, transits through the cytoplasm, then further moves to the nucleus.[27] Unlike most localization studies cited herein, which are mainly based on green fluorescent protein (GFP) fusion and transient expression, RTP1 localization was assessed by immunolocalization during *Uromyces fabae* infection using four independently-raised polyclonal antibodies.[27] RTP1 sequence analyses indicated that the C-terminal domain exhibited similarities to cysteine protease inhibitors, and RTP1 was indeed shown to inhibit proteolytic activity.[26]

Effector Type, Localization, and Function

When dealing with a subject as broad as effectors, it is worthwhile to classify them to the extent that current knowledge in this domain will allow. Therefore, in an attempt to draw clear lines, they can be largely divided into three major groups based on their localization and site of activity: apoplastic, cytoplasmic and nuclear/nucleolar effectors.

As the name suggests, apoplastic effectors are localized to plant extracellular spaces. This class of effectors includes, but is not restricted to, small and cysteine-rich proteins which function primarily by inhibiting host proteases, hydrolases, glucanases, and other lytic enzymes.[13] Recent models suggest that these could be the first effectors to potentially activate the plant defense response (PTI).[13] The architecture of these effectors, often having a signal peptide and a cysteine-rich C-terminus, is highly reminiscent of plant small signaling peptides,[28] which may reflect the prototypic structure that a protein must harbor to survive its passage in apoplastic space. However, apoplastic effectors may have a much more refined mechanism and could exert a long-lasting action in protection of the pathogen cell wall or in chelating/neutralizing antimicrobial compounds being secreted by the host.

On the other hand, cytoplasmic effectors have the duty of dealing with host cells at a much more intricate level. Cytoplasmic effectors are active once they reach the plant cytoplasm and tend to target plant defense signaling components. Effectors from *P. syringae* have been shown to target anti-pathogenic vesicle trafficking and kinase-based recognition activity of the host, a prime defense component.[29] Some effectors may also transit through the cytoplasm to reach their final destination (e.g., organelles).

Nuclear effectors are seemingly ultimate weapons in the inventory of pathogens, since they are thought to suppress the immune response from upstream. Nuclear effectors could potentially shut off master switches of the immune machinery or reprogram host transcription to the benefit of pathogens. A recent investigation of 49 putative effectors from *H. arabidopsidis* revealed that 33% localized strictly to the nucleus, and an additional 33% were nucleo-cytoplasmic.[30] Since several effectors tend to migrate toward the nucleus, it would be logical to assume that some R-proteins act in the nucleus. Indeed, several R-proteins, such as SNC1, N and RPS4, were found to localize to the nucleus.[31-34] Tobacco TIR-NB-LRR R-protein N localizes to the nucleus in the absence of its elicitor, the *Tobacco mosaic virus* p50 helicase fragment,[32] lending support to a default presence of R-proteins in the nucleus to monitor their corresponding effectors rather than being relocalized upon effector binding. However, SNC1 and N nuclear accumulation is reduced at elevated temperatures, making their mode of action temperature-dependent.[35] It was demonstrated recently that ETI is more active at low temperatures (10–23 °C), while PTI takes over at higher temperatures (23–32 °C).[36] It has also been shown that bacterial pathogens strive and multiply at higher temperatures but secrete their effectors more actively at lower temperatures.[37,38] These observations suggest that the immune system of plants is adapted to pathogen physiology. However, some pathogens prefer more temperate environments (around 18 °C) for optimal growth.[39,40]

Nucleolar-Localized Effectors

Computer software, such as NOD, PSORT II, and WoLF PSORT, can predict the subcellular localization of various proteins, but that of very few candidate effectors has been verified experimentally[41-43] relative to the wealth of those from all plant pathogens. A number of plant pathogen-secreted effector proteins have been reported to localize in the nucleus, but most localization studies have been conducted with GFP-tagged assays. It should be noted that GFP fusion may abrogate proper effector localization, either by hiding a sorting signal or by inducing change in the 3D structure of native effectors which could prevent interaction with a protein involved in true effector localization. In addition, most of these experiments are transient assays and do not examine localization during infection. Therefore, although GFP represents a very powerful tool at our disposal to identify subcellular effector localization, care should be taken when analyzing the results. However, since GFP does not diffuse to the nucleolus, it is safe to assume that nucleolar localization is effector-driven. RXLR effectors, such as HaRxLL3b, HaAtr13 Emoy2 and HaRxL44 from *Hyaloperonospora arabidopsidis*, localize to the nucleolus of plant cells.[30] In *Phytophtora capsici*, CRN effectors all localize to the nucleus, and at least two have been found to accumulate in the nucleolus, suggesting that there might be subnuclear localization domains.[44]

The nucleolus is a multifunctional subcellular organelle critically involved in ribosome biogenesis and protein synthesis.[45] Several DNA viruses and retroviruses are known to target the nucleolus. Umbravirus ORF3, potato leafroll virus capsid protein and influenza virus nucleoprotein are some examples of viral proteins localizing to the nucleolus.[46-49] Given that viruses are entirely dependent on the host machinery to translate their genome into proteins, they are expected to target the nucleolus. However, one can wonder why biotrophic filamentous pathogens would target this subnuclear compartment. The effector HaRxL44 from the obligate biotrophic pathogen *H. arabidopsidis* was recently shown to target nucleolar (and nuclear) Mediator subunit 19a (MED19a). This interaction results in MED19a degradation in a proteasome-dependent manner. MED19a degradation appears to shift transcription from salicylic acid-responsive defense to jasmonic acid and ethylene-responsive transcription, thereby conning the host to enhance its susceptibility.[30]

Haustorial Accommodation: Cellular Rearrangements through Reprogramming

What happens once a pathogen gets access to its host? How does the host respond to the pathogen's demands? And what are the overall cellular dynamics in play? Answering such questions becomes a lot more imperative when dealing with obligate biotrophs, because of their intimate relationship with the host and since they can only survive in living cells. Obligate biotrophic pathogens thus have to be subtle when dealing with their host after invasion. First of all, they have to keep host immunity in check at all times by suppressing PTI. Second, they have to continuously feed from plant cells. Finally, they need to steadily propagate and multiply.

Fungal spores grow on plant surfaces upon germination. It has been shown that the rust fungus *Uromyces appendiculatus* uses topographical cues for orientation and the formation of

infection structures.[51] Once *U. appendiculatus* detects a 0.5-μm ridge, which it interprets as the presence of the stomatal lip (its entry point into tissue), it starts producing its infection structure.[51] When the pathogen has forced its way into plant tissue, nutrient acquisition and defense suppression occur primarily through haustoria, although effectors are also released from growing hyphae. Support for such a mechanism is lent by deep sequencing of the biotrophic growth phase of *Colletotrichum higginsianum* during *A. thaliana* infection.[52] In this pathosystem, effector genes are expressed in consecutive waves associated with pathogenic transition, and some are expressed before host invasion at the appressorial stage.[52] In fact, multi-stage transcriptome analysis of *Melampsora larici-populina*, the causative agent of the poplar leaf rust (obligate biotroph), revealed that a number of small-secreted proteins were even expressed in resting urediniospores.[53] Therefore, we can infer that suppression of plant immunity starts prior to the formation of haustorial structures in host tissue. While our understanding of molecular partners at play is progressing, we have made few inroads into the establishment of plant–haustoria interactions and post-invasion events. Dynamic interplay could be mainly driven by the invader, and as we progress in this review, we will examine some important phenomena that may hold clues to these questions.

It should not be difficult to conceptualize massive host cellular reprogramming occurring in response to the development of haustoria. Haustoria are found to be surrounded by endoplasmic reticulum, actin cytoskeleton and cytoplasm, along with the accumulation of Golgi bodies and mitochondria.[54] It has also been observed that a significant amount of tonoplast is present around these complexes.[54] To host such critical appendages, cells have to expand their plasma membrane tremendously. Haustoria are separated from the host cytoplasm by an extrahaustorial matrix (EHM). The EHM has been speculated to be mostly of host origin, sealed from haustoria by a haustorial neck band.[55,56] However, it differs from the plasma membrane in both cytological and biochemical properties.[55,57] The EHM also appears to vary in composition over time.[58,59] Recently, Lu et al.[60] reported that some plasma membrane resident proteins relocalize to the extra-haustorial membrane during infection. For example, the aquaporin PIP1;4 and the calcium ATPase ACA8 remained at the plasma membrane during infection with either *H. arabidopsidis* or *Phytophtora infestans* while the syntaxin PEN1 (penetration deficient 1), the synaptotagmin SYT1 and the remorin StREM1.3 were present in the extra-haustorial membrane around *P. infestans* haustoria. Interestingly, this relocalization appears to be pathogen-dependent since PRR FLS2 localized in the EHM of *P. infestans* but remained at the plasma membrane and was excluded from the EHM in *H. arabidopsidis*. However, the most remarkable feature of this cellular rearrangement is the position of the nucleus. Studies have shown that the *Arabidopsis* nucleus stays close to *H. arabidopsidis* haustoria,[30] and this is presumably driven by the actin cytoskeleton.[61,62] It is possible that proximity of haustoria to the nucleus enables pathogens to deliver their effectors more quickly to the nucleus for cell reprogramming. Proximity of the nucleus to the intruder would thus be driven by the pathogen per se, but one cannot exclude that host plants could steer this process autonomously to respond quickly to pathogen attack.

Vesicular Trafficking as a Possible Pathogen Target

Pathogens are known to target host vesicular trafficking, a key element of plant defense.[30] In *H. arabidopsidis*, 26% of examined effectors have been found to localize to membranes, the majority of them (18%) associating with the endoplasmic reticulum.[63] *Arabidopsis* cells hosting *H. arabidopsidis* haustoria develop bulging vesicular structures compared with non-infected cells,[30] the occurrence of such vesicles being attributed to presence of the pathogen. It is possible that the formation of these vesicles is driven by a particular effector or effectors to upset vesicular movement and disrupt any organized defense response. They may also be pathogen-driven and provide the extra-phospholipid bilayer required at the plasma membrane to accommodate fast-expanding haustoria. Regardless, support for the fact that these are vacuolar structures comes from the observations of very similar structures in cotyledons of transgenic *Arabidopsis* γ-TIP-GFP plants.[64] Other types of membrane structures have been shown to differentially localize around haustoria formed by *H. arabidopsidis* and *P. infestans*.[60]

HaRxL17 localizes to the EHM during infection by *H. arabidopsidis*. However, in the absence of the pathogen, it localizes to the tonoplast where its ability to enhance plant susceptibility is possibly linked with a task in plant cell membrane trafficking.[30] Since tonoplast is located close to the EHM along with the effector HaRxL17 in the event of infection, the effector may be interfering with plant cell membrane trafficking, and interestingly, this also suggests a role for tonoplast in EHM formation. However, no single effector has been reported to cause the bulb-like vesicular structures observed in the presence of growing pathogens,[29] and it is not clear whether it is a plant defense response or an effector-driven process. Surprisingly, our understanding of the detailed mechanism of vacuolar biogenesis is still limited, justifying the need to push the investigation further into such peculiar vesicular structures. It is difficult to elucidate possible pathways being targeted by pathogens to hinder vesicular trafficking and eventually give rise to these bulb-like structures. In *A. thaliana*, a point mutation in the deubiquitinating enzyme AMSH3 renders cells incapable of forming central lytic vacuoles. In addition, *amsh3* mutant cells accumulate autophagosomes and incorrectly sort their vacuolar protein cargo.[65] Vacuoles are important in various plant defense mechanisms, and two vacuole-mediated mechanisms have been postulated to affect programmed cell death.[66] In one of them, vacuolar-processing enzymes mediate vacuolar membrane disruption, thus releasing vacuolar content into the cell cytoplasm (demonstrated for viral infection).[67] In the second proposed mechanism, vacuole fusion with the plasma membrane enables the extracellular release of vacuolar content (demonstrated in bacterial infection).[68] Interestingly and coincidentally, phenotypic similarity between vesicular structures from *amsh3* mutants and cells hosting haustoria can be noticed.[60,65] This concurring vesicular signature suggests that pathogens

could be targeting AMSH3 (or similar components) to alter the vesicular pathway.

Octomeric–exocyst complexes could also be targeted by pathogens, given that the exocyst architecture plays an important role in vesicular tethering and redefining cell polarity, which are integral to plant defense responses.[69] Targeted exocytosis occurs during infection, and freshly-synthesized, defense-related compounds are delivered to infection foci, which eventually leads to asymmetrical plasma membrane development. Small GTPases from the Rab and Rho families are known to be essential in this process which involves delivery, anchoring, and integration of secretory vesicles to the plasma membrane,[70,71] whereas the exocyst complex works as a scaffold in tethering operations.[72,73] The final process of attachment is mediated by the integral membrane proteins v-SNARE and t-SNARE, where plasma membrane and vesicle bilayers are fused together to complete the process.[74,75] It has already been demonstrated that upon mutating, two exocyst subunits—Exo70B and Exo70H1 from *Arabidopsis* plants—are more susceptible to infection, validating their importance in plant immunity.[69]

PEN1 is a classic example of proteins preventing penetration by pathogens. PEN1 encodes a syntaxin known to interact with the SNARE proteins SNAP33 and VAMP72[76] and regulates papillae formation in cells under attack.[77] Papillae are bell-shaped cell wall appositions deposited in epidermal cells. Within papillae, various secondary antimicrobial metabolites accumulate along with lytic enzymes and reactive oxygen species, which stops the pathogen penetration peg. In *Arabidopsis*, PEN1 is found in significant amounts when the non-host fungus *Blumeria graminis* f. sp. *hordei* endeavors an unsuccessful invasion. However, when the host fungus *Erysiphe cichoracearum* successfully penetrates *Arabidopsis* cells, *PEN1* is then downregulated.[77] The *pen1* single mutant allows increased penetration of the non-host fungus *B. graminis* f. sp. *hordei*, thereby showing that PEN1 helps in procuring an effective penetration barrier.[77] Thus, PEN1 could participate actively in polarizing secretion events that lead to papillae formation.[77]

Conclusions

Obligate biotrophic phytopathogens have evolved a robust and elaborate offensive strategy to invade their host by deploying numerous effector proteins. It appears that the effectors inventory of pathogens is organized around different types of molecules, which have unique capabilities and functions. Therefore, most so-called effectors should be considered candidate effectors. A crude way to envision effector deployment is to see apoplastic effectors at the onset of attack, performing all the hullwork and setting the stage for more sophisticated weaponry. True cytoplasmic effectors could act at the intermediate stage by deactivating local surveillance, paving the way for nuclear effectors to enter the nucleus, taking over the entire defensive network and stalling the complete immune set-up. Nucleolar effectors from various pathogens are increasingly being reported,[44,78,79] and it is likely that they have an important function in pathogenesis. Many cellular processes, including plant defenses, depend on the formation of new proteins. Thus, further study needs to be undertaken to understand the task of nucleolar effectors. Some effectors are also involved in disrupting vesicle trafficking and as such, they may be compromising vacuolar integrity, which is believed to play a significant role in plant defense. Plant cells hosting haustoria experience unique cellular rearrangements that are likely influenced by haustoria themselves and driven by secreted effectors.

As genome-sequencing costs are falling, the full sequences of many more genomes are becoming available. Despite the dazzling speed at which effector catalogs can be assembled, functional study of effectors remains a relatively slow and strenuous process. In obligate biotrophs, functional studies of effectors by virulence assays are hindered by the lack of molecular genetic approaches. As a result, alternative tactics with heterologous systems are increasingly being adopted. Given the very large repertoire of effectors observed in obligate biotrophic fungi, such as rusts that encode over 1000 small secreted proteins,[80,81] one could propose that the outcome of each effector may be a lot more subtle than the bacterial effectors of *Pseudomonas syringae* that have roughly 30 or so effectors,[82] and a direct, quantifiable impact on virulence may prove difficult to observe since the cumulative result of many effectors may be required. Alternatively, redundancy could explain the huge number of effectors in filamentous pathogens. In either case, deciphering the interactions of these effectors will likely reveal many unknown components of various plant processes. With these issues in mind, localization remains one of the first aspects to consider when assessing effector functions. In addition, combination of genetic evidence and protein–protein interaction approaches, either yeast two-hybrid assay, co-immunoprecipitation, or bi-molecular fluorescence complexes, may prove to be the best ways of investigating effectors from biotrophic pathogens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ANNEX C

ARABIDOPSIS TAF15B LOCALIZES TO RNA PROCESSING BODIES AND CONTRIBUTES TO SNC1-MEDIATED AUTOIMMUNITY

Oliver X. Dong, Louis-Valentin Meteignier, Melodie Plourde, **BULBUL AHMED**, Ming Wang, Cassandra Jensen, Hailing Jin, Peter Moffett, Xin Li, and Hugo Germain

Annex C contains a published rechearch study, containing findings on MAMP-triggered immunity in plants, which demonstrates the unbiased nuclear proteomics based approach demonstrating that nuclear proteomic is a valid and phenotype-independent approach to uncover factors involved in diverse cellular processes.

I have maintained the plants in the growth chamber. Also conducted the confocal microscopy experiments for the subcellular localization studies under different treatment.

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e-Xtra*

Arabidopsis TAF15b Localizes to RNA Processing Bodies and Contributes to *snc1*-Mediated Autoimmunity

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In both animals and plants, messenger (m)RNA export has been shown to contribute to immune response regulation. The *Arabidopsis* nuclear protein MOS11, along with the nucleoporins MOS3/Nup96/SAR3 and Nup160/SAR1 are components of the mRNA export machinery and contribute to immunity mediated by nucleotide binding leucine-rich repeat immune receptors (NLR). The human MOS11 ortholog CIP29 is part of a small protein complex with three additional members: the RNA helicase DDX39, ALY, and TAF15b. We systematically assessed the biological roles of the *Arabidopsis* homologs of these proteins in toll interleukin 1 receptor–type NLR (TNL)-mediated immunity using reverse genetics. Although mutations in *ALY* and *DDX39* did not result in obvious defects, *taf15b* mutation partially suppressed the autoimmune phenotypes of a gain-of-function *TNL* mutant, *snc1*. An additive effect on *snc1* suppression was observed in *mos11-1 taf15b snc1* triple mutant plants, suggesting that MOS11 and TAF15b have independent functions. TAF15b-GFP fusion protein, which fully complemented *taf15b* mutant phenotypes, localized to nuclei similarly to MOS11. However, it was also targeted to cytosolic granules identified as processing bodies. In addition, we observed no change in *SNC1* mRNA levels, whereas less SNC1 protein accumulated in *taf15b* mutant, suggesting that TAF15b contributes to SNC1 homeostasis through posttranscriptional mechanisms. In summary, this study highlights the importance of posttranscriptional RNA processing mediated by TAF15b in the regulation of TNL-mediated immunity.

The plant immune system relies, in large part, on two distinct but converging molecular recognition mechanisms. In the first, cell surface–localized pattern recognition receptors detect conserved molecular motifs presented by pathogens and induce a low amplitude defense response, termed pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Boller and Felix 2009; Jones and Dangl 2006; Zipfel 2008). Successful pathogens, in turn, produce effector proteins (or virulence factors) that target PTI elements to inhibit this response (Fontes et al. 2004; Xiang et al. 2008) or, alternatively, use its effectors to rewire the host defense mechanism (Caillaud et al. 2013). The second layer of the plant immune system consists of an arsenal of highly polymorphic intracellular receptors that, once activated, triggers a stronger defense response referred to as effector-triggered immunity, which often culminates in the death of the infected cell (Jones and Dangl 2006). These intracellular receptors, commonly referred to as resistance proteins, are typically composed of three domains: an amino terminal domain consisting of either of a coil-coil domain or a toll interleukin 1 receptor (TIR) domain, a central nucleotide binding (NB) domain, and a leucine-rich repeat (LRR) carboxyl terminal domain (Chisholm et al. 2006), although other configurations do exist in the plant kingdom (Collier and Moffett 2009; Germain and Séguin 2011; Li et al. 2015). Remarkably, these NB-LRR receptors (NLR) share significant sequence similarities with animal innate immunity receptors such as Nod proteins, although they were believed to be derived from convergent evolution (Ausubel 2005; Rairdan and Moffett 2007).

One such NLR protein is the TIR-type NLR (TNL) SNC1 (Li et al. 2001). A point mutation, changing a glutamate (E) to a lysine (K) in the linker region located between the NB and LRR domains of SNC1, renders the snc1 protein more stable and constitutively activates TNL-mediated immunity (Cheng et al. 2011; Zhang et al. 2003). The *snc1* mutant phenotypes resulting from this gain-of-function mutation include increased accumulation of the defense hormone salicylic acid (SA), constitutive expression of defense-marker pathogenesis-related (*PR*) genes, and enhanced resistance to the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis* Noco2 and to the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv. *maculicola* ES4326. The morphology of *snc1* plants is also drastically affected, resulting in severely stunted stature, dark green color, and twisted leaves. All of these features are common to plants with elevated SA levels and constitutive expression of *PR* genes (Bowling et al. 1994; Clarke et al. 1998). These autoimmune morphological features have enabled forward genetic screens to be performed to investigate the molecular events surrounding SNC1 activation and homeostasis control (Johnson et al. 2013; Monaghan et al. 2010). Previously identified genetic suppressors, termed *modifier of snc1* (*mos*) mutants, revealed three nucleocytoplasmic trafficking pathways affecting immunity: nuclear localization signal–mediated nuclear import (Palma et al. 2005), nuclear export signal–mediated nuclear export (Cheng et al. 2009), and messenger (m)RNA export (Germain et al. 2010).

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*The *e*-Xtra logo stands for "electronic extra" and indicates that five supplementary figures and one supplementary table are published online.

The contribution of mRNA export to plant immunity was demonstrated in the study of *mos3* and *mos11* mutants (Germain et al. 2010; Zhang and Li 2005). MOS3/SAR3/AtNUP96 is an integral nucleopore component of the conserved Nup107-160 complex and is required for mRNA export (Parry et al. 2006). MOS11 is a conserved nuclear protein with homology to human RNA-binding protein CIP29 (cytokine-induced protein 29kDa), which does not associate with the nuclear pore. We have shown that *mos11* plants display increased nuclear accumulation of mRNAs compared with wild type (WT) (Germain et al. 2010), thereby linking MOS11 to mRNA export. Consistent with this, the mammalian MOS11 ortholog CIP29 was found to interact with ALY, a protein involved in mRNA export via UAP56 (U2AF65-associated protein 56), an RNA helicase with 91% identity to DDX39 (DEAD [Asp-Glu-Ala-Asp] box polypeptide 39B) (Dufu et al. 2010; Meissner et al. 2003). Biochemical analysis performed in human and *Drosophila* spp. further illustrated that CIP29 interacts with FUS/TLS (fused in sarcoma/translocated in sarcoma) and the RNA helicase DDX39 (Dufu et al. 2010; Leaw et al. 2004; Sugiura et al. 2007) to enhance its helicase activity (Sugiura et al. 2007).

Here, we report a systematic reverse genetic analysis of the knockout lines of the closest *Arabidopsis* homologs corresponding to the human/*Drosophila* mRNA export complex composed of hDDX39/hUAP56, hFUS/TLS, and hALY. Our findings demonstrate that one member of the complex, TAF15b (homolog of FUS/TLS), can partially suppress the defense-associated phenotypes of *snc1*. In addition, in triple mutant *mos11-1 taf15b snc1* plants, the morphological and autoimmune phenotypes associated with *snc1* are almost completely abrogated. We also observed that TAF15b localizes to RNA processing bodies (p-bodies), structures involved in mRNA decay, including nonsense-mediated mRNA decay (NMD), AU-rich element-mediated mRNA decay, and micro (mi)RNA-induced mRNA silencing (Kulkarni et al. 2010). TAF15b seems to contribute to SNC1 homeostasis at posttranscriptional levels. Recent findings showed that mutants impaired in NMD have constitutive defense responses (Gloggnitzer et al. 2014) and that PAT1 (a decapping enhancer) is part of the MPK4-mediated defense signaling pathway in response to flagellin (Roux et al. 2015), provide additional evidence that mRNA decay is linked to the regulation of plant immunity.

RESULTS

taf15b partially suppresses the autoimmune phenotypes of *snc1*.

In human and *Drosophila* spp., the CIP29 protein interacts with three partners, namely, DDX39, FUS/TLS, and ALY. DDX39 is an RNA helicase highly similar to UAP56, a RNA helicase well-known for its involvement in mRNA export (Carmody and Wente 2009; Chi et al. 2012; Dufu et al. 2010; Katahira 2012). FUS/TLS is a family of RNA-binding proteins that includes TATA-box binding factors (TAFs). Beyond their RNA binding capacity, TAFs influence the initiation of transcription (Dikstein et al. 1996; Mizzen et al. 1996; Pham and Sauer 2000). ALY is a nuclear protein with nucleic acid–binding ability. ALY has been shown to interfere with silencing in plants (Canto et al. 2006; Uhrig et al. 2004). Through BLAST analysis, we identified the closest homologs of DDX39, FUS/TLS, and ALY in *Arabidopsis* (Table 1). We then obtained homozygous T-DNA knockout lines for each gene and crossed them with *snc1* plants, to monitor if mutations in these members of the CIP29 complex could affect *snc1* signaling similarly as *mos11*.

The stunted morphology of *snc1* was partly suppressed in the *taf15b snc1* double mutant plants (Fig. 1A). However, none of the other double mutants with *snc1* resulted in *snc1* suppression (not shown). In addition to their stunted morphology, *snc1* plants display increased resistance to virulent oomycete pathogen *H. arabidopsidis* Noco2 (Li et al. 2001). To assess if *taf15b* could also suppress this *snc1* phenotype, we performed an infection assay with *H. arabidopsidis* Noco2. The *taf15b snc1* plants did not display a statistically significant difference from *snc1* plants (Fig. 1B). Similarly to *mos11-1*, the single *taf15b* plant did not display enhanced disease susceptibility when compared with WT plants. In contrast to *mos11-1* plants, *taf15b* plants did not display impaired mRNA export (Supplementary Fig. S1). We also assessed the capacity of *taf15b* to alter the constitutive resistance of *snc1* to virulent bacterial pathogen *P. syringae* pv. *maculicola* ES4326. In this case, the suppression of the *snc1* resistance was significant (Fig. 1C). Since *snc1* immune activation can be monitored by the level of expression of *PR* genes (Li et al. 2001), we verified if the *PR* gene expresssion level was affected in *taf15b snc1* plants. The quantitative reverse transcription-polymerase chain reaction (RT-PCR) data demonstrates that *snc1* plants express *PR1* transcript at a level approximately 500 times (486-fold) higher than observed in unstressed WT plants (Fig. 1D). In the double mutant *taf15b snc1* plants, *PR1* expression level was approximately half the level observed in *snc1*, at 233-fold of the WT plants. For *PR2* expression, similar fold changes were observed (Fig. 1D).

In conclusion, among all CIP29 complex component mutants tested, *taf15b* is the only mutant that affects the *snc1* autoimmune phenotypes, acting as a partial suppressor of *snc1*.

TAF15 and TAF15b have different topology and are functionally distinct.

Since *Arabidopsis* contains another FUS/TLS homolog, TAF15, we investigated whether it was redundant to TAF15b. In order to assess whether TAF15 and TAF15b were functionally redundant, we compared the morphology of *taf15* and *taf15b* T-DNA knock-out lines to that of the *taf15 taf15b* double mutant. The *taf15* plants are indistinguishable from WT, whereas the *taf15b* mutant is slightly different from WT, with slightly bigger and rounder leaves, often concave rather than convex, indicating that, in *Arabidopsis*, the TAF15 protein cannot functionally complement *taf15b* morphological phenotypes (Fig. 2A). The *taf15 taf15b* double mutant resembles the *taf15b* single mutant (Fig. 2A). To further substantiate the difference between *taf15b* and WT plants and

Table 1. CIP29 direct and indirect interactors and their putative *Arabidopsis* homolog

Human gene	Gene function	Homolog in *Arabidopsis*	Available T-DNA/location
DDX39	DEAD box RNA helicase	At5g11170	SALK_101221/intron
FUS/TLS TAF15b	Spliceosome assembly and transcriptional control	At5g58470	SALK_061974/intron SAIL_35_B06/intron
ALY (indirect)	Splicing factor linking premRNA splicing to mRNA export	At5g02530	SALK_094909/5′ untranslated region WiscDsLox461-464N10/exon
		At5g59950	SAIL_381_E08/exon WiscDsLox493E08/promoter

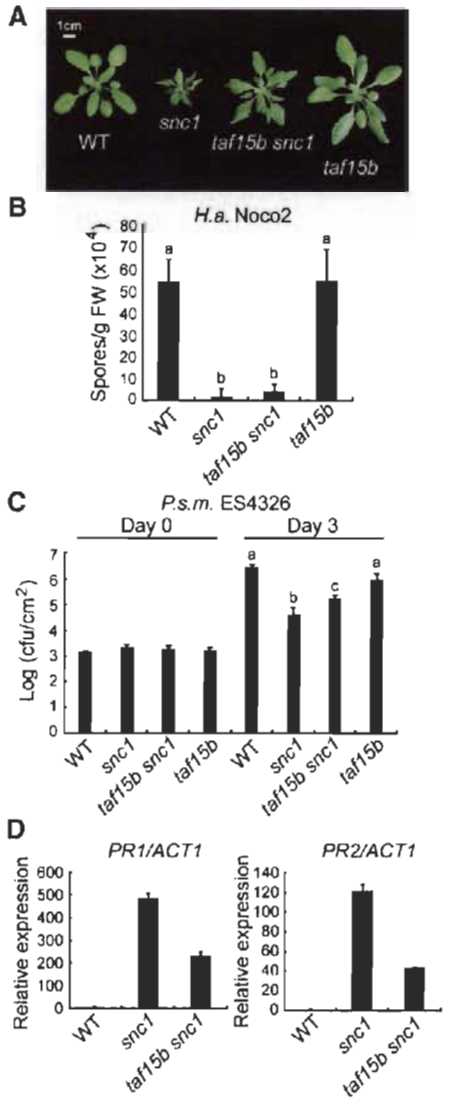

Fig. 1. *taf15b* partially suppresses the autoimmunity of *snc1*. **A**, Morphology of 4-week-old soil-grown wild type (WT), *snc1*, *taf15b snc1*, and *taf15b* plants. **B**, Two-week-old soil-grown plants were inoculated with *Hyaloperonospora arabidopsidis* Noco2 at a concentration of 50,000 conidiospores per milliliter and the number of conidiospores was quantified 7 days after inoculation. Bars represent the mean of four replicates. Statistical significance was established using Student *t* test ($P < 0.05$) and statistically different results are represented by different letters. **C**, Five-week-old soil-grown plants were inoculated with *Pseudomonas syringae* pv. *maculicola* ES4326 at an optical density at 600 nm = 0.0001, and statistical significance was evaluated using Student's *t* test ($P < 0.05$) **D**, *PR1* and *PR2* gene expression was quantified using quantitative reverse transcription-polymerase chain reaction and was normalized with *ACT1*.

between *taf15b* and *taf15*, we evaluated the number of rosette leaves when the plants were 5 weeks old, as the *taf15b* mutant exhibits a late-flowering phenotype. As shown in Figure 1B, *taf15b* and *taf15b taf15* double mutant plants exhibit similar flowering-time defects, confirming that these two genes do not have redundant functions. Furthermore, the phylogenetic tree generated using the full-length amino acid sequences of AtTAF15 and AtTAF15b homologs found in different plant species also shows that AtTAF15 and AtTAF15b form different clades in all species (Fig. 2C).

While TAF15 and TAF15b have similarity in composition of their functional domains, their protein architecture is different. TAF15 possesses a RNA-recognition motif at its amino terminus, followed by a two–zinc finger domain, whereas in TAF15b only one zinc finger is present (Fig. 2D). The human TAF15 protein has the protein topology of AtTAF15, while FUS/TLS shares the protein topology of AtTAF15b. For further analysis, we therefore refer to AtTAF15b as the homolog of FUS/TLS rather than that of human AtTAF15.

With publicly available microarray data (Winter et al. 2007), we asked whether *TAF15*, *TAF15b*, *MOS11*, and *SNC1* gene expression was inducible by virulent bacterial pathogen *Pseudomonas syringae* DC3000, avirulent oomycete pathogen *H. arabidopsidis* Emwa1, virulent oomycete pathogen *H. arabidopsidis* Noco2, and PAMP elicitor flg22. The expression of these genes was not significantly modulated in the assessed conditions (Supplementary Fig. S2).

In order to assess if *taf15b*, *taf15*, or the *taf15 taf15b* mutations would impair the immune capacity of other NLR proteins, we evaluated the response of these mutants against the avirulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 *AvrRpt2* (Supplementary Fig. 3A), *P. syringae* pv. *tomato* DC3000 *AvrRPS4*, and the avirulent oomycete strain *H. arabidopsidis* Emwa1. We also evaluated PTI, using the type III secretion–deficient bacterial strain *P. syringae* pv. *tomato* DC3000 *hrcC–*. Response to the avirulent pathogens tested or *P. syringae* pv. *tomato* DC3000 *hrcC–* was not affected in either single mutants or the double mutant, indicating that neither TAF15b nor TAF15 is a component involved in general NLR signaling. We also assessed the reactive oxygen species (ROS) induction capacity in WT, *taf15*, *taf15b*, and *taf15 taf15b* plants (Supplementary Fig. S4) in response to flg22. Interestingly, we observed mild yet reproducible reduction in ROS induction by flg22 in the *taf15b* single mutant and the *taf15 taf15b* double mutant. Based on the phylogeny and protein topology, we conclude that the closest homolog of AtTAF15b in humans is FUS/TLS. In addition, based on our epistatic analysis (discussed below), we conclude that AtTAF15 and AtTAF15b do not have overlapping functions in plants. Finally, neither TAF15 nor TAF15b appears to be involved in general NLR signaling. However, they do contribute to the ROS induction in PTI.

mos11-1 taf15b snc1 triple mutant analysis.

Since *taf15b*, like *mos11-1*, could partially suppress the *snc1* phenotype, we sought to investigate if the lack of these two proteins would have an additive effect on the suppression of the *snc1* autoimmune phenotypes. While both double mutants were only partial *snc1* suppressors, the triple mutant *mos11-1 taf15b snc1* almost fully suppressed the stunted morphology of *snc1* (Fig. 3A). However, the triple mutant plants had delayed bolting when compared with WT plants (Fig. 3A). Since *snc1* dwarf morphology was fully suppressed in the triple mutant, we verified whether *PR* gene expression was comparable to levels observed in WT. Indeed, we observed that the *PR* gene expression in the triple mutant was very close to that observed in WT plants (Fig. 3B).

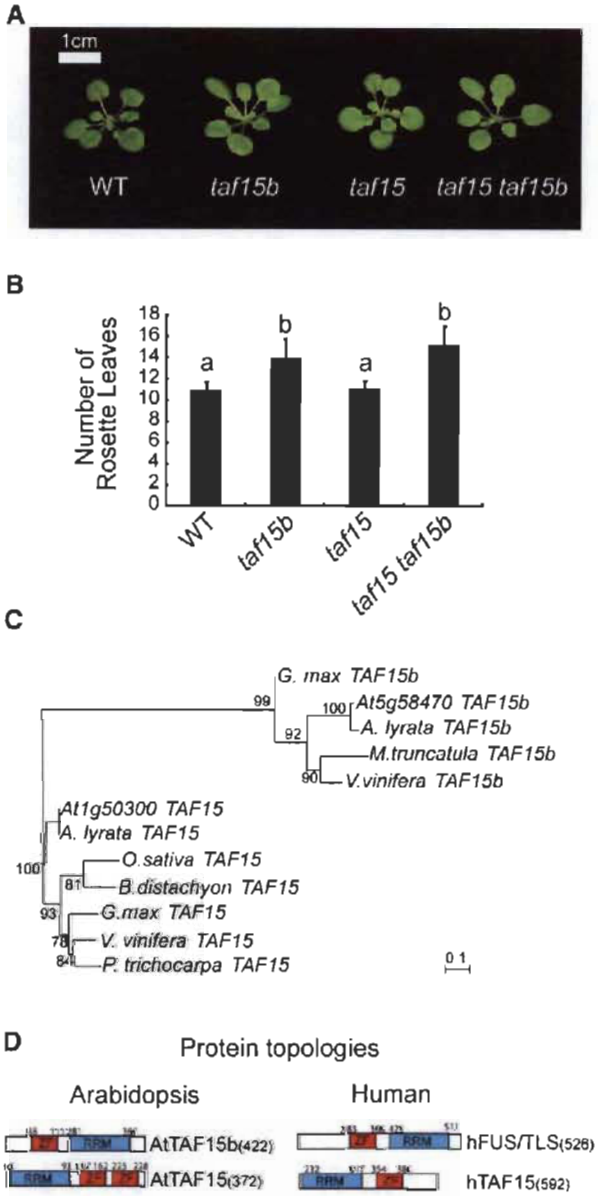

Fig. 2. TAF15 and TAF15b have different topology and are functionally distinct. A, Morphology of 2.5-week-old soil-grown wild type (WT), *taf15b*, *taf15*, and *taf15 taf15b* plants. B, Comparing flowering time in soil-grown WT, *taf15b*, *taf15*, and *taf15 taf15b*. Letters indicate statistical significance as determined by Student's *t* test ($P < 0.0001$). C, Phylogeny of TAF15b-related proteins was inferred, using the full-length amino acid sequences. Alignment and tree was computed using ClustalW and a neighbor-joining alignment was used. D, Protein domain organization was inferred using the National Center for Biotechnology Information conserved domain database software. Small numbers above the proteins indicate the relative position of the domains; the numbers at the end of the protein names indicate the number of amino acids.

these components of RNA regulation and catabolism may affect transcription and translation of specific mRNAs. In addition, plant NLR proteins are known to be regulated by transacting small interfering RNAs (Zhai et al. 2011). Thus, we examined whether the level of *SNC1* transcript or the level of SNC1 protein was affected in *taf15b* and *taf15b snc1*, relative to WT and *snc1* plants. We did not observe significant differences in mRNA levels between WT and *taf15b* and between *snc1* and *snc1 taf15b* (Student's *t*-test, in both cases $P > 0.1$) (Fig. 6A). We then tested whether SNC1 protein accumulation is affected by *taf15b*, through Western blot assay using an anti-SNC1 antibody (L-i et al. 2010). We consistently observed decreased SNC1 protein level in *taf15b* compared with WT, and this difference in maintained in *snc1 taf15b* compared with *snc1* (Fig. 6B and C). As the *SNC1* transcription is not substantially affected by *taf15b* (Fig. 6A), we conclude that *taf15b* affects SNC1 protein accumulation through posttranscriptional mechanisms.

***taf15b* does not affect miRNA accumulation.**

Given the localization displayed by TAF15b and the apparent lack of a direct effect on *SNC1* mRNA level, the suppression of the *snc1* phenotype is likely indirect. Putative targets could be regulated by miRNA and affect TNL-mediated immunity. To test this hypothesis, we assessed the level of known miRNAs in our genetic backgrounds (Fig. 7). Northern blot analysis showed slight differences for some miRNA; however, when multiple repeats were performed, those differences were not found to be statistically significant. Therefore, we conclude that the effect of *taf15b* on *snc1* immunity is not via an obvious alteration of miRNA levels.

DISCUSSION

We have previously observed that *mos11*, through its defect in mRNA export, can partially suppress the phenotypes associated with the autoimmune mutant *snc1*. The human ortholog

Fig. 4. TAF15b does not associate with MOS11 in vivo. **A,** Morphology of *snc1*, *taf15b snc1*, and two *taf15b snc1* transgenic lines expressing *AtTAF15b-FLAG* transgene. Soil-grown plants were photographed 4 weeks after germination. **B,** Immunoprecipitation of AtTAF15b-FLAG using anti–green fluorescent protein (GFP) antibody. Total protein extracts from seedlings grown on ½ Murashige Skoog plates were subjected to immunoprecipitation by anti-GFP agarose beads. Input and eluted samples were detected by anti-FLAG and anti-GFP antibodies.

of MOS11, CIP29, associates with proteins involved in mRNA export: DDX39, ALY and FUS/TLS (TAF15b). Although the *Arabidopsis* genome has been analyzed for the presence of TAFs (Lago et al. 2004), very little is known about TAFs in plants. Here, we have performed a systematic reverse genetics analysis of the knockout lines corresponding to the closest *Arabidopsis* homologs of this complex and found that mutating *TAF15b* can partially suppress *snc1* autoimmunity.

TAFs or TATA-box binding protein–associated factors are main components in the assembly of the general transcription factor IID, required for the initiation of transcription by RNA polymerase II. Therefore, this *sensu stricto* definition would mean that TAF15b would have a role in transcription initiation, which is reinforced by the presence of a RNA-binding domain in the protein (Fig. 2) and a nuclear localization signal (Fig. 5). However, it is not known whether TAF15 proteins participate in the assembly of TFIID in plants. Here, we provide the first functional in planta analysis of *TAF15b*. We show evidence that TAF15b is involved in TNL-triggered immunity. Additionally, we observed that mutation of *TAF15b* enhances the *snc1*-suppressing effect of *mos11*, suggesting that they are not in the same pathway. Furthermore, TAF15b localizes to p-bodies, thereby strengthening the existing link between p-bodies and plant immune responses. SNC1 protein level is reduced in *taf15b* background, suggesting a role of TAF15b in regulating SNC1 accumulation, possibly posttranscriptionally. Its effect on SNC1 homeostasis explains the *snc1*-suppressing phenotypes of *taf15b*.

mRNA metabolism is a highly regulated and complex process. Following synthesis, mRNA molecules are processed in the nucleus, while concomitantly being loaded with proteins that will direct the messenger ribonucleoparticle (mRNP) toward the nucleopore. Upon traveling through the pore, mRNPs are accessible for translation in the cytosol. Stress-related translational slowdown may cause mRNAs to accumulate in stress granules. Alternatively, transcripts may also be stored in p-bodies to be made available later or targeted for degradation, thus providing an additional level of posttranscriptional control (Anderson and Kedersha 2006, 2009). Observed as cytoplasmic foci, p-bodies are the site of mRNA m^7GDP removal, the cap structure present at the 5′ end of all eukaryotic mRNAs. DCP2 (DECAPPING PROTEIN2) in association with DCP1 and VCS (VARICOSE) are sufficient for mRNA decapping (Xu et al. 2006), whereas DCP5 is also required for in vivo activity (Xu and Chua 2009). In mammalian cells, the RNA interference component Argonaute was shown to localize to p-bodies and interacts with GW182. Silencing of GW182 impairs silencing of microRNA reporters (Liu et al. 2005). P-bodies can have an opposite effect on viral replication. It was reported that p-body depletion enhances HIV production (Nathans et al. 2009), while some components of p-bodies represses replication of the *West Nile virus* and *Brome mosaic virus* (Beckham et al. 2007; Chahar et al. 2013). In plants, recovery from virus infection leads to translational repression of viral transcripts and a concomitant increase in p-body numbers and a *dcp2* mutant shows increased virus RNA accumulation and virus-induced gene silencing (Ma et al. 2015). In eukaryotes, the protein PAT1 (protein associated with topoisomerase II) acts as a decapping enhancer and localizes to the p-bodies (Ozgur et al. 2010). It was recently demonstrated that *A. thaliana* PAT1 is a substrate for MPK4, which is activated following flagellin treatment (Roux et al. 2015). Interestingly, *pat1* plants display increased *PR* gene expression that can be suppressed in *pat1 summ2* plants. SUMM2 is a resistance protein thought to guard MPK4 and suppresses autoimmunity caused by loss of different components of the MPK4 cascade (Zhang et al. 2012). It has previously been reported that impairment of NMD can trigger a constitutive immune response in *Arabidopsis* (Riehs-Kearnan

et al. 2012). More recently, Gloggnitzer et al. (2014) showed that NMD contributed to the activation of a defense response through mRNA turnover alteration of a TNL immune receptor (Gloggnitzer et al. 2014). Together, these results point to the crucial roles RNA quality control and catabolism play in plant defense homeostasis.

TAFs are not accessory proteins whose sole role is the assembly of the general transcription factor TFIID. For example,

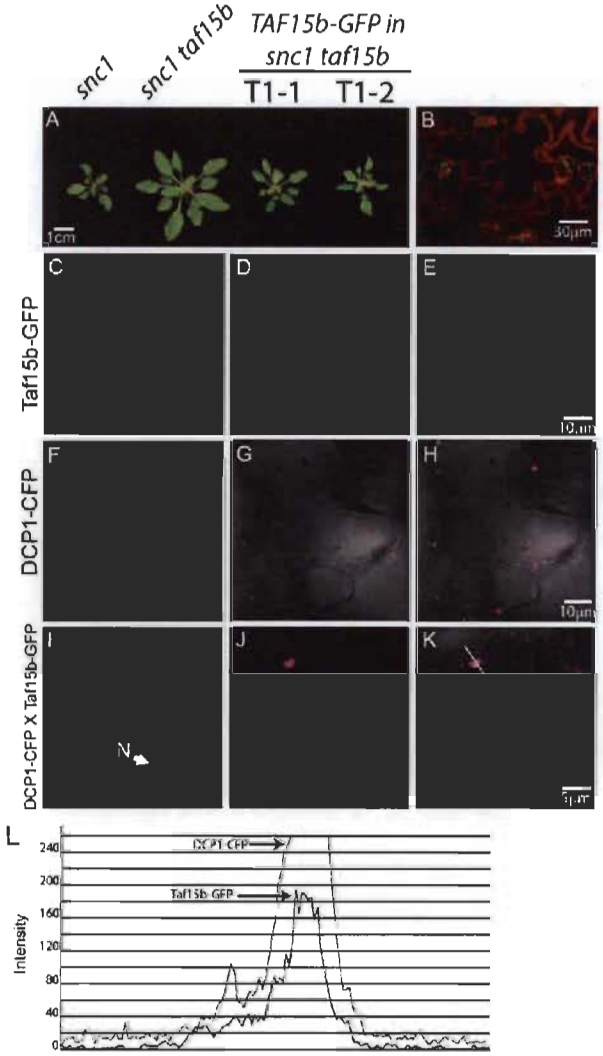

Fig. 5. Subcellular localization of TAF15b-GFP. **A**, Morphology of *snc1*, *taf15b snc1* and two *taf15b snc1* transgenic lines expressing *TAF15b-GFP* transgene. Soil-grown plants were photographed four weeks after germination. **B**, Green fluorescent protein (GFP) fluorescence in TAF15b-GFP transgenic plants. Leaf tissues of 5-day-old seedlings were stained with propidium iodide before imaging. **C**, TAF15b-GFP fluorescence in leaf epidermal cells of *taf15b* complemented with *TAF15b-GFP*. **D**, Differential interference contrast (DIC) image of the same region as in C. **E**, Overlay of the images in C and D. **F**, DCP1-cyan fluorescent protein (CFP) fluorescence in leaf epidermal cells of DCP-CFP plants. **G**, DIC image of the same region shown F. **H**, Overlay of images in F and G. **I**, TAF15b-GFP fluorescence in leaf epidermal cells of *TAF15b-GFP* × *DCP1-CFP* F1 plants. N indicates the nucleus. **J**, DCP1-CFP fluorescence in *TAF15b-GFP* × *DCP1-CFP* F1 plants. **K**, Overlap of images in I and J. **L**, Intensity plot of GFP (TAF15b-GFP) and DCP1 (CFP) in the p-bodies transected by the solid white line in panel K.

the well-characterized protein TAF1 has kinase, acetyltransferase, and ubiquitin-activating or conjugating enzyme activities. As such, it actively regulates transcription by chromatin decondensation and directly phosphorylates transcription factors (Dikstein et al. 1996; Mizzen et al. 1996; Pham and Sauer 2000). However, not all TAFs have been well characterized. In mammalian cells, TAF15 is a member of the highly conserved TET protein family of RNA binding proteins (also known as FET), which comprises FUS and Ewing sarcoma protein. TET proteins are involved in several diseases, including the onset of specific tumors. Human TAF15 was shown to associate with RNA polymerase II (Bertolotti et al. 1996), a more recent report has shown that TAF15 interacts with the U1 snRNP spliceosomal subunit (Leichter et al. 2011). When Marko et al. (2012) examined the subcellular localization of TAF15, they observed that, while a nuclear localization is the prevalent location of TAF15 in some cell types, the carboxy-terminal RGG repeat motif directs TAF15 to cytosolic granules. Interestingly, *Arabidopsis* TAF15b possesses an RGG-rich region in its C-terminus (amino acids 381 to 402 RGGGRGGGGGGYGGGGG) as well. As Marko et al. (2012) observed colocalization of the p-body marker DCP1 and TAF15 and also, importantly, FUS localize to RNA granules in mammals (Han et al. 2012); these results are in direct agreement with our results (Fig. 5), in which we observed AtTAF15b colocalizing with DCP1 in p-bodies. However, the precise role of *Arabidopsis* TAF15b in p-bodies remains unknown. Our results show that TAF15b does not affect the accumulation of *SNC1* mRNA or small RNA; however, it does affect SNC1 protein accumulation. As other TNL-mediated immunity is not affected in *taf15b* plants, we speculate that TAF15b likely specifically affects SNC1-mediated immunity through regulating the stability of its mRNA or its posttranscriptional RNA processing steps in the p-bodies.

Our results indicate that MOS11 and TAF15b act in different pathways. MOS11 regulates mRNA export while the exact role of TAF15b remains to be elucidated. As TAF15b localizes both to the nucleus and p-bodies, we speculate that it is a multifunctional protein. Although we originally hypothesized that it

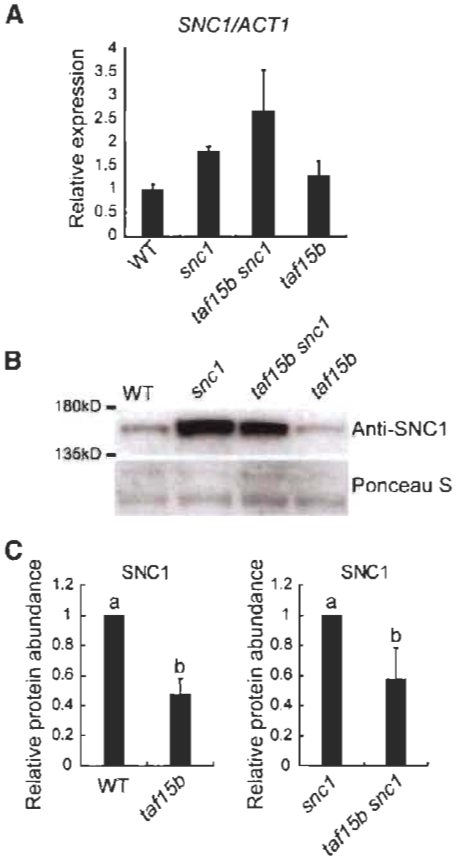

Fig. 6. *taf15b* does not affect *SNC1* transcript but does reduce SNC1 protein level. **A**, Quantitative reverse transcription-polymerase chain reaction was used to quantify the *SNC1* transcript levels in various genotypes. **B**, Western blot using anti-SNC1 antibody to quantify the SNC1 protein levels in wild type (WT), *snc1*, *taf15b snc1*, and *taf15b*. The experiment was repeated four times. The same plants were used for experiments in A and B. Leaf tissue was collected from 4-week-old soil-grown plants. **C**, Quantification of SNC1 band intensity relative to a nonspecific band in Ponceau S in four Western blot repeats as described in B, as determined by using ImageJ. Bars represent mean ± standard deviation of relative SNC1 protein abundance. SNC1 protein abundance in WT (left panel) or *snc1* (right panel) was set as 1. Student's *t* tests were used to calculate the statistical significance between genotypes, as indicated by different letters ($P < 0.05$).

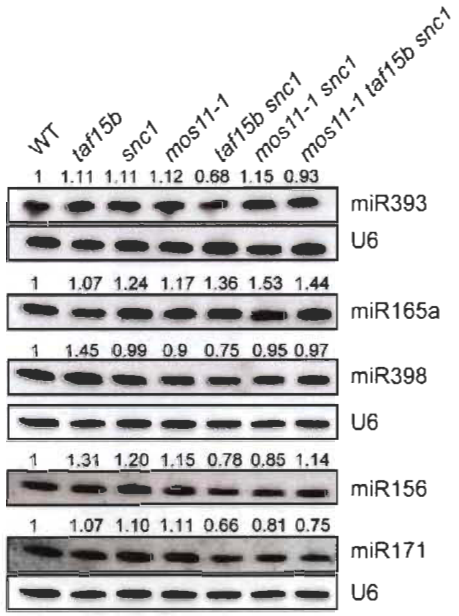

Fig. 7. *taf15b* does alter the level of some micro (mi)RNAs. The expression level of five miRNAs (miR393, miR165a, miR393, miR398, miR156, and miR171) was assessed and quantified by Northern blot for plant genotypes WT (wild type), *taf15b*, *snc1*, *mos11-1*, *taf15b snc1*, *mos11-1 snc1*, and *mos11 taf15b snc1*. Loading control was normalized by U6 RNA. RNA was extracted from the leaves of 4-week-old plants.

may contribute to mRNA export, our data did not support a mRNA export defect in *taf15b*. We cannot exclude the possibility that it still serves a role in mRNA export, but the defect in the mutant is too weak to be detected with the assay we are using. Based on our data, we cannot exclude the possibility that MOS11 and TAF15b still function in a complex in the nucleus for mRNA export in which the two proteins do not interact directly with each other. However, the cytosolic p-body localization of TAF15b suggests its independent function, which is supported by our genetic analysis; whether animal TAF15b behaves similarly in the cytosol remains to be determined. Its localization in the p-bodies indicates its involvement in, perhaps, RNA turnover and mRNA homeostasis control. However, the limited targets we examined, including *SNC1* and some selected miRNAs, did not reveal general striking RNA level defects, only an apparent effect on SNC1 protein accumulation. According to its weak phenotypes, we believe that possible defects in RNAs may also be too small to be detected using the current methods. Further analysis is needed to decipher the exact biochemical role of TAF15b.

MATERIALS AND METHODS

Protein topologies and phylogenetic tree.

Protein topology was searched using the National Center for Biotechnology Information Conserved Domain search tool. The phylogenetic tree was made using Colbalt Protein Alignment software, set to infer the tree using a neighbor-joining method from the full-length amino acid sequences. The species and protein models used to infer the tree are for TAF15b proteins *Arabidopsis thaliana* At5g58470, *Glycine max* XP_003518322, *Arabidopsis lyrata* XP_002866269, *Medicago truncatula* XP_003599332, *Vitis vinifera* XP_002269146, and for TAF, *Arabidopsis thaliana* At1g50300, *Arabidopsis lyrata* XP_002894246, *Oryza sativa* BAD33184, *Brachypodium distachyon* XP_003562545, *Glycine max* NP_001241952, *Vitis vinifera* XP_002273586, and *Populus trichocarpa* XP_002315063. The human protein FUS/TLS and TAF15 used to infer the topologies were AAC35285 and Q92804, respectively.

Plant growth, construction of plasmids, and plant transformation.

Plants were grown at 22°C under 16-h-light and 8-h-dark regime. To create *AtTAF15b-GFP* and *AtTAF15b-FLAG* constructs, a genomic PCR fragment of *AtTAF15b* (At5g58470) with 1,436 bp upstream from the start codon was cloned into the modified binary vector pCam1305 with either C-terminal GFP or FLAG sequence by restriction enzymes *Bam*HI and *Sal*I. The cloning PCR was performed using primers 5′-CGC GGATCCCATTTCTCCAGAGCTATGGC-3′ and 5′-ACGCA CGCGTCGACATATGGACGAGACCGGTTTC-3′. *Agrobacterium*-mediated plant transformation was carried out with slight modification to the procedure described by Clough and Bent (1998). Briefly, the Silwet L-77 was substituted with OFX-309 and floral dip was performed twice with a 1-week interval between dippings (Mireault et al. 2014).

Screen for homozygous T-DNA mutants.

To identify homozygous *taf15b* T-DNA Salk_061974 or Sail_35_B06 mutants, we used primers 5′-CAAAACAATCC ACCACCATTC-3′ and 5′-AAAAAGTCAAGCAGTGCGATG-3′ to perform PCR. Similarly, to check homozygosity for the *mos11-1* mutation, primers 5′-CGGCCGATAATTCGTGGACG-3′ and 5′-CACCAGTAGATAGCCCTCC-3′ were used. For presence of the T-DNA from the generic primers, LBb1.3 (ATTTTG CCGATTTCGGAAC) was used for SALK lines and Sail-F (cgtccgcaatgtgttattaag) for SAIL lines.

RT-PCR analysis and pathogen infections.

RT-PCR analysis of the expression of *PR1* and *PR2* was carried out as previously described (Cheng et al. 2011). *ACT1* was used as loading control. Leaf infiltration of *Pseudomonas syringae* strains was performed on 4-week-old plants as described previously (Li et al. 1999). Spray infection by *H. arabidopsidis* Noco2 or Emwa1 was performed as described previously (Li et al. 1999).

Confocal microscopy and in situ total mRNA hybridization.

Ten-day-old plants grown on ½ Murashige-Skoog (MS) were used for observation with GFP- or CFP-tagged constructs, using a Leica SP8 confocal microscope. In situ poly-A RNA hybridization was performed as previously described (Germain et al. 2010).

Nuclear extraction, coimmunoprecipitation, and Western blot analysis.

F1 plants from a cross between plants homozygous for *MOS11-GFP* in *mos11-1* and *AtTAF15b-FLAG* in Salk_061974 were used to check the association between TAF15b and MOS11 in vivo, while plants homozygous for *AtTAF15b-FLAG* in Salk_061974 were used as the negative control. Plants were grown on MS medium and 5 g of 2.5-week-old plants were harvested from each genotype. Nuclear extraction was performed as previously described (Wiemer et al. 2012). Nuclei (in NE-3 buffer: 20 mM HEPES-KOH, pH 7.9, 2.5 mM $MgCl_2$, 150 mM NaCl, 20% glycerol, 0.2% Triton X-100, 0.2 mM EDTA, and 1 mM dithiothreitol, with protease inhibitors) were sonicated using a 550 Sonic Dismembrator (Fisher) at a power of 2.75 for 4 min with a 10-s pause for each 5 s of operation, to break the nuclear envelope. Nuclear samples were incubated with NHS-activated sepharose beads (GE Healthcare) for 30 min to remove nonspecific binding. Nuclei were pelleted at 5, 000 × *g* for 30 s and were allowed to be incubated with recombinant camel GFP-binding protein–conjugated beads for 3 h. After incubation, the beads were pelleted at 20,000 × *g* for 5 min and were washed three times with NE-3. Subsequent Western blot was performed using anti-GFP and anti-FLAG antibodies respectively (Roche).

ROS assay.

Eight leaf discs (4 × 4 mm) of 4-week-old *Arabidopsis* plants were sampled using a razor blade and were floated overnight under light on sterile water. PAMP-induced ROS produced by the leaf discs were measured by a luminol-based assay (Lu et al. 2010; Trujillo et al. 2008). Luminescence was captured using a Tecan M200 plate reader.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

WoLF PSORT prediction tool: http://www.genscript.com/wolf-psort.html
National Center for Biotechnology Information Conserved Domain search tool: http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi

ANNEX D

AN UNBIASED NUCLEAR PROTEOMICS APPROACH REVEALS NOVEL NUCLEAR PROTEIN COMPONENTS THAT PARTICIPATES IN MAMP-TRIGGERED IMMUNITY

Zainab Fakih, **MD BULBUL AHMED**, Claire Letanneur and Hugo Germain

Annex D contains a published research study-containing findings on MAMP-triggered immunity in plants. It shows an unbiased nuclear proteomics based approach demonstrating that nuclear proteomic is a valid and phenotype-independent strategy to uncover factors involved in diverse cellular processes.

I have performed the gene expression analysis for the verification of chitosan treatment. To do this end, I have treated plants with chitosan, isolated RNA and performed quantitative RT-PCR (RT-qPCR) amplification.

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SHORT COMMUNICATION

OPEN ACCESS

An unbiased nuclear proteomics approach reveals novel nuclear protein components that participates in MAMP-triggered immunity

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ABSTRACT

(MAMP)-triggered immunity (MTI) is the first layer of molecular defense encountered by pathogens. Genetic screens have contributed to our knowledge of MTI, but are limited to phenotype-causing mutations. Here we attempt to identify novel factors involved in the early event leading to plant MTI by comparing the nuclear proteomes of two Arabidopsis genotypes treated with chitosan. Our approach revealed that following chitosan treatment, *cerk1* plants had many nuclear accumulating proteins in common, but also some unique ones, when compared with Col-0 plants. Analysis of the identified proteins revealed a nuclear accumulation of DNA-modifying enzymes, RNA-binding proteins and ribosomal proteins. Our results demonstrate that nuclear proteomic is a valid, phenotype-independent approach to uncover factor involved in cellular processes.

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Plants have evolved a multilayered system to detect and defend against potentially harmful pathogenic microbes. Beyond structural defenses, the first molecular layer is composed of transmembrane pattern recognition receptors (PRR) that detect slowly-evolving microbial components.[1] These microbe-associated molecular patterns (MAMPs), also known as pathogen-associated molecular patterns (PAMPs) include, among many others, the bacterial flagellin (flg22) and elongation factor Tu.[2] MAMP recognition by PRRs triggers ion fluxes, oxidative bursts[3] and mitogen-activated protein kinase (MAPK) pathways activation[4] leading to the transcriptional reprogramming of over 1,200 genes[5] and to the induction of required basal defense responses.[1] The importance of MTI is best illustrated by the pressure exerted by the pathogen to suppress it. One striking example is the HopF2 effector which directly supresses MTI at two different levels of the MAMP-activated MAPK cascades. It can directly target BAK1, which is required for the full elicitation of pathogen-induced defense responses,[6] at the plasma membrane, thereby acting upstream of the MEKK1-MKK1/2- MPK4 pathway. It can also directly block MKK5 of the MEKK-MKK4/5-MPK3/6 cascade.[6]

The chitin receptor is one of the MAMP receptors that has been investigated with some success. Chitin, a major component of the fungal cell wall, is a β-1,4-linked N-acetyl-glucosamine polymer that has long been recognized as a potent MAMP in plant-fungus interactions.[7] In *Arabidopsis*, it is mostly detected by the CHITIN-ELICITED RECEPTOR KINASE 1 (CERK1). *cerk1* knock-out plants lose their response to chitin elicitor, including MAPK activation, reactive oxygen species (ROS) generation and induction of gene expression.[8] Indeed, CERK1 phosphorylates after exposure to chitin or chitosan (acetylated chitin) and can homodimerize when binding to chitin monomers to activate its kinase domain.[9] However, chitin signaling seems to require co-receptors: two additional LysM receptor kinases, AtLYK4 and AtLYK5, are also involved in chitin recognition.[10,11] Supporting the co-receptor theory is the fact that AtLYK5 binds chitin with high affinity and can dimerize with CERK1 in a chitin-dependent manner.[10] Other receptors may also be implicated but are masked by the dominant effect of CERK1.

Despite the importance of MTI, the intracellular modulation that takes place after MAMP recognition, which involves transcriptional reprogramming, is still somewhat unclear. More precisely, the chitin-elicited nuclear proteins involved in the establishment of basal defense responses are not fully known. Two MAPK pathways have been shown to be activated downstream of MAMP signaling. One elicits the activation of the MAPKs MPK3 and MPK6[4] and the second leads to MPK4 activation.[12] Recently, MPK1, MPK11 and MPK13 were also found to be phosphorylated upon flg22 treatment.[13] The absence of MTI defect in these three MAPKs knockout lines suggests functional redundancy, so many more components acting downstream of receptor activation may be missed in phenotype-based screening.

In the present study, we sought to discover proteins that participate in MTI but have escaped phenotype-based screening. Toward this end, we took an unbiased approach based on protein mass spectrometry (MS) of the nuclear proteome of young *Arabidopsis* plants subjected or not to chitosan treatment. Chitosan is known to also bind CERK1[9] and triggers a transcriptional response that overlaps with the response to chitin.[14] Using high performance liquid chromatography-electrospray

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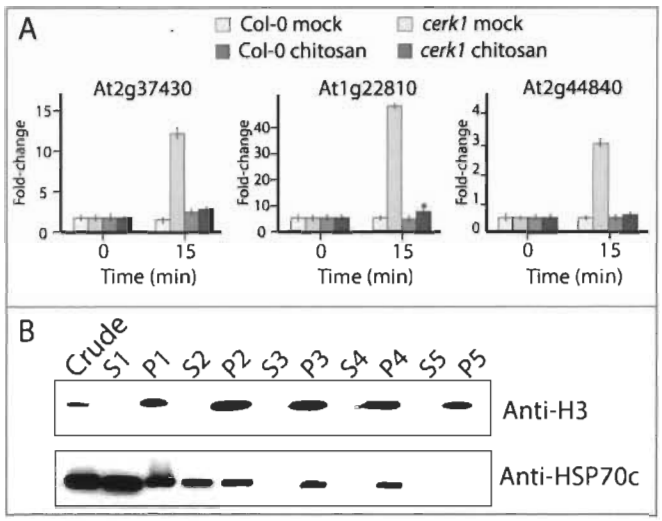

Figure 1. Chitosan treatment elicits MTI responsive gene in planta. (A) Expression of the MAMP-triggered immunity responsive marker genes At2g37430, At1g22810, At2g44840 in Col-0 and *cerk1* following chitosan or mock treatment. Q-RT-PCR was performed on soil grown three-weeks-old plants. ACT1 was used to normalize the transcript levels. (B) Quality control of the fractionation procedure by western blotting using HSP70c and histone H3 as cytosolic and nuclear markers respectively. Crude indicates crude extract, S = supernatant, P = pellet and number indicate the wash number.

ionization tandem mass spectrometry (HPLC-ESI-MS-MS), we identified several plant proteins that accumulate in the nucleus exclusively after chitosan treatment of *Arabidopsis* Columbia-0 (Col-0) or *cerk1* plants.

Before proceeding with the nuclear proteome MS analysis, we assessed if chitosan treatment was efficient in triggering a MAMP-like response. Three genes that are among the most up-regulated after chitin treatment[15] were analyzed by RT-qPCR: At2g37430 (C2H2-ZF), At1g22810 (AP2/ERE) and At2g44840 (AP2/ERE). All three genes were upregulated after chitosan treatment of Col-0 plants, showing respectively 13-fold, 51-fold and 3-fold induction 15 min post-treatment (Fig. 1A). We also observed that At1g22810 was slightly upregulated following chitosan treatment of *cerk1* plants albeit at much lower level than in Col-0 (5-fold).

We assessed the purity of our nuclear fractions by using the cytosolic marker HSP70c and nuclear marker histone H3. HSP70c could not be detected by Western blotting in the nuclear fraction corresponding to pellet five, while the nuclear marker anti-histone H3 was still clearly visible, hence this nuclear fraction was sent for mass spectrometry analysis. Tandem MS identified 1,372 different *Arabidopsis* proteins among a total of 31,416 spectra from our eight samples (duplicates of *cerk1* or Col-0 plants treated or not with chitosan) (PRIDE repository with the dataset identifier PXD003821 and 10.6019/PXD003821). We set very conservative criteria for our analyses: all proteins identified needed a minimum of two spectra to be considered, and all proteins that were present in only one of the duplicates were also rejected.

Our first analysis of the proteomic results was to compare the functional categorization of the 232 proteins found in the nucleus after chitosan treatment (in Col-0 and *cerk1*) with the 182 proteins from the nuclear proteome of cold-treated plants, one of the few studies of *Arabidopsis* nuclear proteomes that can relate to our investigation.[16] In parallel, we performed similar analysis with the SUBA database using only proteins predicted to be nuclear by SUBA bioinformatics tools or confirmed to be nuclear by GFP-tagging (total of 4,421 proteins). Finally, we compared our data to findings on the cytosolic proteome published by Ito et al. (2011) (Fig. 2A). The first observation from this categorization based on predicted cellular components is that only 26% of the nuclear proteins from the SUBA data set were annotated as nuclear proteins by TAIR's gene ontology (GO) annotator (Fig. 2A). In other words, the remaining 74% may be nuclear at some point, but the nucleus was not deemed to be their primary localization in GO. This reflects the fact that proteins may have several putative locations and underlines the weakness of bioinformatic to predict protein localization. The nuclear proteomes of chitosan and cold-treated plants contained only 11% and 16% of predicted nuclear proteins while the cytosolic experimental proteome still showed 9% of nuclear predicted proteins (Fig. 2A). Based on the discrepancies observed with the SUBA dataset, we can assume that a significant proportion of proteins annotated as non-nuclear by GO in these three experimental data sets were indeed at some point nuclear.

In the search for proteins that participate in MTI, categorization by molecular function (Fig. 2B) enables us to identify proteins that have the capacity to modulate transcription or translation during defense responses. Our chitosan-induced nuclear proteome contains 19% of DNA- or RNA-binding proteins, which could alter gene expression through

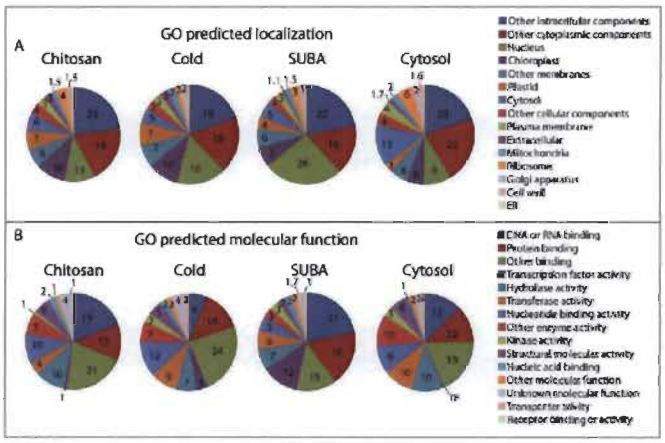

Figure 2. Gene ontologies in chitosan treated plants compared to other datasets. (A) GO predicted subcellular localization. (B) GO predicted molecular function.

DNA-binding, mRNA-processing, and mRNA-export, or could impact translation through mRNA nuclear segregation. Fewer of these proteins (12%) were found in the cytosolic dataset.[17] Proteins with transcription factor activity were most abundant in the SUBA nuclear data set (12%) but still represented 1%, 4% and 0.2% of proteins in chitosan, cold and cytosolic proteomes respectively, confirming that nuclear enrichment does indeed enrich transcription factors. It should also be noted that empirically-obtained proteomes are biased toward abundant proteins which could mask less abundant proteins. Therefore, signaling components such as transcription factors may be under-represented in LC-MS-MS proteomes, as demonstrated by their abundance in the SUBA dataset relative to the three other data sets.

We constructed a Venn diagram comparing the proteins found in each treatment group (Control is the combination of both Col-0 and *cerk1* plants treated with water) (Fig. 3). We identified eight proteins specifically localized to the nucleus of Col-0 plants after chitosan treatment (listed in Table 1). Although most of these are not obvious MTI components, a clear trend toward ribosomal proteins and translation is obvious. Proteins 1 (S19E family ribosomal protein), 4 (ribosomal protein l6), 5 (S19E family ribosomal protein) and 7 (RNAse Z activity involved in tRNA processing) are all involved in translation. Protein 8 (DNA-binding transcriptional regulator) is engaged in transcription regulation while protein 6 (small nuclear ribonucleoprotein G) binds RNA and could be involved in either transcription or translation. Most of these proteins have been reported to be modulated at the transcription level after biotic or abiotic stress, but have not previously been linked with the MAMP response.[18-21]

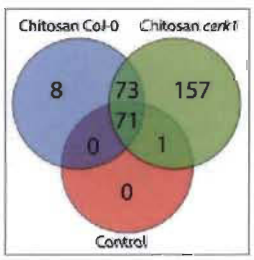

Figure 3. Venn diagram displaying the number of proteins identified in the nucleus for each condition.

157 proteins were only detected in the nucleus of *cerk1* plants after chitosan treatment (reported in Table S2). It is striking that so many protein are unique to *cerk1* as it has an impaired sensing of chitin[8] and as we observed only a weak transcriptional reprogramming in our RT-qPCR results (Fig. 1). On the other hand, it is known that while chitin and chitosan responses largely overlap, 33% of chitosan elicited genes are not elicited by chitin.[14] Table 2 groups the proteins possessing the molecular functions most likely to affect early MTI responses (transcription factor and DNA/RNA-binding protein) and excludes those from metabolisms. Many of those may regulate gene expression or mRNA metabolism, as several additional proteins are RNA helicases that may influence transcription or translation. Interestingly, one resistance protein of the Toll/Interleukin receptor (TIR) family (At4g16990) was found: it is known as RLM3 and is required for resistance to *Leptosphaeria maculans* and other necrophytic pathogens.[22]

We also analyzed the proteins common between Col-0 and *cerk1* nuclei after chitosan treatment (presented at the intersection in Fig. 3). A total of 73 proteins were identified and most of these were either DNA/RNA-binding proteins or ribosomal proteins. Table 3 shortlists the proteins sorted by molecular function, uncovering several DNA/RNA-binding proteins linked with chromatin remodeling and RNA maturation (see full list in Table S3). Receptor for activated C kinase 1 A (RACK1A) was one of the few proteins in Table 3 that was neither ribosomal nor DNA/RNA-binding. This protein was recently shown to act as a scaffold protein

Table 1. Nuclear localized proteins identified by LC-MS-MS in Col-0 plants following chitosan treatment.

Protein description	Uniprot ID	AGI
Ribosomal protein S19e family protein	D7KGE2	AT5G61170
HAD superfamily, subfamily IIIB acid phosphatase	Q9ZWC4	AT1G04040
Galactose mutarotase-like superfamily protein	Q8LFH1	AT3G47800
Ribosomal protein L6 family protein	Q8L9N4	AT1G18540
Ribosomal protein S19e family protein	D7MUI1	AT5G61170
Probable small nuclear ribonucleoprotein G	Q82221	AT2G23930
Encodes a protein with RNase Z activity suggesting a role in tRNA processing	Q8L633	AT2G04530
DNA-binding storekeeper protein-related transcriptional regulator	O23063	AT4G00390

in a new immune signaling pathway.[23] The subset common between the two genotypes (Col-0 and *cerk1*) and the two treatments (water and chitosan) – those at the intersection of the three circles (listed in Table S4) – mostly contained proteins from the chloroplasts and mitochondria as well as many enzymes from primary metabolism that likely contaminated the nuclear preparations, which explains that they were found in all genotypes and treatments. This set of proteins also contained some constitutive nuclear components, such as nucleoporins, spliceosome assembly proteins and polymerases, but few RNA- or DNA-binding proteins and ribosomal components, strengthening the results obtained in chitosan-treated plants in which we observed some specificity among RNA- or DNA-binding proteins and ribosomal components.

The MTI response depends on the recognition of conserved molecular pathogen patterns at the cell surface by pathogen recognition receptors.[1] Genetic screening has largely contributed to our understanding of plant defense[24] and to the molecular dissection of the defense signaling pathways.[25] We used HPLC-ESI-tandem MS, a phenotype-independent approach to discover components participating in the establishment of defense responses resulting from MAMP recognition.

Interestingly, proteins that were either part of the ribosome or actively participated in translation were over-represented following chitosan treatment in both genotypes (Table 1, 2, 3). Since ribosomes are assembled in the nucleus, it is not surprising to observe many ribosomal proteins in our nuclear proteomes, but it is interesting that their identity differed in different genotypes and whether the plants had been exposed to chitosan or not. It is well-known that ribosome composition is highly heterogeneous and varies during plant development to ensure translational regulation.[26] Hence, we could speculate that ribosome subunits, which are highly heterogeneous,[27] may disassemble and reassemble after elicitor detection and triggering of MTI. As is observed in development, such reassembly could promote MTI oriented translational regulation. Recently, JIP60, a barley protein that mediates a translational switch toward stress and defense protein synthesis in the presence of jasmonate and at senescence, was discovered.[28] More recently the ribosomal coding genes *RPL12* and *RPL19* were shown to be involved in nonhost disease resistance in *Nicotiana* and

Table 2. Subset of nuclear localized proteins identified by LC-MS-MS in *cerk1* plants following chitosan treatment.

Protein description	Uniprot ID	AGI
Transcription factor or transcriptional regulator		
MED16, Mediator of RNA polymerase II transcription subunit 16, positive regulation of SAR	F4IGZ1	AT4G04920
Small RNA degrading nuclease 3, regulation of transcription	F4K3N3	AT5G67240
ACT domain-containing small subunit of acetolactate synthase protein	Q93YZ7	AT2G31810
Trihelix transcription factor ASIL2, sequence-specific DNA binding transcription factors	Q9LJG8	AT3G14180
VERNALIZATION INDEPENDENCE 5, regulation of transcription, DNA binding	D7KW58	AT1G61040
Sequence-specific DNA binding transcription factors	Q8LF33	AT3G11100
Short life 1, PHD finger and BAH motif containing putative transcription factor	F4JV93	AT4G39100
Mediator of RNA polymerase II transcription subunit 32	Q84VW5	AT1G11760
CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 2, CAMTA2	Q6NPP4	AT5G64220
EARLY BOLTING IN SHORT DAYS, chromatin assembly or disassembly	O65462	AT4G22140
RNA-binding protein		
NUCLEOSTEMIN-LIKE 1, nucleolar GTP- binding protein involved in RNA methylation	Q93Y17	AT3G07050
RPT2a encodes the 26S proteasome subunit, regulate gene silencing via DNA methylation	Q9SZD4	AT4G29040
EMBRYO DEFECTIVE 2770, RNA-directed DNA methylation, mRNA splicing	Q9ZT71	AT4G03430
Serine/arginine-rich SC35-like splicing factor	Q8L3X8	AT3G55460
RZ1B, Putative RNA-binding involved in cold tolerance	O22703	AT1G60650
WD-40 protein involved in histone deacetylation in response to abiotic stress	Q9FN19	AT5G67320
TOUGH, Interacts with TATA-box binding protein 2. RNA binding	Q8GXN9	AT5G23080
THO complex subunit 7B, component THO/TREX complex	Q9M8T6	AT3G02950
Small RNA degrading nuclease 3, regulation of transcription	F4K3N3	AT5G67240
RNA binding (RRM/RBD/RNP motifs), RNA processing	F4J9U9	AT3G12640
mRNA splicing factor, Cwf18	Q9MAB2	AT3G05070
SWI/SNF complex subunit SWI3C, ATP-dependent chromatin-remodeling complex	Q9XI07	AT1G21700
Splicing factor U2af large subunit B, Necessary for the splicing of pre-mRNA	Q8L716	AT1G60900
Small nuclear ribonucleoprotein	Q9SUM2	AT4G30220
Small nuclear ribonucleoprotein family protein, mRNA splicing	Q9C6K5	AT1G76860
nuclear cap-binding protein, mRNA metabolism	Q9XFD1	AT5G44200
RNA-binding protein-related	F4JM55	AT4G28990

Table 3. Subset of nuclear localized proteins identified by LC-MS-MS in both *cerk1* AND Col-0 plants following chitosan treatment.

Protein description	Uniprot ID	AGI
Miscellanous		
Homologous to the co-chaperon DNAJ protein	Q94AW8	AT3G44110
EPITHIOSPECIFIER MODIFIER 1, defense response to bacterium	Q9LJG3	AT3G14210
RECEPTOR FOR ACTIVATED C KINASE 1 A, MAP-kinase scaffold activity	O24456	AT1G18080
DNA-RNA binding proteins		
Nuclear RNA binding protein A-like protein	Q8LDQ7	AT5G47210
GLYCINE-RICH RNA-BINDING PROTEIN 7, DNA binding, RNA binding	C0Z2N6	AT2G21660
mRNA splicing factor	B3H6J5	AT3G49601
RNA BINDING PROTEIN, RNA modification, RNA processing, RNA stabilization	Q04836	AT4G24770
RNA polymerase I-associated factor PAF67	F4JY76	AT5G25754
ATWTF1, RNA recognition domain	A0MFS5	AT4G01037
GENERAL REGULATORY FACTOR 3, 14-3-3 gene	P42644	AT5G38480
COPPER RESPONSE DEFECT 1, putative ZIP protein, DNA binding	Q9M591	AT3G56940
Histone deacetylase HDT2	Q56WH4	AT5G22650
MAR-binding filament-like protein 1, DNA-binding protein	Q9LW85	AT3G16000
Nucleosome assembly protein 1-like 1	B3H684	AT4G26110
Emsy N Terminus and plant Tudor-like domain, defense response to fungus	Q9C7C4	AT3G12140
Histone deacetylase HD2A	F4J378	AT3G44750
Serine/arginine-rich SC35-like splicing factor	Q9LHP2	AT3G13570
U2 SMALL NUCLEAR RIBONUCLEOPROTEIN B, splicing	O22922	AT2G30260
DEK domain-containing chromatin associated protein	Q84JB7	AT5G63550
ATGRP8, glycine-rich protein with RNA binding domain at the N-terminus	89DFJ8	AT4G39260
MLP-LIKE PROTEIN 423, defense response, mRNA modification	Q93VR4	AT1G24020
Involved in translation		
LOS1, translation elongation factor 2	Q9ASR1	AT1G56070
Ribosomal protein L4/L1 family	F4KDU5	AT5G02870
EMBRYO DEFECTIVE 2184, structural constituent of ribosome	Q9FWS4	AT1G75350
Eukaryotic translation initiation factor 3 subunit E	Q9C5Z3	AT3G57290
Eukaryotic translation initiation factor 3 subunit B	F4K4D5	AT5G27640
40S ribosomal protein S3a-1	Q9CAV0	AT3G04840
40S ribosomal protein S16-3	A8MRX2	AT5G1838
Ribosomal protein L19	Q8W101	AT1G02780
40S ribosomal protein S20-1	P49200	AT3G45030
Translation elongation factor EF1B/ribosomal protein S6	D7KNE3	AT5G19510
Elongation factor 1-β 2	Q9SCX3	AT5G19510
Ribosomal protein S10p/S20e family protein	Q9LK61	AT3G13120
Ribosomal protein L10 family protein	B5X0P0	AT5G13510
50S ribosomal protein L19-2	Q8RXX5	AT5G47190
TRANSLATION INITIATION FACTOR 3 SUBUNIT H1	Q9C5Z2	AT1G10840
RIBOSOMAL PROTEIN S10E B	Q9FFS8	AT5G41520
60S ribosomal protein L36-2	Q9M352	AT3G53740
60S ribosomal protein L17-1	Q93VI3	AT1G27400
40S ribosomal protein S24e	Q9SS17	AT3G04920
Elongation factor 1B β	A8MRC4	AT1G30230

Arabidopsis and also play a minor role in basal resistance against virulent pathogens.[29]

Another type of proteins abundantly observed in our study were DNA-modifying enzymes that have the capacity to affect chromatin remodeling and in doing so to further impact transcription. The role of chromatin remodelling proteins in regulating *Arabidopsis* defense responses has been reviewed by Berr et al.[30] Mutation of chromatin-remodeling enzymes results in pleiotropic phenotypes not specifically associated with MTI or ETI but in which prominent players in transcriptional repression and activation at the onset of these processes are affected.

Various families of RNA-binding proteins, including proteins linked to mRNA splicing, export and maturation, were also identified after elicitation by chitosan. RNA export defects have previously been shown to suppress NB-LRR-mediated immunity,[31,32] basal responses[32] and response to abiotic stress,[33] suggesting that even more proteins involved in RNA metabolism may participate in defense responses.

As reviewed by Boller and Felix (2009), many molecular events unfold during the first 15 min of MAMP recognition and they set a point of no return upon which cells commit to the massive transcriptional reprogramming required for the establishment of the basal response. Consequently, we chose to concentrate our analysis on early nuclear recruitment of molecular components following MAMP detection. While the MTI response is clearly dependant on MAPK pathways, our data indicate that ribosome reorganization, DNA modification and RNA maturation could play major roles during the early MAMP response. Specific proteins affecting translation or switching it to defense mode need to be investigated further. Similarly, the participation of chromatin-remodeling and RNA-modifying enzymes should be studied. Our results demonstrate that nuclear proteomic is a valid, phenotype-independent approach to uncover factors involved in various cellular processes.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana cerk1 seeds (SALK_007193) were obtained from the Arabidopsis Biological Resource Centre (http://abrc.osu.edu/). Col-0 and *cerk1* seeds were sterilized for 2 min in 5% (v/v) bleach solution and 0.1% Tween-20, then rinsed in sterile water. The seeds were incubated at 4°C for 2 days and transferred to a growth room at 22°C under a 16 h light/8 h dark cycle. The plants were grown for 3 weeks in soil before chitosan treatment. *cerk1* plants were genotyped by polymerase chain reaction (PCR) (see Supplementary Table 1 for list of primers).

Chitosan preparation and treatment

Low-viscosity chitosan was purchased from Sigma-Aldrich (Oakville, ON, Can). Chitosan was solubilized in glacial acetic acid, then diluted at 100 μg/ml. *Arabidopsis* leaves were treated by spraying a chitosan solution until fully covered, harvested after 15 min and snap frozen in liquid nitrogen. Control samples were treated with similarly-diluted acetic acid solution.

Gene expression analysis for verification of chitosan treatment

Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. RNA quality was assessed by agarose gel electrophoresis and quantified by spectrophotometry. One μg of each sample was reverse transcribed into cDNA with the High Capacity cDNA Archive Kit (Life Technologies, Burlington, ON, Can). Quantitative RT-PCR (RT-qPCR) amplification was undertaken with a Mx3000P Detection system (Agilent Technologies, Santa Clara, CA, USA) using SYBR Green PCR Master Mix (Bioline, London, U.K.). 100 ng cDNA template and 0.4 μM of each primer (listed in Supplementary Table 1) (were used in a final volume of 20μl. The qRT-PCR thermal profile was: 95°C for 2 min, 40 cycles of 95°C for 5 s, 58°C for 10 s, and 72°C for 5 s. To analyze the quality of dissociation

curves, the following program was added after 40 PCR cycles: 95°C for 1 min, followed by constant temperature increases from 55°C to 95°C. The data were analyzed with MxPRO QPCR software. A threshold of 0.2 was selected to obtain cycle threshold (Ct) values. *Actin 1* served to normalize all RT-qPCR results. The expression levels of each gene were calculated according to the ΔΔCt method. Three technical replicates for each treatment were analyzed. Standard deviation was computed by the error propagation rule.

Preparation of nuclear proteins

To analyze the nuclear proteome content, nuclei were prepared according to the method described by Cheng et al. (2009), with some minor modifications. Briefly, 15 min after treatment, 4 g of 3-week-old *Arabidopsis* leaves were ground in liquid nitrogen. All steps were performed on ice or at 4°C. Tissues were re-suspended in extraction buffer (20 mM Tris-HCl (pH 7.4), 25% glycerol, 2 mM EDTA, 2.5 mM $MgCl_2$ and 1 mM phenylmethanesulfonylfluoride), then passed through nylon filter mesh of 60 μm and 30 μm (Cedarlane Laboratories, Burlington, ON, Can). The rest of the protocol, consisting of serial centrifugations/resuspensions, was undertaken as described previously. Fractions were collected at each step to control for the purity of the extracts. Nuclei-enriched pellets were re-suspended in 50 mM ammonium bicarbonate and 1% sodium deoxycholate for tryptic digestion. Each experimental condition contains two biological replicates. Proteins in solution were sent on dry ice to the Proteomics platform of Centre de Génomique de Québec, where they were further processed. Protein samples were washed 3 times on Amicon 3 kDa column with 50 mM ammonium bicarbonate buffer and then dried down. Prior to digestion, proteins were solubilized in 50 mM ammonium bicarbonate buffer containing 1% sodium deoxycholate. Samples were reduced, alkylated and digested with trypsin. Tryptic peptides were desalted on stage tip (C18) and vacuum dried before MS injection. Lyophilized tryptic peptides were re-dissolved in a 0.1% formic acid solution.

Mass spectrometry

Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES MS/MS). The experiments were performed with a Dionex UltiMate 3000 nanoRSLC chromatography system (Thermo Fisher Scientific / Dionex Softron GmbH, Germering, Germany) connected to an? Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a nanoelectrospray ion source. Peptides were trapped at 20 µl/min in loading solvent (2% acetonitrile, 0.05% TFA) on a 5 mm x 300 µm C18 pepmap cartridge pre-column (Thermo Fisher Scientific/Dionex Softron GmbH, Germering, Germany) during 5 minutes. Then, the pre-column was switched online with a self-made 50 cm x 75 µm internal diameter separation column packed with ReproSil-Pur *C18-AQ* 3-µm resin (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) and the peptides were eluted with a linear gradient from 5-40% solvent B (A: 0,1% formic acid, B: 80% acetonitrile, 0.1% formic acid) in 35 minutes, at 300 nl/min. Mass spectra were acquired using a data dependent acquisition mode using Thermo XCalibur software version 3.0.63. Full scan mass spectra (350 to 1800 m/z) were acquired in the Orbitrap with a resolution of 120 000. Each MS scan was followed by acquisition of fragmentation spectra of the most intense ions for a total cycle time of 3 seconds (top speed mode). The selected ions were isolated using the quadrupole analyzer and fragmented by Higher energy Collision-induced Dissociation (HCD). The resulting fragments were detected by the linear ion trap. Dynamic exclusion was set for a period of 20 sec and a tolerance of 10 ppm.

Protein identification and database searching

Peptide masses were measured as described previously. All MS/MS peak lists (MGF files) were generated using Thermo Proteome Discoverer version 1.4.0.288 (Thermo Fisher). Mgf files were searched against the UniProt Arabidopsis database (release 11/2014 containing 91679 entries) with the Mascot software ((Matrix Science, London, UK; version 2.4.1)). Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine was

specified as a fixed modification. Pyroglutamate of the n-terminus, deamidation of asparagine and glutamine and oxidation of methionine were specified as variable modifications. Two missed cleavages were allowed. Scaffold (version 4.4.6, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve a FDR of less than 1.0% and contained at least two identified peptides. Proteins/peptides FDR rate was set to 1% or less based on decoy database searching (0.1%). Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD003821 and 10.6019/PXD003821 (Vizcaíno JA 2014, Vizcaíno JA 2016).

Three additional datasets were compared: data from the proteomics paper by Bae et al. (2003) on cold-treated Arabidopsis plants and from the Arabidopsis cytosolic proteome report by Ito et al. (2011) were aggregated. We also extracted all Arabidopsis gene identifiers corresponding to predicted/observed nuclear proteins from the Arabidopsis Subcellular Database (SUBA, [4](http://suba3.plantenergy.uwa.edu.au/)(Tanz et al. 2013).

Immunoblotting

The purity of the isolated nuclear fractions was controlled by western blotting. Proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to PVDF membrane, which were incubated with anti-histone H3 and anti-HSP70c antibodies (both from Agrisera, Vännäs, SWEDEN) for 1 h. Antibody-bound proteins were detected by incubation with secondary antibodies conjugated to horseradish peroxidase in an ECL system (BioRad, Mississauga, ON, Can).

ANNEX E

A FUNGAL RUST EFFECTOR TARGETS THE PLANT CELL NUCLEUS AND MODULATES TRANSCRIPTION

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Annex E contains a submitted manuscript on functional studies of an *M. larici-populina* effector, Mlp124478. We demonstrated that Mlp124478 accumulates in the nucleus and nucleolus of host cells and binds the TGA1a promoter to suppress genes induced in response to pathogen infection.

A fungal rust effector targets the plant cell nucleus and modulates transcription

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Abstract

The basidiomycete *Melampsora larici-populina* causes poplar leaf rust, invading leaf tissue and secreting effector proteins through specialized feeding structures known as haustoria. The mechanisms by which rust effectors promote pathogen virulence are poorly understood. The present study characterized Mlp124478, a candidate effector of *M. larici-populina*. We investigated the plant models *Arabidopsis thaliana* and *Nicotiana benthamiana* and established that Mlp124478 accumulates in the nucleus and nucleolus, and promotes growth of the oomycete pathogen *Hyaloperonospora arabidopsidis*. Stable constitutive expression of *Mlp124478* in *A. thaliana* altered leaf morphology, observed through increased waviness of rosette leaves and repressed expression of genes involved in immune responses. Our results indicate that Mlp124478, which contains a DNA-binding domain, interacts with the TGA1a-binding sequence. Taken together, our results suggest that Mlp124478 accumulates in the nucleus and nucleolus of host cells and binds the TGA1a promoter to suppress genes induced in response to pathogen infection.

Introduction

Plant pathogens secrete molecules (e.g. proteins) into host tissues, known as effectors, to promote parasitic growth. Effectors target various host cell compartments and interact with molecules, such as proteins and DNA, to modulate their location, stability and function (Chaudhari et al. 2014, Lewis et al. 2009, Vargas et al. 2016, Win et al. 2012). Nowadays, molecular plant pathologists employ effectors as probes to identify and understand the plant processes targeted by pathogens and exploit this insight to develop resistant crops. Genomic approaches coupled with heterologous expression studies of *Arabidopsis thaliana* and *Nicotiana benthamiana* are commonly undertaken to decipher the mechanisms by which effectors promote pathogen virulence (Fabro et al. 2008, Gaouar et al. 2016, Kunjeti et al. 2016, Rafiqi et al. 2012, Sohn et al. 2007).

Many effectors target the host nucleus and interfere with transcription to alter plant immune responses (Boch et al. 2009, Motion et al. 2015, Rivas and Genin 2011). For instance, bacterial transcription activator-like effectors (TAL) function as transcription factors and alter host gene expression levels, which may result in substantial influence on host phenotypes (Gu et al. 2005, Yang et al. 2006). *Hyaloperonospora arabidopsidis*, a filamentous obligate biotrophic pathogen, has effectors that target the nucleus. One of them, HaRxL44, goes to the nucleus and interacts with the Mediator complex MED19a, inducing its proteasome-mediated degradation. This, in turn, leads to transcriptional changes resembling jasmonic acid and ethylene induction with repressed salicylic acid signaling enhancing susceptibility to biotrophs (Caillaud et al. 2013). Similarly, global expression profiling of the fungal biotroph *Ustilago maydis*-maize interaction demonstrated early induction (within the first 12 h post-infection) of the defense response genes which are later quenched (between 12-24 h pots-infection) (Doehlemann et al. 2008), indicating that transcriptional reprogramming is a conserved mechanism amongst obligate biotroph.

Rust fungi (order *Pucciniales*) are notorious plant pathogens and are among the most studied obligate biotrophic fungal pathogens (Dean et al. 2012). *Melampsora larici-populina* causes poplar leaf rust disease, which is a threat to poplar plantations worldwide (Pinon and Frey 2005). Genome analysis of *M. larici-populina* has predicted 1,184 small secreted proteins (SSPs) (Duplessis et al. 2011a). Several features, such as expression in poplar leaves during infection, homology to other known rust effectors, signature of positive selection and specificity to Pucciniales order, and lack of predicted function were considered to select candidate secretory effectors proteins (CSEPs) (Hacquard et al. 2012, Petre et al. 2015a). Recently, twenty *M. larici-populina* candidate effectors were shown to accumulate in multiple leaf cell compartments and target several protein complexes when expressed heterologously in *N. benthamiana* (Petre et al. 2015b). Of the effectors analyzed by Petre et al. (2015b) and effectors screened in our laboratory, (Germain et al. In revision) Mlp124478 is the only one to localize to the nucleus and nucleolus in *Nicotiana benthamiana* and *Arabidopsis*. *Mlp124478* is part of the CPG2811 gene family with nine members, which are specific to the Pucciniales

(Duplessis *et al.*, 2011a, Hacquard *et al.*, 2012). *Mlp124478* expression is strongly enhanced during infection and reaches 50-fold induction at 96 h after infection. Given the kinetics of *M. larici-populina* infection, this corresponds to the biotrophic growth stage in mesophyll cells (Duplessis et al. 2011b). In addition, the CPG2811 family presents a rapid evolution signature, a feature of pathogen effector families (Hacquard et al. 2012). Mlp124478 is part of a multigenic family specific to rust fungi with nine members in *M. larici-populina* genome (Duplessis et al. 2011a; Hacquard et al. 2012). Its unique localization as well as other interesting features observed in Mlp124478 (described thereafter) prompted us to investigate more precisely the functional role of Mlp124478.

Here, we confirm that Mlp124478 accumulates in the nucleus and nucleolus of leaf epithelial cells, identified the sequence responsible for the nucleolar accumulation and investigate its cellular function *in planta*. Since the *in planta* constitutive expression of *Mlp124478* affects plant morphology and susceptibility to the oomycete pathogen *H. arabidopsidis*, we took the transcriptomics route to ascertain whether it induces transcriptional reprogramming. Our results indicate that Mlp124478 localizes to the nucleus to target DNA and reprogram normal transcriptional responses to pathogenic attack, thereby altering host susceptibility.

Materials and Methods

Plant material and growth conditions

A. thaliana and *N. benthamiana* plants were soil-grown in a growth chamber under a 14 h/10 h light/dark cycle with temperature set at 22°C and relative humidity of 60%. The plants were grown in Petri dishes for the selection of single-insertion homozygous transgenic *Mlp124478* with ½ Murashige and Skoog medium containing 0.6% agar and 15 mg/ml Basta.

Growth of *Pseudomonas syringae* pv. *tomato*, *H. arabidopsidis* Noco2 and infection assay

Pseudomonas syringae strain DC3000ΔCEL (Alfano et al. 2000) containing *Mlp124478* was grown overnight and infiltrated the leaves of 4-week-old Col-0 and transgenic *Mlp124478* plants at optical density at 600 nm (OD_{600})=0.001. Prior to inoculation, bacterial growth was assessed at different times by OD_{600} measurements. *Pst* infections were produced by syringe infiltration of 4-week-old *Arabidopsis* plant leaves, and *H. arabidopsidis* Noco2 spray infections were induced, as described previously (Li et al. 1999).

Plasmid construction

Constructs were developed via Gateway cloning systems (Invitrogen, Life Technologies). The *Mlp124478* coding sequence without the signal peptide (lacking amino acids 1-27, hereafter referred to as *Mlp124478*) was ordered from GenScript in lyophilized form, and primer pairs (Supplementary Table 1, Primer Nos. 1-3) amplified the open reading frame (ORF) of *Mlp124478* from pUC57. The polymerase chain reaction (PCR) amplicons were then cloned into pDONR^{TM}221 entry vector by Gateway BP recombination, followed by recombination with Gateway LR reaction either into pVSP*Ps*Spdes vector for *Pst* infection assay (effector delivery) or pB7FWG2.0 vector (Karimi et al. 2002) to express C-terminal green fluorescent protein (GFP)-tagged *Mlp124478* fusion *in planta*. pVSP*Ps*Spdes harbors the AvrRpm1 secretion signal (Rentel et al. 2008).

Transient expression

Solutions of *A. tumefaciens*-carrying recombinant plasmids were infiltrated into leaf pavement cells of 6-week-old *N. benthamiana* plants (Sparkes et al. 2006). Briefly, *A. tumefaciens* AGL1-competent cells were transformed with pB7FWG2-containing *Mlp124478* and grown overnight in yeast extract peptone medium supplemented with spectinomycin (50 mg/L). The cells were precipitated by centrifugation at 300 *g* and

adjusted to OD_{600} of 0.5 in infiltration buffer (10 mM $MgCl_2$ and 150 μM acetosyringone). After 1 h, the agro-suspension was infiltrated into the abaxial side of leaves, and the plants were returned to the growth chamber. At 4 days post-infiltration (dpi), water-mounted slides of epidermal peels from agro-infected leaves were visualized by confocal microscopy.

Microscopy

Cells were viewed by Leica TCS SP8 confocal laser scanning microscopy (Leica Microsystems). Images were obtained with HC PL APO CS2 40X/1.40 oil immersion objective, acquired sequentially to exclude excitation and emission crosstalk (when required). Leaf peels were immersed in water containing 0.2 μg/ml DAPI for 15 min for nuclei staining at room temperature. The samples were then observed at excitation/emission wavelength of 405/444-477 nm and 488/503-521 nm for DAPI and eGFP, respectively. Images were annotated with LAS AF Lite software.

Chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) assay

ChIP assays were conducted, as described previously, with minor modifications (Yamaguchi et al. 2014). Briefly, 300 mg of 2-week-old *A. thaliana Mlp124478* stable transgenics and Col-0 were collected in tubes containing 10 mL of phosphate-buffered saline (PBS), which were replaced by 10 mL of 1% formaldehyde to cross-link tissue under vacuum infiltration. To quench the cross-linker, 0.125 M glycine was added after removal of formaldehyde, followed by vacuuming, incubation for 5 min, and tissue-rinsing with 10 mL cold PBS. Cross-linked tissues were dried on paper towel for nuclei isolation. Sheared chromatin was immunoprecipitated with 50 μL/mL anti-GFP microbeads (MACS, Miltenyi Biotec Inc.) and incubated for 2 h at 4°C. The beads were placed in the μ-column, in the magnetic field of a μMACS separator, and washed twice. After reverse crosslinking of DNA-protein, ChIP samples underwent DNA purification according to a previously-described method (Yamaguchi et al. 2014), followed by PCR amplification with specific primer pairs listed in Supplementary Table 1 (Primer Nos. 4-38).

Electrophoretic mobility shift assay (EMSA)

EMSA was undertaken, as described earlier (Kass et al. 2000), with minor modifications. Unlabeled and digoxigenin (DIG)-labeled forward TGA1a oligonucleotides were ordered from Integrated DNA Technologies. Double-stranded (DS) oligonucleotides were annealed by heating 1 nmol of each oligonucleotide at 95°C for 10 min, then slowly cooled down to 20°C. DS oligonucleotides were diluted in TEN buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA, pH 8, 100 mM NaCl) to a final concentration of 50 pmol/μL. Dot blotting was carried out by serial dilutions and spotted on positively-charged nylon membranes to test efficiency of the DIG-labeled probe. 3 pmol of probe was found to be efficient for detection with anti-DIG primary antibody. Gel shift reaction was performed with 3 pmol of DS oligonucleotides and 100 ng of synthetic peptide in binding buffer (100 mM HEPES, pH 7.6, 5 mM EDTA, 50 mM $(NH_4)_2SO_4$, 5 mM DTT, 1% Tween 20 and 150 mM KCl). After binding reaction at 25°C for 15 min, the samples were placed on ice for 15 min, and the mixtures were electrophoresed immediately through 0.25X TBE 20% polyacrylamide gel at 12.5 volts/cm. Bio-Rad semi-dry transfer cells were electroblotted on positively-charged nylon membranes at 25 volts for 10 min. DNA was then cross-linked to the membrane by baking at 80°C for 40 min. For DIG detection, the membranes were blocked in TBS (50 mM Tris and 150 mM NaCl) + 1% BSA], followed by 2 washes with TBS for 10 min and 1 wash with TBST (TBS and 1% Tween 20), then incubated overnight at 4°C with anti-DIG monoclonal antibody diluted 1:1,000 in TBS + 1% BSA. The membranes were washed 4 times in TBS for 5 min and once in TBST. Finally, they were incubated with HRP-conjugated secondary antibody diluted 1:3,000 in TBST + 5% milk at room temperature for 45 min. The membranes were washed 4 times in TBS and once in TBST for 5 min. Bio-Rad's Clarity Western ECL blotting substrate was then applied for detection. EMSA was performed at least 3 times with independent dilution of synthetic peptides and freshly-hybridized DIG probe.

RNA extraction and transcriptome analysis

Total RNA was extracted from 4-day-old *A. thaliana Mlp124478* stable transgenics and Col-0 with RNeasy Plant Mini Kit (Qiagen, Inc.), according to the manufacturer's specifications. Control and transgenic plants were extracted in triplicate. Eluted total RNA was quantified, sent to the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (Université Laval, Quebec City, Canada) for library construction, and sequenced with Ion Torrent Technology. Differential expression was analyzed with green line workflow of the DNA subway in the iPlant collaborative pipeline (now CYVERSE) (Cold Spring Harbor Laboratory), including *A. thaliana*-Ensembl TAIR 10 as reference genome. Regulated genes with significant expression differences that is with Q-value ≤ 0.05 and fold-change ≥ 2 were further investigated with GO enrichment analysis. The Cytoscape software (version 3.1.1) (Shannon et al. 2003) with the plug-in ClueGO and CluePedia (Bindea, Galon and Mlecnik 2013) was used to visualize functions enriched in the deregulated genes. The GO terms presented are significantly enriched in up-regulated and down-regulated genes with FDR≤ 0.05 (Benjamini-Hochberg p-value correction).

Bioinformatics analyses

Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) aligned sequences of the 9-members of CPG2811 effector family and annotated them later manually. Phylogenetic trees were generated by COBALT (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi). SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) predicted signal peptides. NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus/) forecast nuclear-localizing signals. Transcription factor-binding sites (TFBS) were identified and analyzed with the AthaMap (http://www.athamap.de/search_gene.php) (Steffens et al. 2005), Pscan (http://159.149.160.88/pscan/) (Zambelli et al. 2009) and PlantPan (http://plantpan2.itps.ncku.edu.tw/index.html) (Chang et al. 2008) databases. Consensus TFBS sequences were retrieved from the Pscan database. Promoter sequences were obtained individually with TAIR's SeqViewer

(http://tairvm09.tacc.utexas.edu/servlets/sv), and TFBS-specific primers (Supplementary Table 1, Primer Nos. 4-38) were designed with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Genevestigator (http://genevestigator.com/gv/doc/intro_plant.jsp) provided gene expression data under different biological conditions. Expression values were copied from Genevestigator, and a heatmap was created in Excel. Protein DNA-binding sites were predicted by MetaDBSite (http://projects.biotec.tu-dresden.de/metadbsite/) (Si et al. 2011). ChIP-PCR-positive genes on the up-regulated gene list from the Genevestigator expression dataset were searched with the National Center for Biotechnology Information (NCBI) protein blast search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to identify similar genes in poplars. PLEXdb (http://www.plexdb.org/modules/PD_probeset/annotation.php?genechip=Poplar) (Dash et al. 2012) visualized gene expression profiles in poplars.

Results

Mlp124478 accumulates in the nucleus and the nucleolus of *A. thaliana* cells and affects the shape of leaves

Mlp124478 is part of a multigenic family, CPG2811 specific to rust fungi with nine members: *Mlp124478, Mlp124479, Mlp124480, Mlp124481, Mlp124482, Mlp124483, Mlp124484, Mlp124485 and Mlp124486*; each is composed of a predicted signal peptide followed by 2 exons encoding short peptides (75-96 amino acids) (Fig. 1A). Except for the 6 conserved cysteine residues, amino acid conservation is low in the family. Amino acid identity ranges from 28% to 60% between Mlp124478 and the other family members (Fig. 1B). Mlp124478 is the only member of the CPG2811 family that has a putative nuclear localization signal (NLS) (amino acids 29-38) and a putative DNA-binding domain (amino acids 58 to 80) (Supplementary Fig. 1). Because *MLP124478* is express during infection and appears to harbor specific features, making it unique within this family, we decided to investigate if it played a role *in planta* during pathogen growth.

To evaluate the biological consequences of Mlp124478's presence in plant cells, we used functional genomic assays as summarized in Fig. 2. We generated a stable transgenic *A. thaliana* line expressing the mature form of *Mlp124478* (i.e., without the signal peptide) fused to GFP under the control of a 35S promoter (pro35S::*Mlp124478-GFP*) in the Col-0 background (Fig. 2E; Supplementary Fig. 2). Interestingly, the transgenic lines manifested altered leaf morphology, characterized by waviness of leaf margins, while no curvature in the margins was evident in Col-0 plants (Fig. 3A). Anti-GFP immunoblotting for proteins extracted from Mlp124478-GFP and Col-0 lines revealed a band signal at the expected size of 37 kDa only in the transgenic line (Fig. 3B), indicating that constitutive *in planta* expression of Mlp124478-GFP fusion alters plant morphology.

To ascertain the subcellular localization of Mlp124478, we undertook confocal laser scanning microscopy of leaves from 4-day-old *A. thaliana* seedlings expressing *Mlp124478-GFP* fusion. We detected the GFP signal in the nucleolus, with a weaker signal in the nucleoplasm and cytosol of epithelial cells (Fig. 3C) similar to the localization observed in *N. benthamiana* by Petre et al. (2015b). In contrast, in control plants expressing free GFP, the fluorescent signal accumulated only in the nucleoplasm and cytosol, with none in the nucleolus (Fig. 3C). We conclude that Mlp124478-GFP specifically accumulates in the nucleolus of leaf cells, with weaker accumulation in the nucleoplasm and cytosol.

Mlp124478 carries a Nuclear Localization Signal (NLS) required for nucleolus translocation

Mlp124478 carries a predicted NLS consisting of 10 amino acids within the N-terminal part of the mature form ($Mlp124478_{29\text{-}38}$::RHKNGGGSRK) (Supplementary Fig. 1). To assess whether the predicted NLS was required for nuclear localization, we designed a GFP tagged construct lacking the predicted NLS, hereafter named $Mlp124478_{\Delta 29\text{-}38}$-GFP, and expressed it transiently in *N. benthamiana* leaf cells by agro-infiltration (Fig. 4A). Consistent with our *A. thaliana* observation, Mlp124478-GFP fusion accumulated in both the nucleus and nucleolus of *N. benthamiana* epithelial cells

(Fig. 4B). However, Mlp124478$_{\Delta 29\text{-}38}$-GFP accumulated solely in the nucleus, and its signal was mostly excluded from the nucleolus (Fig. 4B). To quantify the changes in subcellular distribution, we generated intensity plots of the fluorescent signals, which clearly showed decreased fluorescence in the nucleus between the two Mlp124478 constructs (Fig. 4C). Moreover, we noted average distribution ratios by comparing fluorescence intensities in the nucleus and nucleolus from confocal images acquired under identical settings (Supplementary Fig. 3). Mlp124478-GFP had a significantly higher nucleolar/nuclear ratio of 5.55 compared to a Mlp124478$_{\Delta 29\text{-}38}$ protein ratio of 0.8 (Fig. 4C). Taken together, these results suggest that the predicted nuclear localization signal acts as a nucleolar localization signal.

In planta Mlp124478 presence increases *H. arabidopsidis* growth

We conducted pathogen assays to test whether Mlp124478 alters pathogen virulence on *A. thaliana*. Since no rust fungi infects *A. thaliana*, we used the obligate biotrophic oomycete pathogen *H. arabidopsidis* as a proxy for filamentous pathogen. We inoculated transgenic *Mlp124478-GFP* and Col-0 (negative control) and *enhanced disease susceptibility 1-1 (eds1-1)* plants (positive controls hypersensitive to *H. arabidopsidis*). After 7 days, we quantified the number of spores and observed 10,000, 25,000 and 85,000, respectively, on average, for each genotype. We noted significantly increased susceptibility in *Mlp124478* transgenic plants compared to Col-0 (P<0.0001), although not as strong as that encountered in *eds1-1* plants (Fig. 5A). This finding demonstrates that *Mlp124478* can augment plant susceptibility to obligate biotrophic filamentous pathogens.

To investigate whether Mlp124478 could enhance susceptibility to bacterial pathogens, we infiltrated 4-week-old Col-0 leaves with *Pst*DC3000ΔCEL bacteria carrying *Mlp124478* or carrying an empty vector (Fig. 5B & 5C). In this system, the effector is expressed in bacteria and delivered *in planta* via the type three secretion system (T3SS). No significant difference was observed between bacterial strains carrying *Mlp124478* and the empty vector strain (Student's t-test, P-value 0.066)

(Fig. 5B). We also undertook infection assays in transgenic plants overexpressing *Mlp124478* and Col-0 with *Pst*DC3000ΔCEL. The effector was expressed *in planta* in this system. Bacterial growth was not significantly different between Mlp124478 and control plants (*P*-value 0.4368) (Fig. 5C). From this experiment set, we conclude that Mlp124478 enhances the growth of a filamentous pathogen such as *H. arabidopsidis* but not bacterial pathogen *P. syringae* in *A. thaliana*.

The expression of Mlp124478 plant cells alters *A. thaliana* transcriptome

To better understand how Mlp124478 functions in plant cells, and since alters plant morphology and susceptibility to pathogen and localizes to the nucleus and nucleolus, we investigated whether Mlp124478 can alter gene expression patterns in *A. thaliana* transgenics. We performed transcriptome profiling of the 4-days-old *A. thaliana* Mlp124478 stable transgenic line and Col-0 expressing GFP only. From a total of 108 million reads (80 M corresponding to control and 28 M to Mlp124478 plants), and after applying filters for modulated genes with fold-change greater than 2.0 and Q-values of $P \leq 0.05$, we obtained 98 and 294 up- and down-regulated genes, respectively (Fig. 6A, Supplementary Tables 2 & 3). To validate the expression level of deregulated genes from the transcriptome, real-time PCR (qRT-PCR) was performed for randomly selected 3 up-regulated (AT1G24580, AT2G15020, AT2G37770) and 7 down-regulated genes (AT2G47780, AT4G12480, AT2G35980, AT4G12470, AT2G43000, AT5G15120, AT5G02760). Real-time PCR showed similar expression tendency as observed in transcriptome, despite some quantitative differences in the level of expression (supplementary Fig. 4). Deregulated genes were considered for gene ontology (GO) term enrichment to assess their involvement in biological processes. GO revealed 7 functional groups (groups 0-6) of 15 GO terms significantly enriched (i.e. response to virus, response to bacterium, response to brassinosteroid, indole-containing compound biosynthetic process, cell wall organization, response to red or far red light signaling and negative regulation of ethylene-activated signaling pathway) (Fig. 6B). Comparing to down-regulated genes, only few GO terms corresponds to up-regulated genes. The up-regulated genes are three expansin genes involved in bacterial response,

CRK21 (Cysteine-rich receptor-like protein kinase 21), ESM1 (Epithiospecifier modifier 1) and LOX2 (transcriptional activator) involved in defense response. Only two other up-regulated genes were found enriched in response to ethylene and negative regulation of signal transduction (Supplementary Table 4, Fig. 6B). We noticed that out of 15 GO terms, only 7 GO terms were enriched among up-regulated genes, but all of the 15 GO terms of 7 functional groups enriched among down-regulated genes. Among the 294 down-regulated genes and out of the 42 genes of the "cell wall organization", 37 belong to the xyloglucan transglycolase XTH, XRT and EXT families (Eklöf and Brumer 2010). The defense-related transcription factors WRKY18, WRKY27, WRKY33, MYB51 (Pandey and Somssich 2009, Gigolashvili et al. 2007), the defense-related proteins NHL3 (Varet et al. 2003), RPP8 (Mohr et al. 2010), YLS9 (Yoshida et al. 2001), AZI1 (Atkinson, Lilley and Urwin 2013), CRK11 (Chen et al. 2004) and the jasmonate pathway and regulation genes JAZ1 (Demianski, Chung and Kunkel 2012), ASA1, ASB1 (Sun et al. 2009) were down-regulated in the Mlp124478-GFP transgenic lines compared to the GFP transgenic plants. Other genes involved in diverse mechanisms were down-regulated such as the chitinase CHI, the brassinosteroid-related genes BAS1 (Neff et al. 1999), BES1 (Jiang, Zhang and Wang 2015) PAR1 (Bou-Torrent et al. 2008), BEE1 (Friedrichsen et al. 2002), the salicylic acid-related genes NPR3 (Fu et al. 2012), the ethylene-related response genes ARGOS and ARGOS-like (ARL) (Hu, Xie and Chua 2003), EBF2 (Binder et al. 2007), ERF6 (Dubois et al. 2015), ETR2, RTE1 (Qiu et al. 2012), the carbon metabolism-related genes EXO (Schroder et al. 2009) and also the red/far red light signalization-related genes FAR1 (Lin and Wang 2004), GA2OX2 (Rieu et al. 2008), PAR1 (Bou-Torrent et al. 2008), PIF3 (Leivar et al. 2008), PKS4 (de Carbonnel et al. 2010), RR5. The changes in Mlp124478-GFP *A. thaliana* transgenic line transcriptomes occur mostly by a down-regulation of the expression of genes involved in diverse functions, frequently related to defense response regulation.

Mlp124478 is predicted to possess a DNA-binding domain. We inferred that it might interfere with transcription through direct interaction with DNA. Thus, we screened for Transcriptional Factor Binding Sites (TFBS) within the promoter sequences

of all up- and down-regulated genes. We identified four different TFBS which were very abundant among the up- (43 genes out of 98) and down-regulated genes (30 genes out of 294) (Supplementary Tables 5 and 6). TFBS abundant in the up-regulated gene set included ABF1 and TGA1a belongs to the basic region/leucine zipper motif (bZIP) transcription factor (TF) family; and TCP16 belongs to the TCP (TEOSINTE BRANCHED 1, CYCLOIDEA and PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR 1) TF family. The TFBS ATHB5 and TCP16 were abundant among the down-regulated genes, bound by homeodomains associated with the leucine zipper (HD-ZIP) and TCP TF families. We observed that some TFBS are abundant in up-regulated and down-regulated genes.

Next, we analyzed the gene expression profiles of up- and down-regulated genes during different biotic perturbations. We accessed Genevestigator (http://www.genevestigator.com) towards this end. Expression levels in 5 different biotic conditions (*Golovinomyces orontii*, *Phytophtora infestans*, *H. arabidopsidis*, *G. cichoracerum*, *Plectosphaerella cucumerina*) were retrieved for all up- and down-regulated genes with in the *Mlp124478*-expressing line (Fig. 7A & B). Most genes up-regulated in the *A. thaliana* transgenic line overexpressing *Mlp124478* were down-regulated in response to these pathogens. Only one gene (At3g51660) appeared up-regulated (maximum fold change of 2.4) in most conditions analyzed (Fig. 7A) and also up-regulated in the transgenic line expressing Mlp124478. The same conditions were imposed to analyze the expression pattern of down-regulated genes from our transcriptome (Fig. 7B). Of the 30 down-regulated genes, 8 were up-regulated in almost all conditions considered (At2g37130, At3g25600, At5g13190, At5g57910, At2g39200, At1g50740, At5g39610, At2g35980). We further analyzed the identity of these genes. At2g37130 encoded a peroxidase which was strongly up-regulated in response to fungal infection. At5g13190 encoded a plasma membrane protein regulating cell death. At2g39200 encoded *MILDEW RESISTANCE LOCUS O 12* (*AtMLO12*) whereas the product of the At2g35980 gene was very similar to *Arabidopsis NON-RACE-SPECIFIC DISEASE RESISTANCE 1* (*NDR1*), a central integrator of defense responses downstream of the coiled-coil-nucleotide-binding leucine-rich repeat (CC-NLR).

These results are analogous to the results observed using Cytoscape and further confirm that Mlp124478 rewires host transcription specifically to induce genes not normally expressed during defense against those five biotrophic pathogens while more importantly down-regulating genes normally up-regulated in response to such pathogens.

Mlp124478 binds DNA

The nuclear localization of Mlp124478, the presence of a DNA-binding motif and alterations at the transcriptional, morphological and defense levels prompted us to investigate whether Mlp124478 associates with DNA molecules. For this purpose, we performed a ChIP-PCR experiment. More precisely, we cross-linked proteins and DNA using formaldehyde, and then immunoprecipitated (IP) Mlp124478-GFP fusion with anti-GFP beads to pulldown DNA bound to GFP-tagged proteins from transgenic plants. We designed 32 primer pairs that could amplify the promoter regions most abundant among de-regulated genes containing either TCP16, ATHB5, TGA1a and ABF1. We also tested Col-0 genomic DNA as PCR-positive control and subjected Col-0 to the same ChIP procedure for negative control. Only 1 of the primer sets resulted in specific amplification, revealing interaction of Mlp124478 with the promoter of a HMG-box (high mobility group) DNA-binding family gene (AT2G34450) containing a TGA1a-binding site among the most strongly upregulated genes in Mlp124478-expressing plants. We did not observe any band in the IP with Col-0 DNA, which served as negative control, but a band was produced with *A. thaliana* genomic DNA as positive control (Fig. 8). AT2G34450 was up-regulated in the presence of Mlp124478 and showed down-regulation against biotrophic pathogens (Fig. 7A). We attempted EMSA with a synthetic peptide encompassing the DNA-binding domain of Mlp124478 and a double-stranded oligonucleotide displaying the consensus TGA1a sequence, but did not discern any interaction (Supplementary Fig. 5). Since poplars are *M. larici-populina's* natural host, and not *Arabidopsis*, we searched for the sequence immunoprecipitated in the ChIP experiment and presence in poplars. Hence, poplars contained a promoter with 57% similarity to the promoter of AT2G34450 (gene model POPTR_0004s13630.1).

Both gene models have similar exon-intron structures (6 exons and 5 introns), and their promoter regions contain TGA1a regulatory sequences (Supplementary Fig. 6), indicating that DNA interaction in *Arabidopsis* could occur in poplars.

Discussion

Recently, several groups reported on the use of heterologous systems to investigate the function, localization and interaction of effectors from biotrophic pathogens (Caillaud et al. 2012a, Caillaud et al. 2012b, Du et al. 2015, Gaouar et al. 2016, Petre et al. 2015a, Petre et al. 2016, Petre et al. 2015b, Kunjeti et al. 2016). It has also been shown that many effectors target the nucleus and, in some cases, alter transcription (Canonne and Rivas 2012, McLellan et al. 2013, Rennoll-Bankert et al. 2015). Here, we undertook functional genomics to study Mlp124478, a small secreted peptide from the poplar leaf rust pathogen *M. larici-populina*. We conducted *in planta* pathogen assays, live-cell imaging, comparative transcriptomics, and protein-nucleic acid interaction to assess Mlp124478 function.

Our pathogenicity assays – with either a bacterial delivery system in which Mlp124478 was translated in *P. syringae* and translocated via the T3SS to the host cell, or when Mlp124478 was constitutively expression *in planta* – did not reveal significant bacterial growth alteration. However, when *Arabidopsis* was exposed to a filamentous pathogen that possesses a lifestyle similar to rusts, we observed more susceptibility to pathogen growth, which indicates that this effector may target an immunity component specifically affected by pathogens with filamentous lifestyles. Although it was previously reported that independently-evolved effectors, arising in different kingdoms, can converge onto molecular hubs (Mukhtar et al. 2011), our results support kingdom effector specificity directed towards pathogen lifestyle. Effector screening of 16 effectors in our laboratory suggests some degree of kingdom specificity in the effector repertoire (Germain et al. In revision).

Expression of candidate effectors in *N. benthamiana* has been the method of choice to assess localization, since it represents a fast and robust system for studying protein subcellular localization and the effect of ectopic expression (Caillaud et al. 2012a, Du et al. 2015, Gaouar et al. 2016, Lim et al. 2015, Petre et al. 2016, Petre et al. 2015b, Wang et al. 2016). Since the default GFP distribution in plant cells is nucleo-cytoplasmic, the localization of a GFP-tagged effector displaying nucleo-cytoplasmic distribution is considered non-informative. However, in the case of Mlp124478, the localization in nucleoli indicates that GFP is not masking the Mlp124478 localization sequence, thus localization is driven by the effector sequence. The nucleolus has long been recognized as a hallmark of virus infection (Salvetti and Greco 2014, Hiscox 2002, Hiscox 2007), essentially to recruit nucleolar proteins and facilitate virus replication (Hiscox 2007). While viral lifestyle easily explains the need to target the nucleolus, the reasons why a rust effector would do so is not as intuitive. Given that little is known in plants about what could link the nucleolus and the biological processes of plant defenses it would be highly speculative at this point to suggest a reason why we observed Mlp124478 accumulation in the nucleolus. Interestingly, however, the amino acid sequence, predicted to act as a nuclear localization sequence, in fact served as a nucleolar localization sequence. Thus, Mlp124478 localization in the nucleus and nucleolus and the putative DNA-binding domain in the protein sequence prompted us to further investigate its role *in planta*.

Our stable transgenic line overexpressing the effector was subjected to pathogenicity assays, comparative transcriptomics and phenotype analysis, insightful approaches taken more easily than adopting *N. benthamiana* as a heterologous expression system. Stable transgenic plants could provide clues with regard to putative interacting proteins, if a well-described phenotype is copied. In our case, although plants overexpressing Mlp124478 clearly displayed wavy leaf margins, it did not enable us to speculate about a putative interacting protein because of the plethora of mutants displaying similar phenotypes (Abe et al. 2010, Graciet et al. 2009, Koyama et al. 2010, Reed 2001). Recent studies on quantitative measurements of phenotypes of leaf margins in *Arabidopsis* showed that leaf waviness is associated with oscillating normal curvature

along the leaf margins provided by either as an outcome of induction level of gene misexpression or as an action of time (Armon et al. 2014). However, the clear leaf morphology phenotype of Mlp124478-expressing plants confirmed that Mlp124478 expression *in planta* affected plant development and prompted us to investigate the effect of Mlp124478 expression on the plant transcriptome.

The first step in transcriptome analysis, after sorting genes through fold-induction/repression, is usually to assess whether transcript levels relative to a specific biological function or process are altered under certain conditions. As for the transgenic lines of *A. thaliana* expressing Mlp124478, most of the changes occur in down-regulation of defense-response associated genes. Interestingly, the genes that were found enriched down-regulated in our study corresponds to GO terms that were also reported recently (Hacquard et al. 2016a). Indeed, Hacquard and collaborator recently shown a transcriptomic analysis of the *A. thaliana* responses during colonization of two species of *Colletotrichum tofieldiae* (symbiont) and *C. incanum* (parasite). This study highlighted eight similar GO terms as in our case, except that genes are activated during the colonization of *C. incanum* and down regulated in Mlp124478 transgenic lines. Few deregulated genes are similar under the same GO terms as illustrated in the supplementary Table 7. Hence, the expression of this single effector (Mlp124478) appears to bear broad transcriptional impact as it appears to counter the normal defense output described by Hacquard (2016) using a very similar analysis.

We analyzed the promoter regions of de-regulated genes in transgenic plants, we observed that TGA1a, ABF1, TCP16 and ATHB5 regulatory sequences were very abundant in the promoters of deregulated genes. Moreover, we observed that the list of up-regulated genes did not specifically contain genes involved in plant defense, but rather genes involved in defense were among the most repressed. The presence of a DNA-binding domain in Mlp124478 and the fact that we could confirm Mlp124478 interaction with DNA in a sequence-specific manner suggest that it may alter gene expression to deceive plant immune systems. We named this effector *SHAM TO DECEIVE*. The fact that our EMSA –with synthetic peptide encompassing the DNA-

binding domain – showed no *in vitro* binding with an oligonucleotide representing the consensus TGA1a sequence indicates that a longer peptide may be needed to adopt proper DNA-binding conformation, or DNA interaction may require host factors or co-effectors. Recently, effectors that bind DNA have started to emerge. CgEP1, a *Colletotrichum graminicola* effector with DNA-binding properties has been shown to enhance anthracnose development in maize (Vargas et al. 2016). Like Mlp124478, the oomycete effector PsCRN108 exhibits a DNA-binding domain, localizes to the nucleus and it binds with the HhH promoter motif to downregulate the expression of defense-related genes (Song et al. 2015).

Taken together, our results indicate that SHAM TO DECEIVE manipulates plants by targeting DNA, remodeling transcription likely via direct DNA-binding, to suppress normal transcriptional responses to pathogens, and mislead the host into up-regulating the expression of genes unrelated to defense. While our current results are consistent with SHAM TO DECEIVE in the nucleus, we cannot rule out that it could also have a distinct function in the nucleolus.

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Legend of figures

Fig. 1. Sequence alignment and phylogenetic tree.

(A) Multiple sequence alignment of 9 members of CPG2811 showing sequence similarity. Signal peptide is marked by a box. Identical/highly conserved residues (*); semi conserved residues (:) and designate conserved residues (.). NLS is indicated by solid black underline. (B) Phylogenetic tree of 9 effectors members of the CPG2811 family obtained with COBALT using Kimura distance value and neighbor joining tree method.

Fig. 2. Overview of functional studies of Mlp124478.

(A) Mlp124478 effector was mined from the set of candidate effectors of *M. larici-populina.* (B) Mature form of Mlp124478 (*Mlp124478*ΔSP) was cloned using Gateway Cloning Technology. (C) *Mlp124478*ΔSP was further recombined into pVSPPsSpdes vector for *Pst* infection assay (effector delivery) and pB7FWG2.0 for subcellular localization. *Mlp124478* recombined into pB7FWG2.0 was then transformed into *A. tumefaciens* strain C58C1 to develop stable transgenic *A. thaliana* plants expressing *Mlp124478* and transient expression in *N. benthamiana.*

Fig. 3. Mlp124478 accumulates in the nucleus and the nucleolus of *A. thaliana* cells and affects the shape of leaves.

(A) Morphology of 4-weeks old soil grown Col-0 and stable transgenic *Mlp124478* plant grown at 22°C under 14 h/10 h photoperiod in growth chamber. (B) Immunodetection of GFP protein in Col-0 and stable transgenic seedlings from 12 days old plantlets grown on ½ Murashige Skoog agar plates. (C) Live cell imaging using confocal microscope with sequential scanning of epidermal cells of 4-days old stable transgenic *Mlp124478-GFP* plantlets. GFP in the Col-0 background was used as control. Fluorescence in the green channel (left panel), DIC (middle panel) and merge of all channels (right panel) of the same region are shown. Nucleoli are pointed with white arrow heads.

Fig. 4. Mlp124478 carries a Nuclear Localization Signal (NLS) required for nucleolus translocation.

(A) Schematic representation of the constructs (Mlp124478 and Mlp124478$\Delta_{29\text{-}38}$) used for transient expression. For both constructs GFP is tagged to the C-terminus. (B) Subcellular accumulation of Mlp124478-GFP and Mlp124478$\Delta_{29\text{-}38}$-GFP in *N. benthamiana* epidermal cells at 4-days post infiltration, the nucleus was stained by DAPI staining dye and epidermal cells were observed under the blue channel (left panel), green channel (middle panel) and merge of all channels (right panel). Arrowheads point the nucleolus. A solid yellow line is transected in the merge channel over the nuclear and nucleolar region to draw the intensity plot in C. (C) Nuclear-nucleolar distribution of the fluorescent fusion proteins according to the fluorescent intensity ratios: nucleolar intensity (I_{No}) divided by nuclear intensity (I_N). Average fluorescence intensity ratios (± SD) were determined from the fluorescent intensities on the nucleus and nucleolus in confocal images with the Leica LAS X software.

Fig. 5. *In planta* Mlp124478 presence increases *H. arabidopsidis* growth.

(A) Four weeks old soil grown Col-0, stable transgenic *Mlp124478* and *eds1-1* plants were spray inoculated with *Hyaloperonospora arabidopsidis* Noco2 (50,000 conidiospores/mL) and number of conidiospores were quantified at 7 days after inoculation. Statistical significance was evaluated using student's *t* test. Data were represented with the experiments repeated three times with similar results. (B) Quantification of *Pst*DC3000ΔCEL growth in Col-0 and stable transgenic *A. thaliana* expressing *Mlp124478*. Growth of bacteria was measured on days 0 and 3 (cfu: colony forming unit/mL inoculum). Statistical significance was evaluated using student's *t* test. Data were represented with the experiments repeated three times with similar results. (C) Quantification of growth of *Pst*DC3000ΔCEL carrying or not *Mlp124478* in Col-0. Four weeks old plants were syringe infiltrated with bacteria at OD_{600}=0.001. Growth of bacteria was measured on days 0 and 3 (cfu: colony forming unit/mL inoculum). Statistical significance was evaluated using student's *t* test. Data were represented with the experiments repeated three times with similar results.

Fig. 6. The expression of Mlp124478 plant cells alters *A. thaliana* transcriptome.

(A) Schematic illustration of transcriptomics work flow. RNA was isolated from 4-days old *A. thaliana* Mlp124478 stable transgenic and Col-0 plants and sequenced using ion torrent. Transcripts were analyzed using iPlantCollaborative DNA subway and deregulated genes were considered for further analysis. (B) Go term enrichment was performed with deregulated genes filtered with Q-value ≤ 0.05 and fold-change ≥ 2 using the Cytoscape software (version 3.1.1). Cytoscape was performed with the plug-in ClueGO and CluePedia to visualize functions enriched in the deregulated genes. The GO terms presented are significantly enriched in up-regulated and down-regulated genes with FDR≤ 0.05 (Benjamini-Hochberg p-value correction) and revealed 15 GO terms belongs to 7 functional groups.

Fig. 7. Regulation of gene expression level.

Heat map of biotrophic pathogens response of genes in two groups: (A) upregulated genes and (B) down regulated genes. Genevestigator was used for differential expression analysis and heat map was created on Microsoft Excel.

Fig. 8. TFBSs determining DNA-binding ability.

Two weeks old plants tissues of Col-0, stable transgenic *Mlp124478* and *A. thaliana* genomic DNA were used for chromatin preparation using ChIP assay with antibody against GFP as described in the material and methods section. TGA1a associated site was PCR amplified with TGA1a specific primer pair. Expected bands (211 bp) was obtained from transgenic and *Arabidopsis* genomic DNA for TGA1a at the promoter region of AT2G34450 gene. AT4G08870 and AT3G63160 showed band of 248 and 229 bp with all three types of DNA (Mlp124478, Col-0 and genomic DNA); whereas AT2G39250 showed amplification with only genomic DNA, and AT2G47750 showed amplification with Col-0 and genomic DNA. Col-0 DNA: negative control; *A. thaliana* genomic DNA: positive control.

Supplementary Fig. 1. Mlp124478 represents SP, NLS and DNA-binding domain.

The cartoon represents signal peptide (SP) and nuclear localization sequences (NLS) within the peptide sequence of Mlp124478. Both SP and NLS reside to the N-terminus region and stretch of NLS amino acids are very close to the SP.

Supplementary Fig. 2. Schematic representation of Mlp124478 construct.

Open reading frame (ORF) of Mlp124478 was cloned at the N-terminus of eGFP in pB7FWG2.0 vector using Gateway cloning technology. 35S promoter and basta resistance gene (*bar*) shown.

Supplementary Fig. 3. Intensity plot of Mlp124478-GFP and Mlp124478$\Delta_{29\text{-}38}$-GFP.

Intensity plot represents intensities strongly differ between nucleus (N) and nucleolus (No). The corresponding fluorescent intensity profiles of the merge images of fig. 3B. The overlapping signals are shown in different colors. Intensity of BF, GFP and DAPI represented by gray, green and blue lines, respectively. BF: bright field; GFP: Green Fluorescent Protein; DAPI: 4',6-diamidino-2-phenylindole; Nu; nucleolar and N: nuclear.

Supplementary Fig. 4. Binding activity of TGA1a and DNA-binding domain of Mlp124478 by EMSA.

EMSA was carried out with DNA-binding domain specific synthetic peptide and digoxigenin labelled TGA1a probe. 10-fold excesses of oligonucleotides were added as competitors including wild type (TGA1a) and mutated (Mut-1 and Mut-2).

Supplementary Fig. 5. Real-time PCR confirmation of the selected differentially expressed genes deregulated from transcriptome of *A. thaliana* expressing Mlp124478.

Relative transcript levels (log of fold change) of selected deregulated genes determined using real-time PCR, shown by black bar. Actin was used as the reference control. The fold change of deregulated genes from transcriptome data is shown by gray bars. The minus value means the gene is down-regulated; while the positive value means the up-regulation of genes.

Supplementary Fig. 6. Complete exon and intron structure with TFBSs at the upstream of gene.

A. thaliana gene AT2G34450 showing the generalized structure of exons and introns with TGA1a at the upstream of transcription start site (TSS) (upper one). Lower image represents Ptp.5659.1S1_at, the homolog of AT2G34450 in poplar represents similar number of exons and introns.

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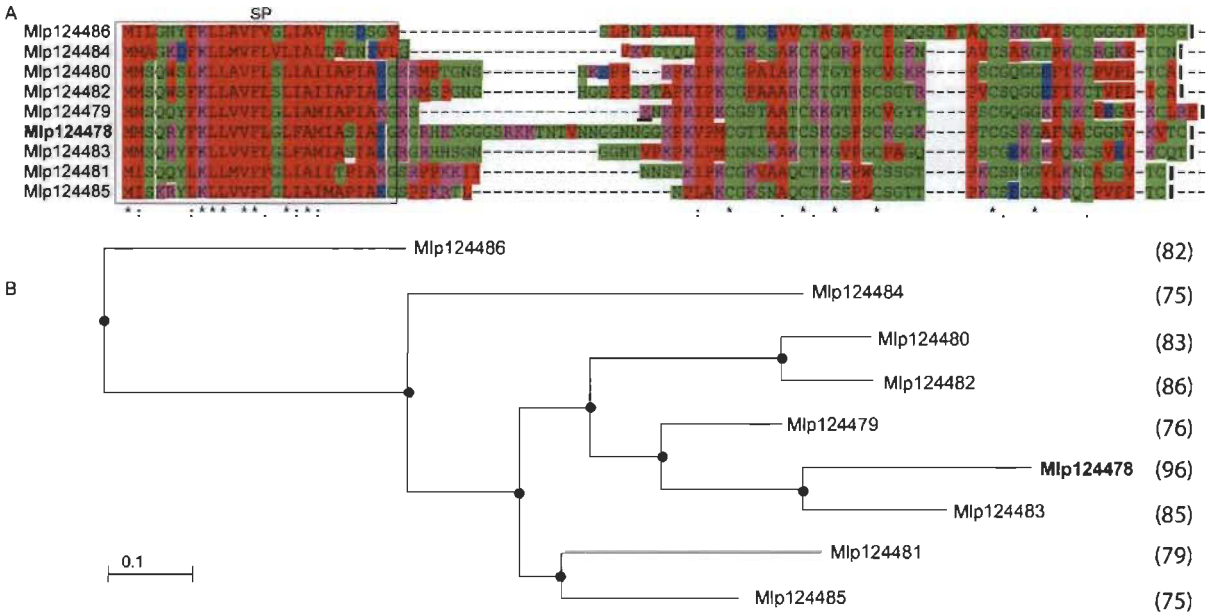
Fig. 1.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Mlp124479
Mlp124478
Mlp124483
Mlp124481
Mlp124485
B
Mlp124486
Mlp124484
Mlp124480
Mlp124482
Mlp124479
Mlp124478
Mlp124483
Mlp124481
Mlp124485
(82)
(75)
(83)
(86)
(76)
(96)
(85)
(79)
(75)
0.1

Fig. 2.

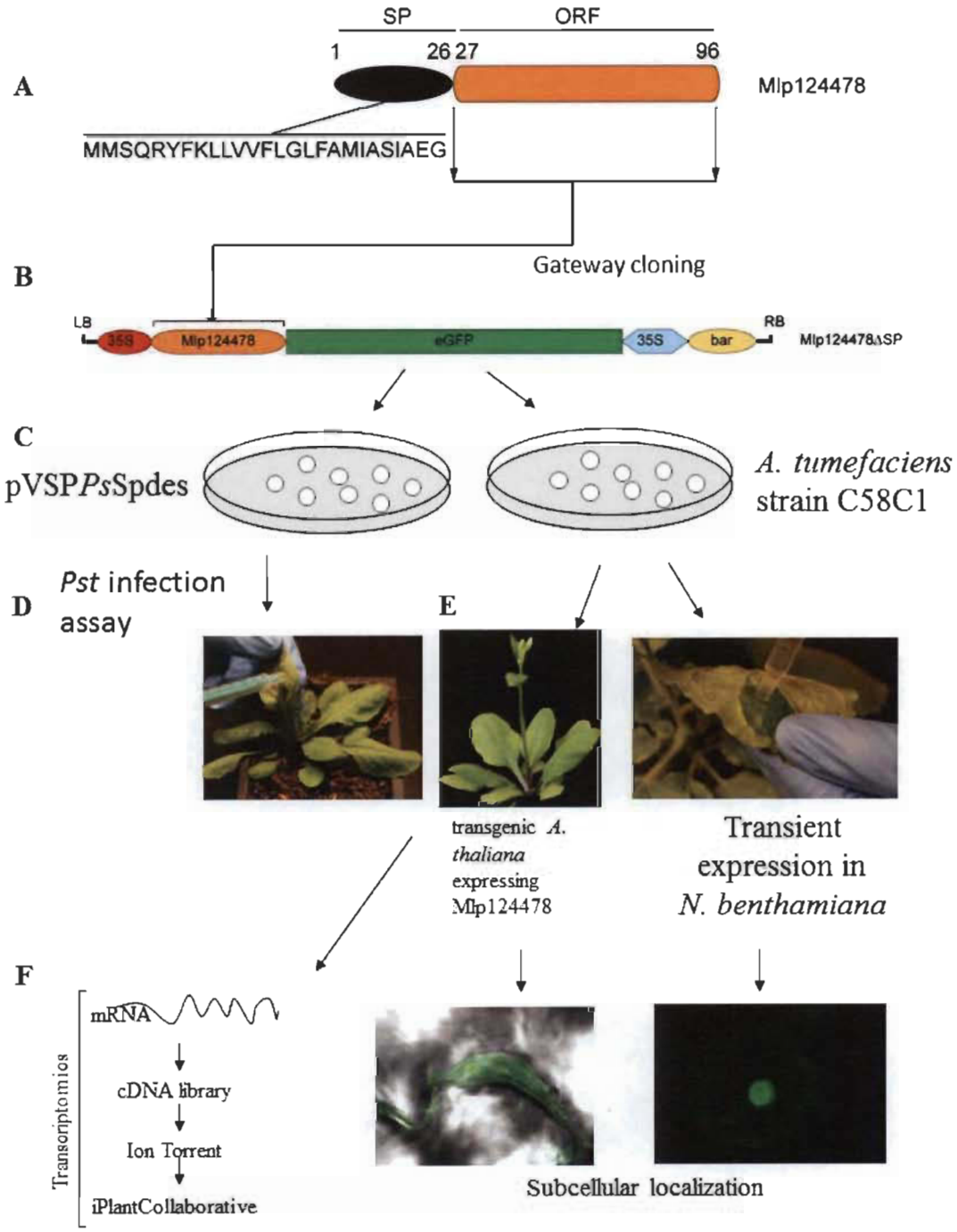

A
SP
ORF
1
26 27
96
Mlp124478
MMSQRYFKLLVVFLGLFAMIASIAEG
B
Gateway cloning
LB
35S
Mlp124478
eGFP
35S
bar
RB
Mlp124478ΔSP
C
pVSPPsSpdes
A. tumefaciens
strain C58C1
D
Pst infection
assay
E
transgenic A.
thaliana
expressing
Mlp124478
Transient
expression in
N. benthamiana
F
Transcriptomics
mRNA
cDNA library
Ion Torrent
iPlantCollaborative
Subcellular localization

Fig. 3.

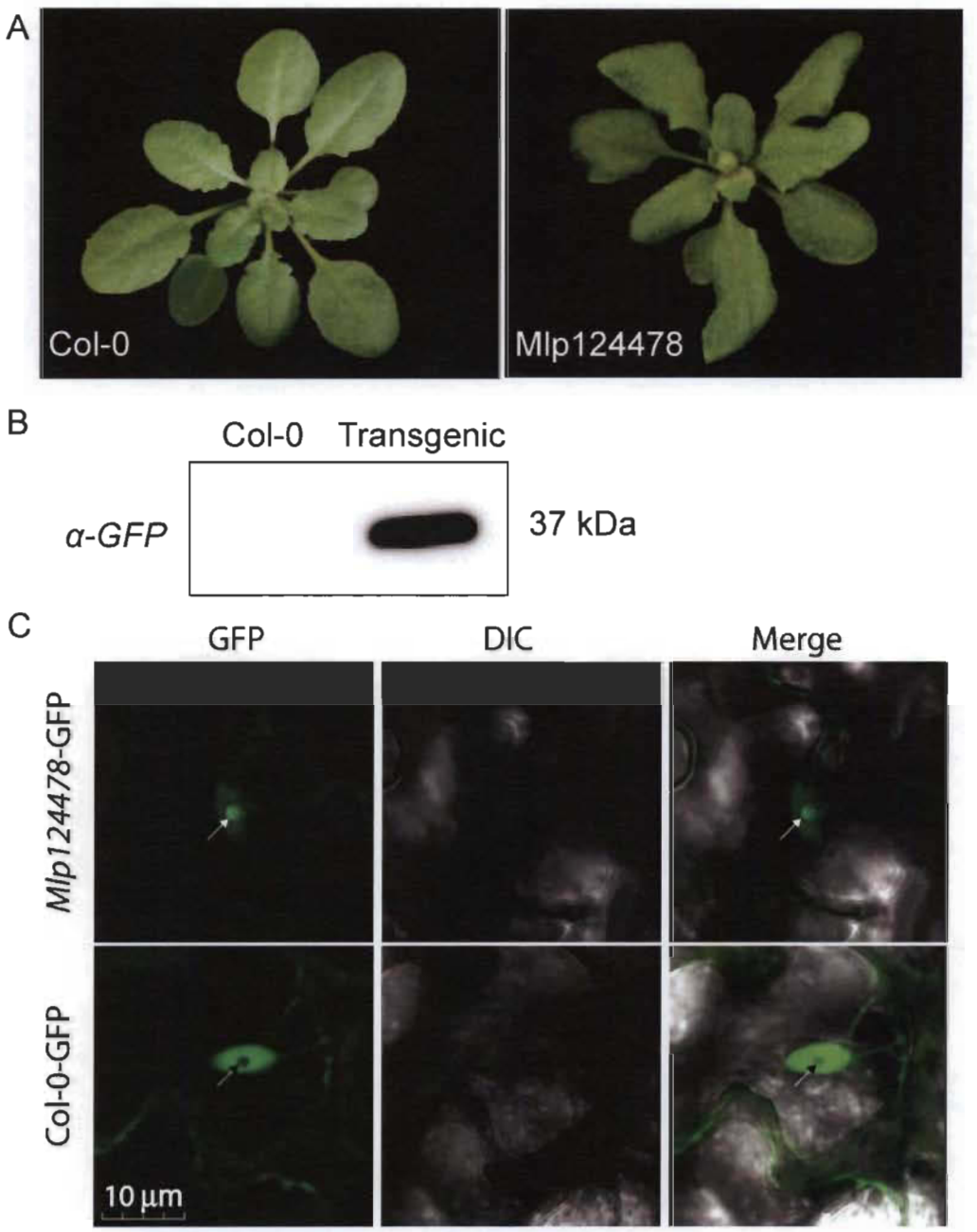

A
Col-0
Mlp124478
B
Col-0
Transgenic
α-GFP
37 kDa
C
GFP
DIC
Merge
Mlp124478-GFP
Col-0-GFP
10 µm

Fig. 4.

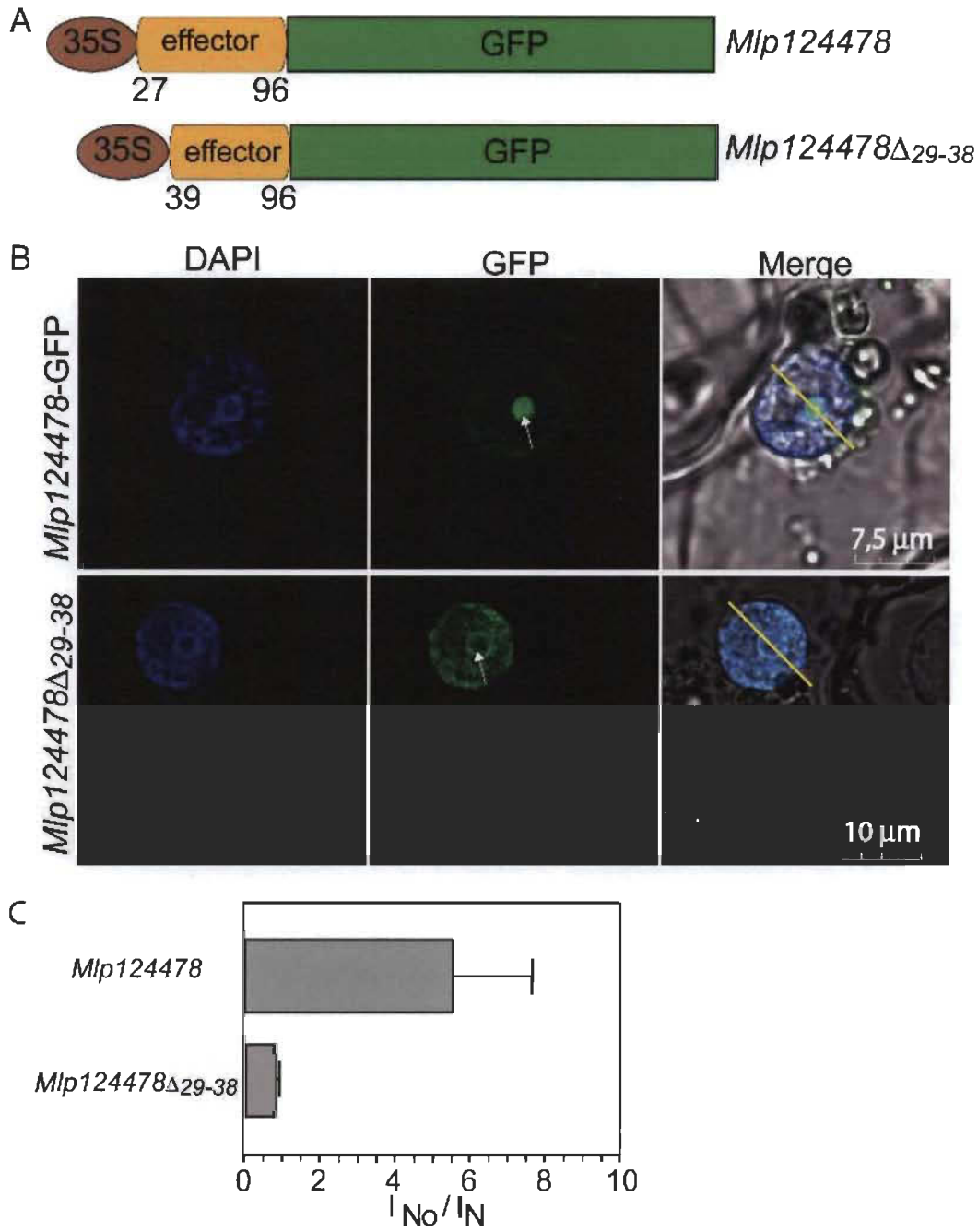

A
35S
effector
GFP
Mlp124478
27
96
35S
effector
GFP
Mlp124478Δ29-38
39
96
B
DAPI
GFP
Merge
Mlp124478-GFP
Mlp124478Δ29-38
7,5 µm
10 µm
C
Mlp124478
Mlp124478Δ29-38
0
2
4
6
8
10
INo / IN

Fig. 5.

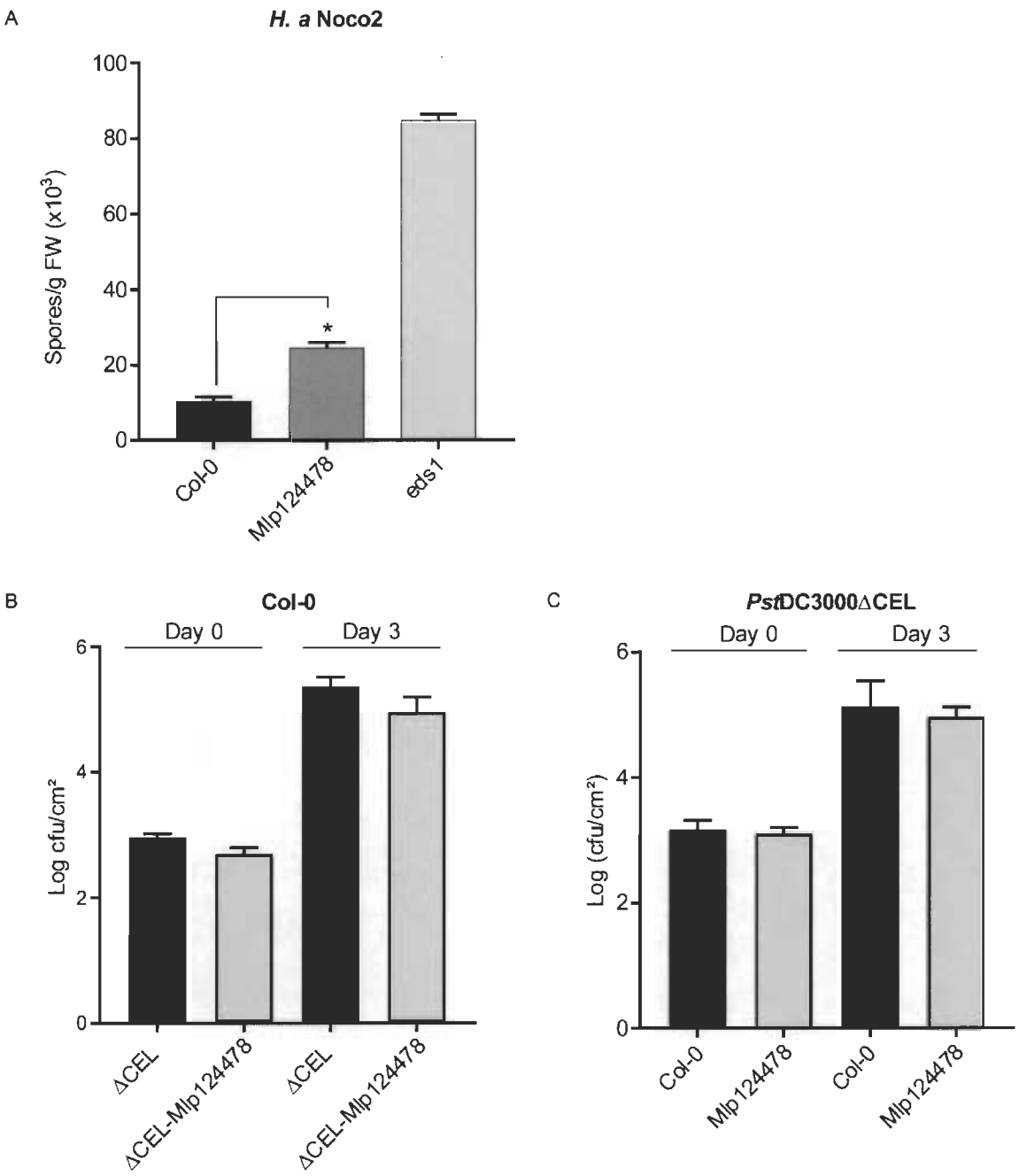

A
H. a Noco2
Spores/g FW (x10³)
100
80
60
40
20
0
*
Col-0
Mlp124478
eds1
B
Col-0
Day 0
Day 3
Log cfu/cm²
6
4
2
0
ΔCEL
ΔCEL-Mlp124478
ΔCEL
ΔCEL-Mlp124478
C
PstDC3000ΔCEL
Day 0
Day 3
Log (cfu/cm²)
6
4
2
0
Col-0
Mlp124478
Col-0
Mlp124478

Fig. 6.

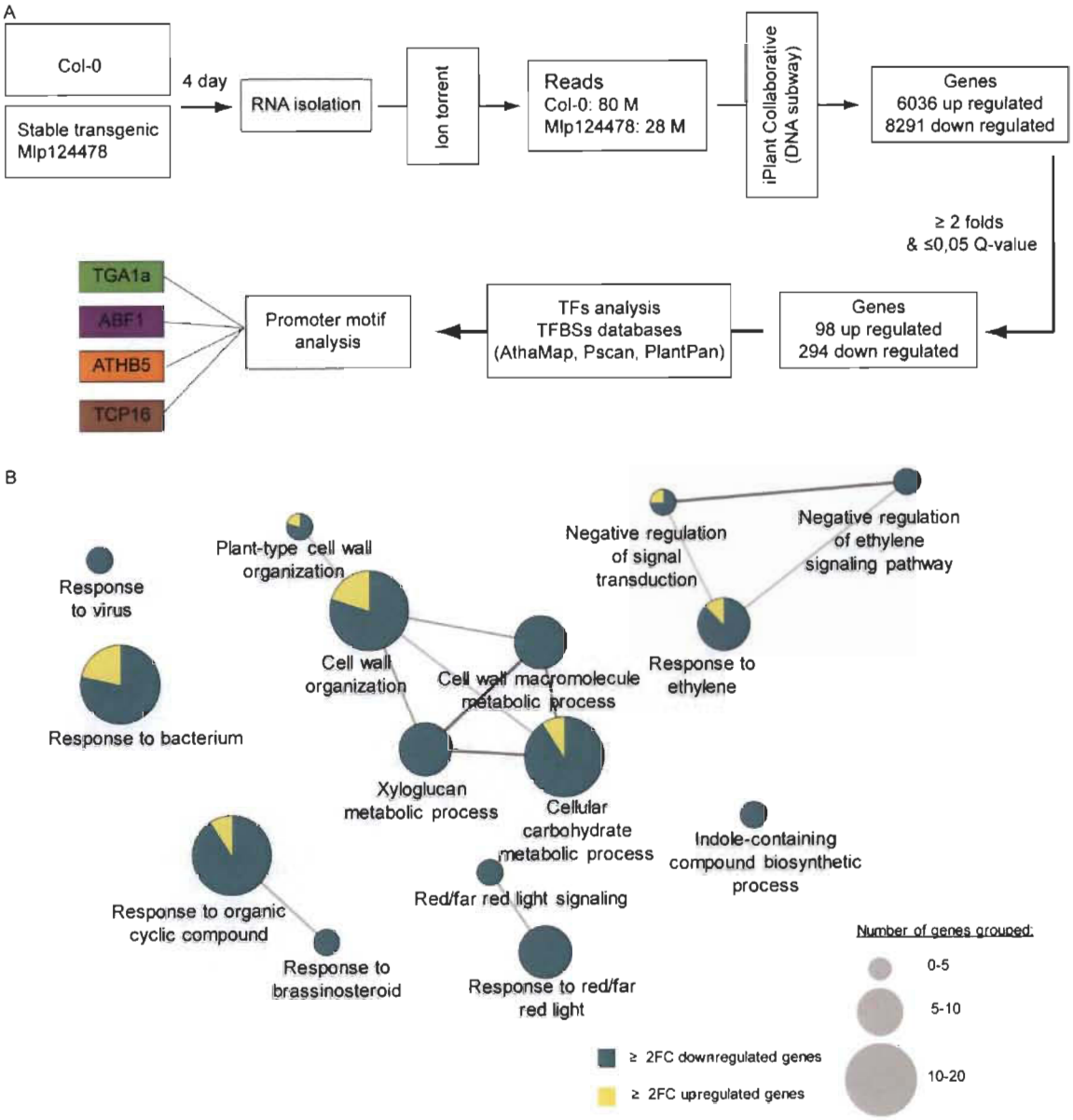

A
Col-0
Stable transgenic Mlp124478
4 day
RNA isolation
Ion torrent
Reads
Col-0: 80 M
Mlp124478: 28 M
iPlant Collaborative (DNA subway)
Genes
6036 up regulated
8291 down regulated
≥ 2 folds
& ≤0,05 Q-value
Genes
98 up regulated
294 down regulated
TFs analysis
TFBSs databases
(AthaMap, Pscan, PlantPan)
Promoter motif analysis
TGA1a
ABF1
ATHB5
TCP16
B
Response to virus
Response to bacterium
Plant-type cell wall organization
Cell wall organization
Cell wall macromolecule metabolic process
Xyloglucan metabolic process
Cellular carbohydrate metabolic process
Negative regulation of signal transduction
Negative regulation of ethylene signaling pathway
Response to ethylene
Indole-containing compound biosynthetic process
Response to organic cyclic compound
Response to brassinosteroid
Red/far red light signaling
Response to red/far red light
Number of genes grouped:
0-5
5-10
10-20
≥ 2FC downregulated genes
≥ 2FC upregulated genes

Fig. 7.

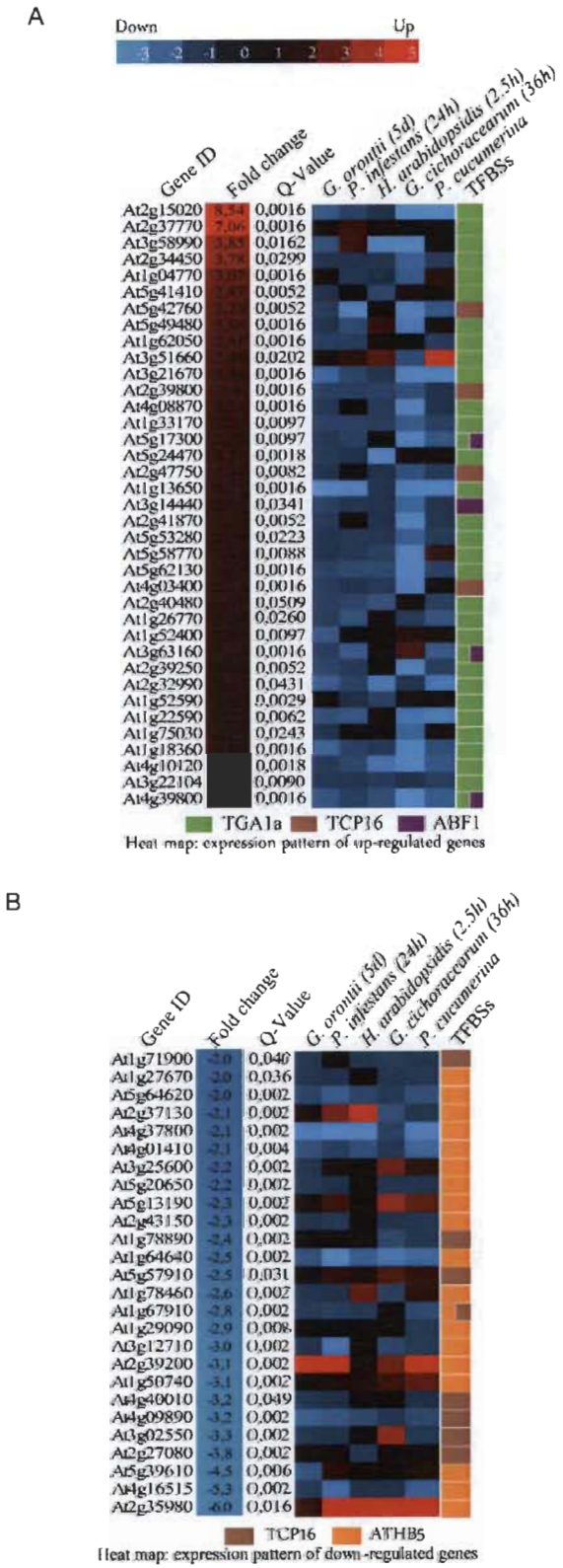
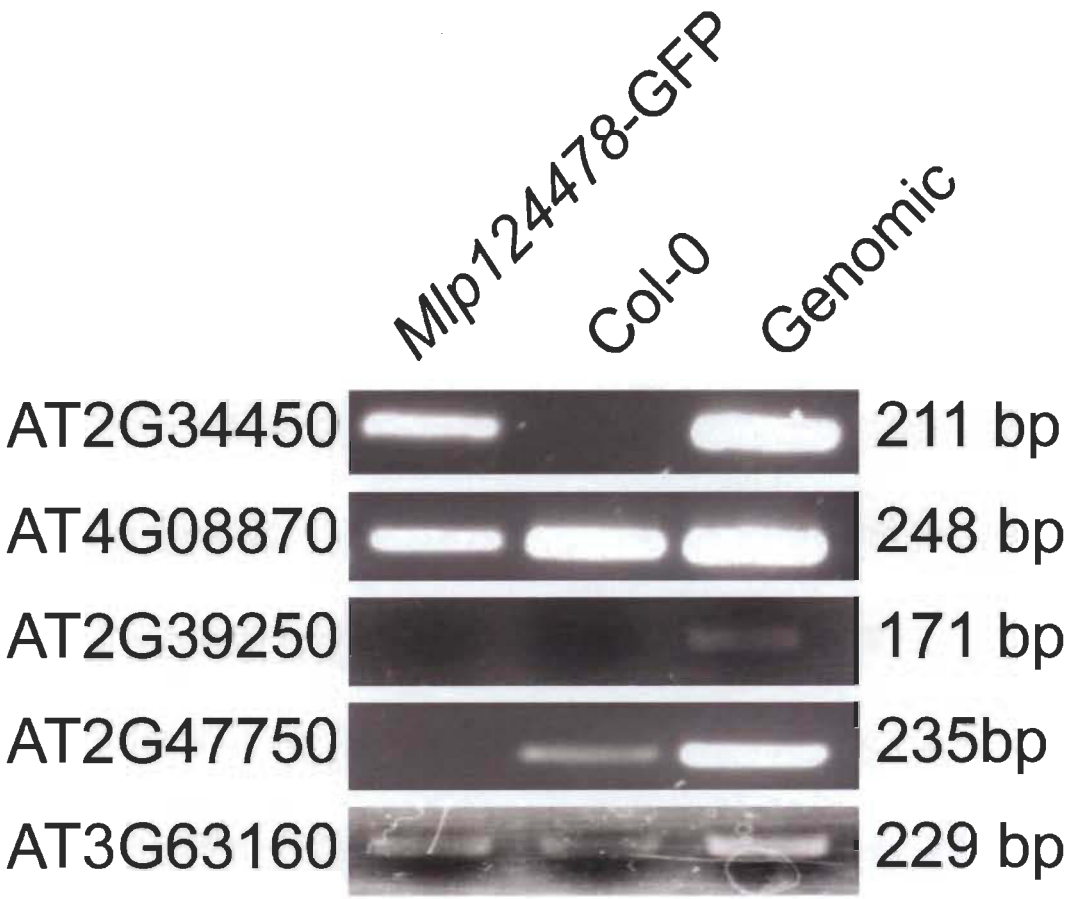
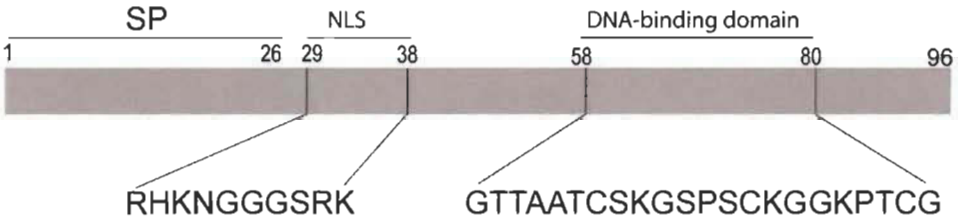

A
Down
Up
-3 -2 -1 0 1 2 3 4 5
Gene ID
Fold change
Q-Value
G. orontii (5d)
P. infestans (24h)
H. arabidopsidis (2.5h)
G. cichoracearum (36h)
P. cucumerina
TFBSs
At2g15020 8,54 0,0016
At2g37770 7,06 0,0016
At3g58990 3,83 0,0162
At2g34450 3,78 0,0299
At1g04770 3,55 0,0016
At5g41410 3,47 0,0052
At5g42760 3,2 0,0052
At5g49480 3,1 0,0016
At1g62050 3 0,0016
At3g51660 2,9 0,0202
At3g21670 2,8 0,0016
At2g39800 2,8 0,0016
At4g08870 2,8 0,0016
At1g33170 2,8 0,0097
At5g17300 2,8 0,0097
At5g24470 2,8 0,0018
At2g47750 2,8 0,0082
At1g13650 2,8 0,0016
At3g14440 2,8 0,0341
At2g41870 2,8 0,0052
At5g53280 2,8 0,0223
At5g58770 2,8 0,0088
At5g62130 2,8 0,0016
At4g03400 2,8 0,0016
At2g40480 2,8 0,0509
At1g26770 2,8 0,0260
At1g52400 2,8 0,0097
At3g63160 2,8 0,0016
At2g39250 2,8 0,0052
At2g32990 2,8 0,0431
At1g52590 2,8 0,0029
At1g22590 2,8 0,0062
At1g75030 2,8 0,0243
At1g18360 2,8 0,0016
At4g10120 2,8 0,0018
At3g22104 2,8 0,0090
At4g39800 2,8 0,0016
TGA1a
TCP16
ABF1
Heat map: expression pattern of up-regulated genes
B
Gene ID
Fold change
Q-Value
G. orontii (5d)
P. infestans (24h)
H. arabidopsidis (2.5h)
G. cichoracearum (36h)
P. cucumerina
TFBSs
At1g71900 -2,0 0,040
At1g27670 -2,0 0,036
At5g64620 -2,0 0,002
At2g37130 -2,1 0,002
At4g37800 -2,1 0,002
At4g01410 -2,1 0,004
At3g25600 -2,2 0,002
At5g20650 -2,2 0,002
At5g13190 -2,3 0,002
At2g43150 -2,3 0,002
At1g78890 -2,4 0,002
At1g64640 -2,5 0,002
At5g57910 -2,5 0,031
At1g78460 -2,6 0,002
At1g67910 -2,8 0,002
At1g29090 -2,9 0,008
At3g12710 -3,0 0,002
At2g39200 -3,1 0,002
At1g50740 -3,1 0,002
At4g40010 -3,2 0,049
At4g09890 -3,2 0,002
At3g02550 -3,3 0,002
At2g27080 -3,8 0,002
At5g39610 -4,5 0,006
At4g16515 -5,3 0,002
At2g35980 -6,0 0,016
TCP16
ATHB5
Heat map: expression pattern of down-regulated genes

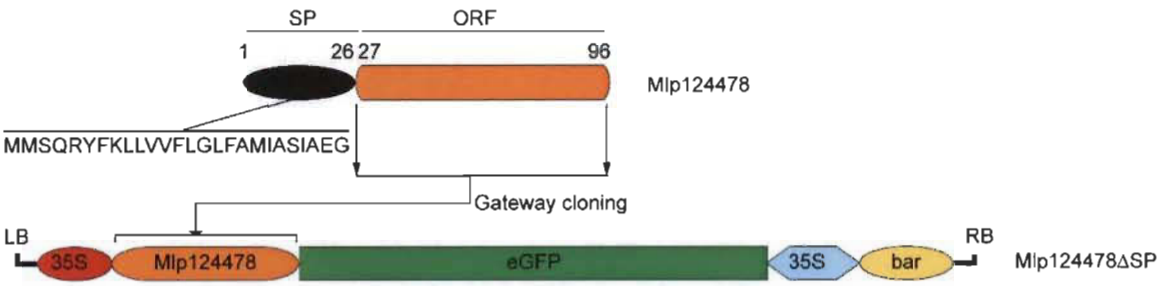
Fig. 8.


Mlp124478-GFP
Col-0
Genomic
AT2G34450
211 bp
AT4G08870
248 bp
AT2G39250
171 bp
AT2G47750
235bp
AT3G63160
229 bp

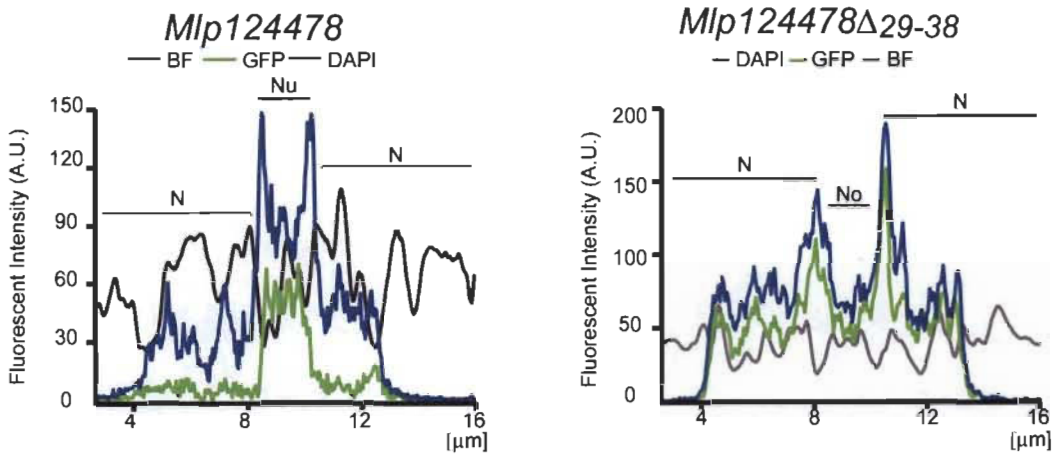
Supplementary Fig. 1.



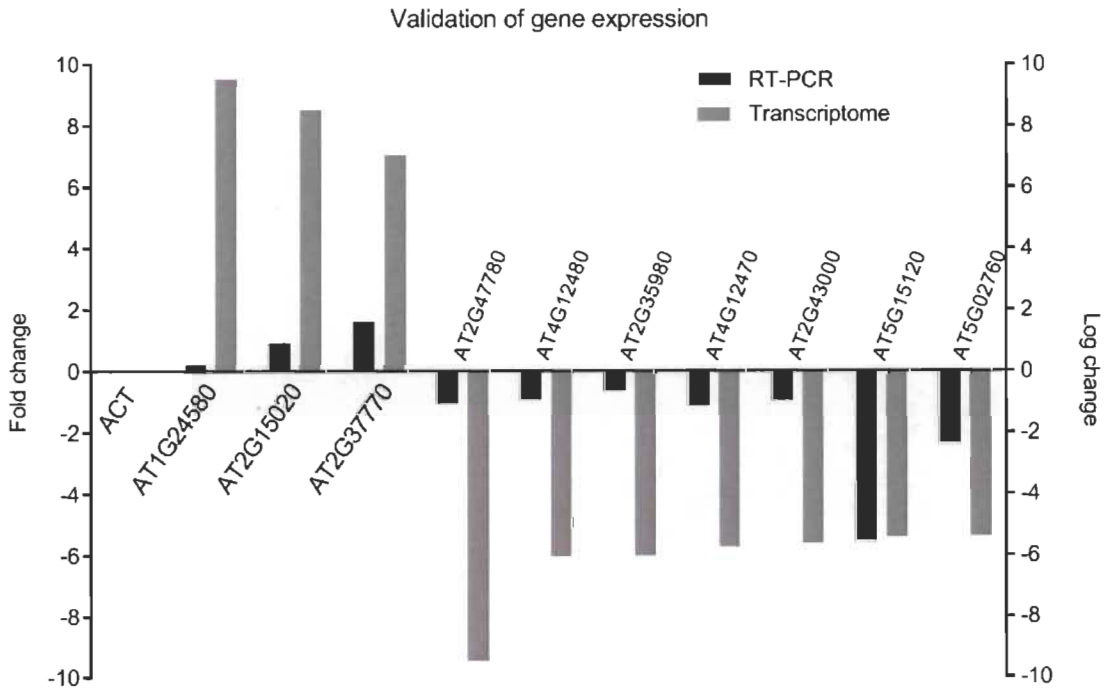
Supplementary Fig. 2.



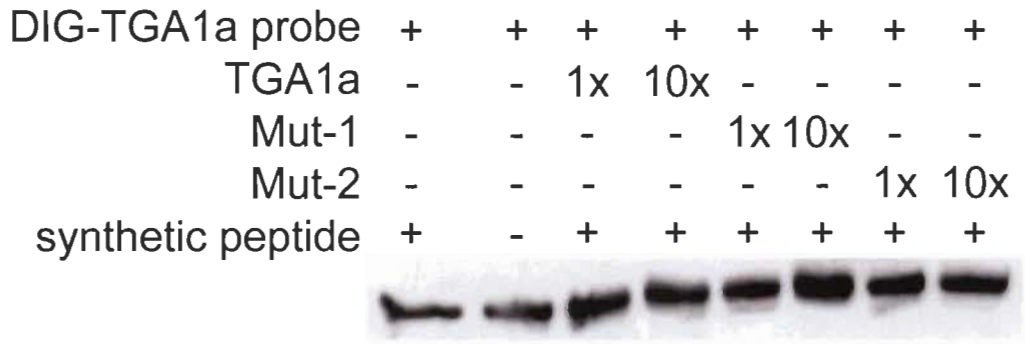
Supplementary Fig. 3.



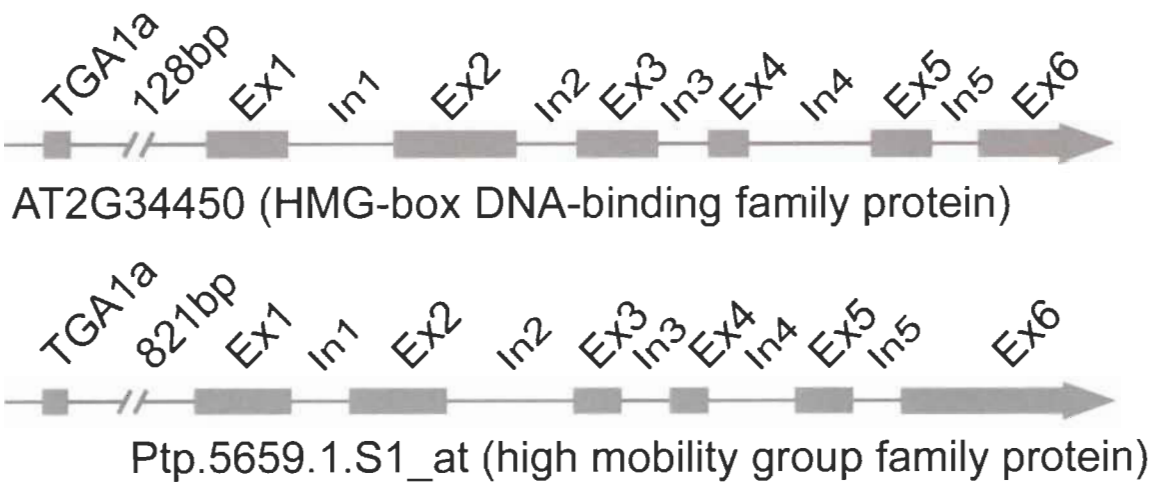
Supplementary Fig. 4.



Supplementary Fig. 5.



Supplementary Fig. 6.



Supplementary Table 1. List of primers.

No	Name	Sequence			Tm
1	GW-GFPc-Mlp124478-ΔSP_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAGGTCGACACAAAAATGGGGGT			60.6
2	GW-GFPc-Mlp124478$\Delta_{29\text{-}38}$_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGAGGACAGCTATGACAAACACCGTAAATAACGG			58.7
3	GW-GFPc-Mlp124478+$\Delta_{29\text{-}38}$_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCACATGTAACTTTCACGTTCCCCC			61.9
No	**Name**	**Left primer sequence**	**Tm**	**Right primer sequence**	**Tm**
4	Ch_U_ABF1.1	accgtgaaacgttgagtcttc	59,25	agactcatctcccacaatttga	58.65
5	Ch_U_ABF1.2	tttgcgtcttatcttccttgg	59,35	aaaaacagcgatacatttcaa	55,16
6	Ch_U_ABF1.3	ccttgaaatcatcggcattt	59,9	tttggcgcgtgagatagtta	59,47
7	Ch_U_ABF1.4	ggatggtggtgaagaaaagg	59,38	aggaggcgtaagacgtgaaa	59,88
8	Ch_U_TGA1a.1	cacgcataggcttctgtgat	58,9	cactagaccaatcagaaagagga	57,62
9	Ch_U_TGA1a.2	tggttgggaaagatttgaga	58,12	gcccatttttctttgtcagtg	58,2
10	Ch_U_TGA1a.3	tcataaaacaccctctcacaca	58,13	ggagacttttgctttttcca	57,07
11	Ch_U_TGA1a.4	gatggaagtaaaggagcgaaaa	59,74	agattgtcggagacctttgc	59,29
12	Ch_U_TGA1a.5	tctcccctccacatacaaaa	57,99	ttggtagaccaaatcgggta	57,94
13	Ch_U_TGA1a.6	acgtcgtcgtttgattgatt	58,08	cgtgatgacgtggtgaatta	57,97

No	Name	Left primer sequence	Tm	Right primer sequence	Tm
14	Ch_U_TGA1a.7	atggcttgttcacagcaaaa	59,32	aattagacgcatccctggttt	59,85
15	Ch_U_TGA1a.8	tgatcacagccattttgatg	58,04	ctcatgaatcttcacgacgta	56,37
16	Ch_U_TGA1a.9	attcatttgaccgctgcac	59,67	aatggtgaagctttttaggc	56,18
17	Ch_U_TGA1a.10	caacgaaagagtccacgttt	57,86	gaagcaggaaacgaaacaga	58,08
18	Ch_U_TGA1a.11	agcacaaactccacctttga	58,34	accacaaaaacttgggcata	58
19	Ch_U_TGA1a.12	cccgagttggtaccagtgta	58,5	tgttgggaccatgaatttct	57,84
20	Ch_U_TGA1a.13	acgtacgtaccatccttttga	58,56	ccgcatataaaatgcctcac	58,14
21	Ch_U_TGA1a.14	aaattaggagacgtggacga	57,27	tttctttcctccacagatgg	57,71
22	Ch_U_TCP16.1	ttggctaaaatctgggagtg	57,82	tagcgaaacaagagccaaac	58,17
23	Ch_U_TCP16.2	aaaagaatccacacccaaca	57,89	aagcaatcacaaccatccat	57,86
24	Ch_U_TCP16.3	cagggaaagcagttgaaaga	58,08	gggaattaccgtccacaaat	58,62
25	Ch_U_TCP16.4	cgccttgtttggtaagaaaa	57,95	ccaaagtttcatgacgatcc	57,99
26	Ch_D_ATHB5.1	cgcttgttgcatgatgttagta	58,91	agatctcatgatttcgtcaaa	54,85
27	Ch_D_ATHB5.	cttacctctctcctctttgga	58,98	gcaattgtaggcttgtgatgtt	59,16
28	Ch_D_ATHB5.3	cggataaggccctttagaga	59,7	tgacccacctttgcttctt	59,71
29	Ch_D_ATHB5.4	gcttcctacccaatttccaa	59,02	cgcgtttttggagaagaagt	59,49
30	Ch_D_ATHB5.5	cgcttgtttgttaccgtcaa	59,77	cacacttaatgggccttttgt	59,01
31	Ch_D_ATHB5.6	gagcaacgaagctcctctctat	59,29	ttaaacgcgaaacctcatgt	58,29
32	Ch_D_ATHB5.7	acgtactccccaaaataagca	58,64	ccatgaccagtcaaggcata	59,52

No	Name	Left primer sequence	Tm	Right primer sequence	Tm
33	Ch_D_ATHB5.8	aattgtcttgccaactgaacc	59,09	tcgtagatccgaattggtaatg	58,97
34	Ch_D_ATHB5.9	ctttttaggccaccaccaaa	59,97	caagcgataaaccgaggtaaa	59,26
35	Ch_D_ATHB5.10	atacttgcaccgcccatatt	59,32	aatggaataccgccgtgtag	59,85
36	Ch_D_ATHB5.11	agtgcaagcacaagggaact	59,91	ccggttggaaccttgaatta	59,79
37	Ch_D_TCP16.1	ccggaaaccataaaaccaga	59,79	aatcgccacgtaagtcatcc	59,96
38	Ch_D_TCP16.2	aaaaccttgcaccagtttcc	59,08	cttgtgttccattgtcgtctg	59,2

Supplementary Table 2. List of up-regulated genes.

Gene	Fold Change	Description
AT1G24580	9.53	RING/U-box domain-containing protein
AT2G15020	8.54	Uncharacterized protein
AT2G37770	7.06	NAD(P)-linked oxidoreductase-like protein
AT2G19650	4.62	Cysteine/histidine-rich C1 domain-containing protein
AT5G24660	4.41	Response to low sulfur 2
AT3G47420	4.28	Phosphate starvation-induced protein
AT4G12320	4.26	Cytochrome P450
AT3G58990	3.85	Isopropylmalate isomerase 1
AT2G34450	3.78	High mobility group (HMG1/2) domain-containing protein
AT4G14020	3.49	Rapid alkalinization factor (RALF) family protein
AT2G27420	3.28	Cysteine proteinase-like protein
AT3G45140	3.12	Lipoxygenase 2
AT3G52740	3.08	Uncharacterized protein
AT1G04770	3.07	Male sterility MS5 family protein
AT5G13530	3.03	E3 ubiquitin-protein ligase KEG
AT1G18773	3.02	Uncharacterized protein
AT4G15490	2.98	UDP-glycosyltransferase-like protein
AT4G23290	2.91	Cysteine-rich receptor-like protein kinase 21
AT5G41410	2.87	Homeobox protein BEL1-like protein
AT5G46690	2.87	Transcription factor bHLH71
AT3G54600	2.78	Class I glutamine amidotransferase domain-containing protein
AT5G42760	2.73	Leucine carboxyl methyltransferase
AT5G14760	2.69	L-aspartate oxidase
AT5G49480	2.68	Ca2+-binding protein 1
AT3G14210	2.68	Epithiospecifier modifier 1

Gene	Fold Change	Description
AT4G34950	2.64	Major facilitator family protein
AT5G39710	2.62	Pentatricopeptide repeat-containing protein
AT1G62050	2.61	Ankyrin repeat-containing protein
AT4G23290	2.59	Cysteine-rich receptor-like protein kinase 22
AT2G46810	2.56	Transcription factor bHLH70
AT5G23040	2.50	CELL GROWTH DEFECT FACTOR 1
AT4G16880	2.48	Leucine-rich repeat (LRR) family protein
AT3G51660	2.48	Macrophage migration inhibitory factor family protein
AT3G04140	2.47	Ankyrin repeat-containing protein
AT3G21670	2.44	Major facilitator protein
AT1G29640	2.43	Uncharacterized protein
AT1G06180	2.41	Myb proto-oncogene protein
AT2G39800	2.40	Gamma-glutamyl phosphate reductase
AT1G18810	2.40	Protein phytochrome kinase substrate 3
AT4G08870	2.34	Putative arginase
AT4G09770	2.32	TRAF-like family protein
AT4G39510	2.31	Cytochrome P450
AT1G57610	2.31	Uncharacterized protein
AT1G33170	2.30	Putative methyltransferase PMT18
AT5G54130	2.28	Calcium-binding endonuclease/exonuclease/phosphatase family protein
AT5G17300	2.27	Myb family transcription factor
AT5G24470	2.27	Two-component response regulator-like APRR5
AT1G10920	2.26	*NB-ARC domain-containing disease resistance protein*
AT1G60590	2.26	Pectin lyase-like protein
AT2G47750	2.24	Putative indole-3-acetic acid-amido synthetase GH3,9
AT2G43920	2.24	Putative thiol methyltransferase 1

Gene	Fold Change	Description
AT3G48310	2.24	Cytochrome P450 71A22
AT3G14200	2.23	Chloroplast import apparatus 2 protein
AT1G13650	2.23	Uncharacterized protein
AT3G48320	2.23	Cytochrome P450 71A21
AT4G12830	2.22	Hydrolase
AT1G65190	2.22	Protein kinase domain-containing protein
AT4G38620	2.21	Transcription repressor MYB4
AT1G65900	2.21	Uncharacterized protein
AT3G14440	2.19	9-cis-epoxycarotenoid dioxygenase NCED3
AT3G05830	2.18	Uncharacterized protein
AT4G31870	2.17	Glutathione peroxidase 7
AT4G26860	2.17	Putative pyridoxal phosphate-dependent enzyme
AT5G17550	2.16	Peroxin 19-2
AT5G53280	2.16	Plastid division protein 1
AT5G62130	2.13	Per1-like family protein
AT5G13170	2.13	Senescence-associated protein 29
AT5G58770	2.13	Dehydrodolichyl diphosphate synthase 2
AT4G03400	2.12	Auxin-responsive GH3 family protein
AT1G62630	2.12	CC-NBS-LRR class disease resistance protein
AT2G01290	2.12	Ribose 5-phosphate isomerase A
AT5G50950	2.11	Fumarate hydratase 2
AT2G40480	2.11	Uncharacterized protein
AT5G25120	2.10	Cytochrome P450 71B11
AT5G16980	2.09	2-alkenal reductase
AT1G26770	2.09	Expansin A10
AT1G52400	2.09	Beta glucosidase 18
AT3G26310	2.09	Cytochrome P450 71B35

Gene	Fold Change	Description
AT1G74640	2.09	Putative alpha/beta-hydrolase-like protein
AT2G39250	2.08	AP2-like ethylene-responsive transcription factor SNZ
AT4G28250	2.08	Expansin B3
AT3G63160	2.08	Uncharacterized protein
AT3G11090	2.08	LOB domain-containing protein 21
AT5G25130	2.07	Cytochrome P450 71B12
AT2G32990	2.07	Endoglucanase 11
AT1G52590	2.06	Putative thiol-disulfide oxidoreductase DCC
AT1G75900	2.05	GDSL esterase/lipase EXL3
AT1G22590	2.05	Protein AGAMOUS-like 87
AT1G75030	2.05	Thaumatin-like protein 3
AT1G18360	2.04	Alpha/beta-hydrolase domain-containing protein
AT4G10120	2.04	Sucrose-phosphate synthase
AT3G22104	2.03	Phototropic-responsive NPH3 family protein
AT1G64500	2.02	Glutaredoxin-like protein
AT4G39800	2.02	Inositol-3-phosphate synthase isozyme 1
AT1G02010	2.01	Protein transport sec1a
AT2G41870	2.01	Remorin-like protein
AT1G73870	2.01	Zinc finger protein CONSTANS-LIKE 7
AT3G09440	2.00	Protein heat shock protein 70-3

Supplementary Table 3. List of down-regulated genes.

Gene	Fold Change	Description
AT5G25250	-38.92	Flotillin-like protein 1
AT4G22470	-16.61	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT3G49620	-13.50	2-oxoglutarate-Fe(II)-dependent oxygenase domain-containing protein
AT3G59900	-12.02	ARGOS protein
AT2G47780	-9.41	Rubber elongation factor protein (REF)
AT1G12290	-8.68	CC-NBS-LRR class disease resistance protein
AT3G54590	-6.04	Hdroxyproline-rich glycoprotein
AT4G12480	-5.97	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT2G35980	-5.95	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT2G36690	-5.88	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein
AT4G12470	-5.68	Azelaic acid induced 1
AT2G43000	-5.55	NAC domain-containing protein 42
AT4G25200	-5.51	Small heat shock protein 23,6
AT5G67060	-5.48	Transcription factor HEC1
AT5G15120	-5.35	Uncharacterized protein
AT5G02760	-5.33	Putative protein phosphatase 2C 67
AT4G16515	-5.31	Uncharacterized protein
AT3G62680	-5.00	Proline-rich protein 3
AT4G16515	-5.00	Uncharacterized protein
AT5G53250	-4.98	Arabinogalactan protein 22
AT1G18400	-4.86	Transcription factor BEE 1
AT3G54580	-4.74	Proline-rich extensin-like family protein

Gene	Fold Change	Description
AT5G02540	-4.69	Rossmann-fold NAD(P)-binding domain-containing protein
AT1G10550	-4.56	Xyloglucan:xyloglucosyl transferase
AT3G29370	-4.55	Uncharacterized protein
AT5G39610	-4.50	NAC domain containing protein 6
AT5G25440	-4.39	Protein kinase family protein
AT5G57240	-4.38	OSBP(oxysterol binding protein)-related protein 4C
AT2G20835	-4.29	Uncharacterized protein
AT3G23150	-4.23	Putative ethylene receptor
AT4G02270	-4.12	Protein root hair specific 13
AT2G32190	-4.11	Uncharacterized protein
AT1G27020	-4.06	Uncharacterized protein
AT4G30280	-4.05	Xyloglucan:xyloglucosyl transferase
AT1G06350	-4.01	Delta-9 desaturase-like 4 protein
AT1G05135	-4.00	Pseudogene
AT2G22860	-3.88	Phytosulfokine-beta
AT5G14920	-3.82	Gibberellin-regulated protein
AT3G20395	-3.81	RING-finger domain-containing protein
AT2G38530	-3.77	Non-specific lipid-transfer protein 2
AT2G27080	-3.75	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT5G22500	-3.72	Fatty acyl-CoA reductase 1
AT4G24275	-3.68	Uncharacterized protein
AT4G15090	-3.54	Protein FAR-RED IMPAIRED RESPONSE 1
AT1G69490	-3.52	NAC domain-containing protein 29
AT5G05500	-3.51	Pollen_Ole_e_I-domain containing protein
AT1G14120	-3.47	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein
AT2G41990	-3.46	Uncharacterized protein

Gene	Fold Change	Description
AT5G13860	-3.44	Protein ELC-like protein
AT3G05150	-3.44	Sugar transporter ERD6-like 8
AT4G09030	-3.43	Arabinogalactan protein 10
AT5G65390	-3.42	Arabinogalactan protein 7
AT5G43270	-3.40	Squamosa promoter-binding-like protein 2
AT4G13400	-3.35	Dioxygenase domain-containing protein
AT3G02550	-3.32	LOB domain-containing protein 41
AT5G60460	-3.31	Protein transport protein SEC61 subunit beta
AT5G26622	-3.28	Beta-galactosidase related protein
AT2G44080	-3.24	ARGOS-like protein
AT5G03150	-3.24	Zinc finger protein JACKDAW
AT3G18200	-3.23	EamA domain-containing protein
AT5G40780	-3.18	Lysine histidine transporter 1
AT5G15230	-3.17	Gibberellin-regulated protein 4
AT4G09890	-3.17	Uncharacterized protein
AT4G40010	-3.15	Serine/threonine-protein kinase SRK2F
AT3G46280	-3.14	Protein kinase-like protein
AT1G29465	-3.12	Uncharacterized protein
AT5G04960	-3.11	Putative pectinesterase/pectinesterase inhibitor 46
AT4G08040	-3.10	1-aminocyclopropane-1-carboxylate synthase 11
AT1G50740	-3.10	Transmembrane proteins 14C
AT2G39200	-3.06	MLO-like protein 12
AT1G73330	-3.05	Drought-repressed 4 protein
AT3G12710	-3.02	DNA-3-methyladenine glycosylase I
AT1G32920	-3.00	Uncharacterized protein
AT1G18570	-2.99	Myb domain protein 51
AT1G11210	-2.98	Uncharacterized protein

Gene	Fold Change	Description
AT2G19620	-2.94	N-MYC downregulated-like 3 protein
AT1G60060	-2.93	Serine/threonine-protein kinase WNK (With No lysine)-related protein
AT1G29090	-2.92	Cysteine proteinase-like protein
AT5G03860	-2.92	Malate synthase
AT5G22310	-2.90	Uncharacterized protein
AT5G64410	-2.86	oligopeptide transporter 4
AT2G15292	-2.86	Unknown gene
AT1G17620	-2.83	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family
AT1G73830	-2.83	Transcription factor BEE 3
AT5G24210	-2.81	Lipase class 3 family protein
AT3G19680	-2.81	Uncharacterized protein
AT2G46330	-2.78	Arabinogalactan protein 16
AT3G11550	-2.78	Uncharacterized protein
AT1G67910	-2.76	Uncharacterized protein
AT1G19350	-2.74	Protein brassinazole-resistant 2
AT1G19180	-2.74	Protein TIFY 10A
AT5G67520	-2.73	Adenylylsulfate kinase
AT5G37660	-2.73	Cysteine-rich repeat secretory protein 60
AT2G34930	-2.71	Disease resistance-like protein/LRR domain-containing protein
AT3G15540	-2.70	Auxin-responsive protein IAA19
AT2G42870	-2.70	Phy rapidly regulated 1
AT2G41100	-2.68	Calmodulin-like protein 12
AT4G14560	-2.67	Auxin-responsive protein IAA1
AT4G01950	-2.66	Glycerol-3-phosphate acyltransferase
AT4G29140	-2.63	Delta-9 acyl-lipid desaturase 1

Gene	Fold Change	Description
AT1G76930	-2.62	Extensin 4
AT1G78460	-2.62	SOUL heme-binding protein
AT5G25930	-2.61	Protein kinase family protein with leucine-rich repeat domain
AT4G19810	-2.60	Glycosyl hydrolase family protein with chitinase insertion domain
AT5G52830	-2.60	WRKY DNA-binding protein 27
AT1G63860	-2.60	TIR-NBS-LRR class disease resistance protein
AT5G64310	-2.59	Arabinogalactan protein 1
AT2G23810	-2.59	Tetraspanin8
AT1G19610	-2.58	Defensin-like protein 19
AT5G06930	-2.57	Uncharacterized protein
AT5G05380	-2.56	PRA1 family protein B3
AT5G25350	-2.55	EIN3-binding F-box protein 2
AT4G38400	-2.55	Expansin-like A2
AT4G17260	-2.55	L-lactate dehydrogenase
AT1G57990	-2.55	Purine permease 18
AT1G65310	-2.55	Xyloglucan:xyloglucosyl transferase
AT3G46700	-2.54	Glucuronosyl transferase-like protein
AT2G27260	-2.54	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT5G06860	-2.52	Polygalacturonase inhibitor 1
AT1G17020	-2.52	Protein SRG1
AT1G21310	-2.51	Extensin 3
AT2G17880	-2.50	DNAJ heat shock N-terminal domain-containing protein
AT5G62920	-2.50	Two-component response regulator ARR6
AT2G25735	-2.50	Uncharacterized protein
AT5G57910	-2.49	Uncharacterized protein
AT1G50040	-2.49	Uncharacterized protein

Gene	Fold Change	Description
AT1G30750	-2.49	Uncharacterized protein
AT3G45730	-2.48	Uncharacterized protein
AT4G25810	-2.48	Xyloglucan:xyloglucosyl transferase
AT2G41010	-2.47	Calmodulin binding protein 25
AT1G30040	-2.47	Gibberellin 2-beta-dioxygenase 2
AT1G64640	-2.46	Early nodulin-like protein 8
AT5G13890	-2.45	Uncharacterized protein
AT4G31800	-2.45	WRKY transcription factor 18
AT2G26070	-2.45	Uncharacterized protein
AT1G19540	-2.44	NmrA-like negative transcriptional regulator-like protein
AT4G36500	-2.44	Uncharacterized protein
AT3G45970	-2.43	Expansin-like A1
AT1G03870	-2.43	Fasciclin-like arabinogalactan protein 9
AT4G35840	-2.43	NEP1-interacting protein 1
AT5G04190	-2.43	Protein PHYTOCHROME KINASE SUBSTRATE 4
AT1G78890	-2.43	Uncharacterized protein
AT3G05890	-2.42	Hydrophobic protein RCI2B
AT4G12720	-2.42	Nudix hydrolase 7
AT3G11550	-2.42	Uncharacterized protein
AT4G08950	-2.41	Phosphate-responsive 1 family protein
AT5G13890	-2.40	Uncharacterized protein
AT3G27220	-2.39	Kelch repeat-containing protein
AT1G74340	-2.38	Dolichyl-phosphate mannosyltransferase polypeptide 2
AT3G04290	-2.37	GDSL esterase/lipase LTL1
AT2G26710	-2.37	PHYB activation tagged suppressor 1 protein
AT1G48320	-2.37	Thioesterase-like protein
AT4G17500	-2.36	Ethylene-responsive transcription factor 1A

Gene	Fold Change	Description
AT3G63380	-2.36	Ca2+-transporting ATPase
AT4G23190	-2.35	Cysteine-rich receptor-like protein kinase 11
AT1G25220	-2.35	Anthranilate synthase beta subunit 1
AT2G38470	-2.34	Putative WRKY transcription factor 33
AT5G25940	-2.34	Early nodulin-related protein
AT4G32460	-2.33	Uncharacterized protein
AT2G43290	-2.32	Calmodulin-like protein 5
AT1G25230	-2.32	Purple acid phosphatase 4
AT5G08150	-2.32	Suppressor of phytochrome b 5
AT2G38870	-2.32	Serine protease inhibitor
AT1G14870	-2.31	Cadmium resistance protein 2
AT4G33050	-2.31	Calmodulin-binding protein
AT2G43150	-2.30	Proline-rich extensin-like family protein
AT3G13435	-2.30	Uncharacterized protein
AT5G15830	-2.30	Basic leucine-zipper 3
AT3G26760	-2.30	Rossmann-fold NAD(P)-binding domain-containing protein
AT2G47930	-2.29	Arabinogalactan protein 26
AT1G52190	-2.29	Putative peptide transporter
AT1G65845	-2.29	Uncharacterized protein
AT4G02800	-2.29	Uncharacterized protein
AT5G44260	-2.28	Zinc finger CCCH domain-containing protein 61
AT5G05730	-2.27	Anthranilate synthase component I-1
AT2G44500	-2.27	Axi 1 protein-like protein
AT4G22300	-2.27	Carboxylesterase
AT4G27280	-2.27	EF-hand
AT1G48930	-2.27	Endoglucanase 5
AT4G18760	-2.27	Receptor like protein 51

Gene	Fold Change	Description
AT1G71970	-2.27	Uncharacterized protein
AT2G05510	-2.27	Glycine-rich protein
AT4G34250	-2.26	3-ketoacyl-CoA synthase 16
AT2G14890	-2.25	Arabinogalactan protein 9
AT4G37450	-2.25	Lysine-rich arabinogalactan protein 18
AT4G16330	-2.25	Oxidoreductase
AT2G42840	-2.25	Protodermal factor 1
AT4G01720	-2.25	Putative WRKY transcription factor 47
AT5G13190	-2.25	Uncharacterized protein
AT1G11545	-2.24	Xyloglucan:xyloglucosyl transferase
AT4G14130	-2.24	Xyloglucan:xyloglucosyl transferase
AT2G34380	-2.24	Putative adipose-regulatory protein (Seipin)
AT5G43190	-2.23	F-box/kelch-repeat protein
AT2G21140	-2.23	Proline-rich protein 2
AT1G09530	-2.23	Transcription factor PIF3
AT4G21570	-2.21	Uncharacterized protein
AT4G26220	-2.21	Putative caffeoyl-CoA O-methyltransferase
AT4G17490	-2.20	Ethylene-responsive transcription factor 6
AT4G33960	-2.20	Uncharacterized protein
AT1G23080	-2.19	Auxin efflux carrier component 7
AT4G18340	-2.19	Glycosyl hydrolase family 17 protein
AT5G65200	-2.19	U-box domain-containing protein 38
AT1G12080	-2.19	Vacuolar calcium-binding protein-like protein
AT2G01180	-2.18	Lipid phosphate phosphatase 1
AT1G69690	-2.18	Transcription factor TCP15
AT3G62800	-2.18	Double-stranded-RNA-binding protein 4
AT2G19970	-2.17	Putative pathogenesis-related protein

Gene	Fold Change	Description
AT1G78260	-2.17	RNA recognition motif-containing protein
AT3G62570	-2.17	Tetratricopeptide repeat-containing protein
AT1G51430	-2.17	Uncharacterized protein
AT1G06850	-2.16	Basic leucine-zipper 52
AT5G24280	-2.16	Gamma-irradiation and mitomycin c induced 1
AT3G20820	-2.16	Leucine-rich repeat-containing protein
AT5G20650	-2.16	Copper transporter 5
AT5G43470	-2.16	Disease resistance protein RPP8
AT1G27770	-2.15	Autoinhibited Ca2+-ATPase 1
AT3G25600	-2.15	Putative calcium-binding protein CML16
AT3G50340	-2.15	Uncharacterized protein
AT4G07841	-2.15	Zinc ion binding protein
AT5G62280	-2.14	Uncharacterized protein
AT5G27610	-2.14	Protein ALWAYS EARLY 1
AT3G57930	-2.14	Uncharacterized protein
AT5G01950	-2.13	Leucine-rich repeat protein kinase-like protein
AT2G47760	-2.12	Alpha-1
AT4G29240	-2.12	Leucine-rich repeat-containing protein
AT3G49220	-2.12	Pectinesterase
AT3G16660	-2.12	Pollen Ole e 1 allergen and extensin family protein
AT4G35220	-2.12	Cyclase family protein
AT1G14440	-2.12	Homeobox protein 31
AT5G06320	-2.11	NDR1/HIN1-Like protein 3
AT4G01410	-2.11	Late embryogenesis abundant hydroxyproline-rich glycoprotein
AT2G42840	-2.11	Protein phosphatase 2A subunit A2
AT5G51730	-2.11	RNA-binding (RRM/RBD/RNP motifs) family protein

Gene	Fold Change	Description
AT1G26570	-2.11	UDP-glucose dehydrogenase 1
AT3G22800	-2.11	Leucine-rich repeat extensin-like protein 6
AT2G17230	-2.10	Protein exordium like 5
AT5G02020	-2.10	Uncharacterized protein
AT1G04240	-2.09	Auxin-responsive protein IAA3
AT5G13270	-2.09	Pentatricopeptide repeat-containing protein
AT1G80820	-2.09	Cinnamoyl-CoA reductase
AT3G57450	-2.08	Uncharacterized protein
AT3G55850	-2.08	Amidohydrolase family protein
AT4G16765	-2.08	Oxidoreductase
AT5G64260	-2.08	Protein EXORDIUM like 2
AT5G03360	-2.07	DC1 domain-containing protein
AT1G33590	-2.07	Leucine-rich repeat-containing protein
AT2G06850	-2.07	Xyloglucan endotransglucosylase/hydrolase protein 4
AT4G37800	-2.07	Xyloglucan:xyloglucosyl transferase
AT3G21720	-2.06	Isocitrate lyase
AT4G36410	-2.06	Putative ubiquitin-conjugating enzyme E2 17
AT2G23170	-2.06	Indole-3-acetic acid-amido synthetase GH3,3
AT1G07570	-2.06	Protein kinase APK1A
AT1G09560	-2.06	Germin-like protein subfamily 2 member 1
AT1G03850	-2.06	Monothiol glutaredoxin-S13
AT2G37130	-2.05	Peroxidase
AT5G45110	-2.05	NPR1-like protein 3
AT5G49700	-2.04	Predicted AT-hook DNA-binding family protein
AT2G45050	-2.04	GATA transcription factor 2
AT3G48100	-2.04	Ttwo-component response regulator ARR5
AT1G15430	-2.04	Uncharacterized protein

Gene	Fold Change	Description
AT4G12520	-2.04	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT3G05880	-2.04	Hydrophobic protein RCI2A
AT3G05320	-2.04	O-fucosyltransferase family protein
AT3G60530	-2.03	GATA transcription factor 4
AT5G04720	-2.03	ADR1-like 2 protein
AT5G64620	-2.03	Cell wall / vacuolar inhibitor of fructosidase 2
AT1G69840	-2.03	Hypersensitive-induced response protein 2
AT2G45180	-2.02	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT5G62200	-2.02	Embryo-specific protein 3
AT1G27670	-2.02	Uncharacterized protein
AT1G02660	-2.02	Alpha/beta-hydrolase domain-containing protein
AT3G02640	-2.02	Uncharacterized protein
AT2G22680	-2.01	C3HC4-type RING finger domain-containing protein
AT2G14900	-2.01	Gibberellin-regulated protein
AT2G24150	-2.01	Heptahelical protein 3
AT1G72200	-2.01	RING-H2 finger protein ATL11
AT1G22330	-2.01	RNA recognition motif-containing protein
AT1G66160	-2.01	U-box domain-containing protein 20
AT3G06070	-2.01	Uncharacterized protein
AT2G43570	-2.01	Chitinase class 4-like protein
AT1G80080	-2.01	Protein TOO MANY MOUTHS
AT1G15670	-2.01	Putative F-box/kelch-repeat protein
AT5G57887	-2.01	Uncharacterized protein
AT5G44680	-2.00	DNA-3-methyladenine glycosylase I
AT5G09440	-2.00	Protein exordium like 4
AT4G19030	-2.00	Aquaporin NIP1-1

Gene	Fold Change	Description
AT1G49050	-2.00	Aspartyl protease
AT1G22500	-2.00	E3 ubiquitin-protein ligase ATL15
AT3G28200	-2.00	Peroxidase 31
AT5G13330	-2.00	Ethylene-responsive transcription factor ERF113
AT3G06750	-2.00	Hydroxyproline-rich glycoprotein family protein
AT5G02290	-2.00	Putative serine/threonine-protein kinase NAK
AT3G07800	-2.00	Thymidine kinase
AT1G71900	-2.00	Uncharacterized protein

Supplementary Table 4. GO enrichment of down-regulated (cluster 1) and up-regulated genes of Mlp124478 versus Col-0 lines.

GO Term	GO Group	Nr. Gene	Genes Down-regulated (cluster 1)	Genes up-regulated (cluster 2)	% Genes (cluster 1)	% Genes (cluster 2
Response to virus	0	4	[CHI, NHL3, RPP8, YLS9]	[]	100,00	0,00
Response to bacterium	1	14	[AZI1, BAS1, BES1, CRK11, JAZ1, MYB51, NHL3, NPR3, WRKY18, WRKY27, WRKY33]	[CRK21, ESM1, LOX2]	78,57	21,43
Response to organic cyclic compound	2	11	[ARL, BAS1, BES1, CHI, EXO, LTL1, MYB51, PAR1, RPP8, WRKY18]	[MYB4]	90,91	9,09
Response to brassinosteroid	2	5	[ARL, BAS1, BES1, EXO, PAR1]	[]	100,00	0,00
Indole-containing compound biosynthetic process	3	4	[ASA1, ASB1, MYB51, WRKY33]	[]	100,00	0,00
Cellular carbohydrate metabolic process	4	11	[ICL, MLS, XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[MIPS1]	90,91	9,09
Cell wall organization	4	15	[EXT3, EXT4, MYB51, PRP2, XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[AT1G60590, EXPA10, EXPB3]	80,00	20,00
Cell wall macromolecule metabolic process	4	9	[CHI, XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[]	100,00	0,00

GO Term	GO Group	Nr. Gene	Genes Down-regulated (cluster 1)	Genes up-regulated (cluster 2)	% Genes (cluster 1)	% Genes (cluster 2
Plant-type cell wall organization	4	5	[EXT3, EXT4, PRP2, XTH33]	[EXPA10]	80,00	20,00
Xyloglucan metabolic process	4	8	[XTH15, XTH17, XTH18, XTH33, XTH4, XTH7, XTH8, XTR6]	[]	100,00	0,00
Response to red or far red light	5	8	[BEE1, FAR1, GA2OX2, PAR1, PIF3, PKS4, RR5]	[]	93,50	0,00
Red or far-red light signaling pathway	5	4	[FAR1, PIF3, PKS4]	[]	94,64	0,00
Response to ethylene	6	8	[ARGOS, EBF2, ERF6, ETR2, MYB51, PRP3, RTE1]	[SNZ]	87,50	12,50
Negative regulation of signal transduction	6	4	[EBF2, ETR2, RTE1]	[KEG]	75,00	25,00
Negative regulation of ethylene-activated signaling pathway	6	3	[EBF2, ETR2, RTE1]	[]	100,00	0,00

Supplementary Table 5. Up-regulated genes represent abundant TFBSs.

Gene ID	Fold Change	Q-Value	Description
At1g24580	9.53	0.015	RING/U-box domain-containing protein
At2g15020	8.54	0.0016	Uncharacterized protein
At2g37770	7.06	0.0016	NAD(P)-linked oxidoreductase-like protein
At3g58990	3.85	0.0162	Isopropylmalate isomerase 1
At2g34450	3.78	0.0299	High mobility group (HMG1/2) domain-containing protein
At1g04770	3.07	0.0016	Male sterility MS5 family protein
At1g18773	3.02	0.0478	Uncharacterized protein
At5g41410	2.87	0.0052	Homeobox protein BEL1-like protein
At3g54600	2.78	0.0029	Class I glutamine amidotransferase domain-containing protein
At5g42760	2.73	0.0052	Leucine carboxyl methyltransferase
At5g49480	2.68	0.0016	Ca2+-binding protein 1
At1g62050	2.61	0.0016	Ankyrin repeat-containing protein
At3g51660	2.48	0.0202	Macrophage migration inhibitory factor family protein
At3g04140	2.47	0.0041	Ankyrin repeat-containing protein
At3g21670	2.44	0.0016	Major facilitator protein
At2g39800	2.4	0.0016	Gamma-glutamyl phosphate reductase
At4g08870	2.34	0.0016	Putative arginase
At1g33170	2.3	0.0097	Putative methyltransferase PMT18
At5g17300	2.27	0.0097	Myb family transcription factor
At5g24470	2.27	0.0018	Two-component response regulator-like APRR5
At3g48310	2.24	0.0018	Cytochrome P450 71A22
At2g47750	2.24	0.0082	Putative indole-3-acetic acid-amido synthetase GH3,9
At1g13650	2.23	0.0016	Uncharacterized protein
At3g14440	2.19	0.0341	9-cis-epoxycarotenoid dioxygenase NCED3

Gene ID	Fold Change	Q-Value	Description
At2g41870	2.17	0.0052	Glutathione peroxidase 7
At5g17550	2.16	0.0131	Peroxin 19-2
At5g53280	2.16	0.0223	Plastid division protein 1
At5g58770	2.13	0.0088	Dehydrodolichyl diphosphate synthase 2
At5g62130	2.13	0.0016	Per1-like family protein
At4g03400	2.12	0.0016	Auxin-responsive GH3 family protein
At2g40480	2.11	0.0509	Uncharacterized protein
At1g26770	2.09	0.026	Expansin A10
At1g52400	2.09	0.0097	Beta glucosidase 18
At3g63160	2.08	0.0016	OEP6, OUTER ENVELOPE PROTEIN 6
At2g39250	2.08	0.0052	AP2-like ethylene-responsive transcription factor SNZ
At2g32990	2.07	0.0431	Endoglucanase 11
At1g52590	2.06	0.0029	Putative thiol-disulfide oxidoreductase DCC
At1g22590	2.05	0.0062	Protein AGAMOUS-like 87
At1g75030	2.05	0.0243	ATLP-3, THAUMATIN-LIKE PROTEIN 3, TLP-3
At1g18360	2.04	0.0016	Alpha/beta-hydrolase domain-containing protein
At4g10120	2.04	0.0018	Sucrose-phosphate synthase
At3g22104	2.03	0.009	Phototropic-responsive NPH3 family protein
At4g39800	2.02	0.0016	Inositol-3-phosphate synthase isozyme 1

Supplementary Table 6. Down-regulated genes represent abundant TFBSs.

Gene ID	Fold Change	Q-Value	Description
At1g71900	2	0.0398	Uncharacterized protein
At5g57887	2.01	0.0069	Uncharacterized protein
At1g27670	2.02	0.0358	Uncharacterized protein
At5g64620	2.03	0.0016	Cell wall / vacuolar inhibitor of fructosidase 2
At2g37130	2.05	0.0016	Peroxidase
At4g37800	2.07	0.0018	Xyloglucan:xyloglucosyl transferase
At4g01410	2.11	0.0041	Late embryogenesis abundant hydroxyproline-rich glycoprotein
At3g25600	2.15	0.0018	Putative calcium-binding protein CML16
At5g20650	2.16	0.0016	Copper transporter 5
At5g65200	2.19	0.0314	U-box domain-containing protein 38
At5g13190	2.25	0.0018	Uncharacterized protein
At2g43150	2.3	0.0018	Proline-rich extensin-like family protein
At1g78890	2.43	0.0018	Uncharacterized protein
At1g64640	2.46	0.0018	Early nodulin-like protein 8
At5g57910	2.49	0.0314	Uncharacterized protein
At1g78460	2.62	0.0018	SOUL heme-binding protein
At1g67910	2.76	0.0018	Uncharacterized protein
At1g29090	2.92	0.0079	Cysteine proteinase-like protein
At3g12710	3.02	0.0018	DNA-3-methyladenine glycosylase I
At2g39200	3.06	0.0018	MILDEW RESISTANCE LOCUS O 12, MLO12
At1g50740	3.1	0.0018	Transmembrane proteins 14C
At1g29465	3.12	0.0079	Uncharacterized protein
At4g40010	3.15	0.0486	Serine/threonine-protein kinase SRK2F
At4g09890	3.17	0.0018	Uncharacterized protein
At3g02550	3.32	0.0018	LOB domain-containing protein 41

Gene ID	Fold Change	Q-Value	Description
At2g27080	3.75	0.0018	Late embryogenesis abundant hydroxyproline-rich glycoprotein
At2g20835	4.29	0.0357	Uncharacterized protein
At5g39610	4.5	0.0057	NAC domain containing protein 6
At4g16515	5.31	0.0016	CLE-LIKE 6,
At2g35980	5.95	0.0156	ARABIDOPSIS NDR1/HIN1-LIKE 10, ATNHL10

Supplementary Table 7. Similar de-regulated genes found between the transcriptome of Mlp124478 and Hacquard et al (2016).

GO term	**Deregulated in both transcriptomes**	
Response to virus	CHI	YLS9
	RPP8	
Response to bacterium	JAZ1	
	MYB51	
Response to organic cyclic compound	ARL	MYB51
	CHI	RPP8
	EXO	MYB4
Response to brassinosteroid	ARL	EXO
Indole-containing compound biosynthetic process	ASA1	MYB51
	WRKY33	
Cell wall organization	MYB51	PRP2
Cell wall macromolecule metabolic process	CHI	
Plant-type cell wall organization	PRP2	