Recognition of the flax rust fungal effector, AvrM,

by the M flax rust resistance protein

Thi Danh Vu

2188339

A thesis submitted for the Master degree of Biotechnology studies

Supervisor: Assoc. Prof. Peter Anderson

The College of Science and Engineering, Flinders University

June 2019

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ABSTRACT

Plant that live in the natural environment must deal with many stressful factors, none more so than disease causing micro-organisms. Crop losses affect the incomes of many families whose livelihoods depend on agricultural production and they also pose a major threat to food security worldwide. In order to contribute to our understanding of the plant pathogen interface, the research presented here has focussed on the interaction between the flax rust fungal effector (AvrM) and the flax rust resistance protein M. Resistance proteins, like M, have evolved to recognise the presence of pathogen effectors that are secreted into plant cells. In this research, we have used two flax rust effector proteins, AvrM and avrM, in which the *AvrM* effector-containing strains of flax rust elicits a disease resistance response when inoculated onto flax plants that contain the *M* flax rust resistance gene. Both AvrM and avrM were isolated from *Melampsora lini*. The avrM protein, despite sharing 96% similarity to the AvrM protein, does not elicit a response when *avrM*-containing strains are inoculated in the same way.

With the isolation and cloning of the *AvrM* and *avrM* effector genes, and the flax rust resistance gene *M*, this interaction can be uncoupled from its natural plant/pathogen system and the effectors expressed transiently via *Agrobacterium* infiltration into transgenic tobacco stably transformed with the *M* gene. That is, when *Agrobacterium tumefaciens* carrying either the *AvrM* or *avrM* genes is infiltrated into transgenic *M*-containing tobacco plants, a characteristic disease resistance response or hypersensitive cell death response (HR) may be generated in the tobacco tissue dependent on whether the AvrM/M proteins are interacting. We have used this experimental system to investigate the specific amino acid differences between AvrM and avrM that control recognition and activation of the M rust resistance protein. The work to identify mutants in *AvrM* that can knockdown M resistance protein-induced HR or knock-in that of *avrM* was part of the PhD project of Motiur Rahman (Rahman, 2016). One limitation of Rahman's study was that all 19 mutants of both *AvrM* and *avrM* were shown the change of HR *in planta.* However, the

expression of these proteins in the tissue was not confirmed by western blot analysis. This was the aim of this study.

From the structure of the AvrM and avrM proteins (Ve et al, 2011), a small charged pocket was found at the interface of the AvrM homodimer that was unique to AvrM and not found in the avrM structure. Residues E237, E309 and R313 were non-polymorphic between AvrM and avrM and yet were responsible for this charge difference. Based on this, Rahman (2016) made seven mutant forms of AvrM, namely AvrM E237A, AvrM E309A, AvrM R313A, AvrM E237A+ E309A, AvrM E237A+R313A, AvrM E309A + R313A, AvrM E237A + E309A + R313A, and found that all single mutants and some double mutants were recognised by M, while the AvrM E237A+ E309A double and all triple mutants were not. Rahman (2016) concluded from this, that the charged pocket in the AvrM structure is essential for M recognition. A minor difference between the study of Rahman and this study was that here only the triple mutant, AvrM E237A + E309A + R313A, had the ability to knock down the recognition of M protein, whereas Rahman showed that the double mutant (AvrM E237A+ E309A) was able to do this.

Of the 13 residues that were polymorphic between AvrM and avrM, Rahman (2016) made eight single mutants in the avrM effector, and in a number of double, triple, and quadruple combinations. The avrM mutants were created by changing the identity of amino acid in avrM to that of AvrM. This was done in order to see what polymorphic residues were important in M recognition. Work presented here, showed that M recognition was partially restored in the avrM R170K + S179L and avrM R170K + T247I double mutants. To further investigate the role of key residues involved in M recognition, Rahman (2016) made the reciprocal changes to those discussed above in AvrM, to see if M recognition could be knockout. For four mutants of polymorphic residue in the AvrM effector: AvrM K232R + L241S, AvrM K232R + I310T, AvrM L241S + I310T and AvrM K232R + L214S + I310T, all were recognised by the M protein to same level as that of AvrM. These changes could not knock down the M recognition. Work presented here showed

by diluting the Agro-infiltration of the mutants, (avrM S179L + T247I and avrM R170K + S179L + T247I) had a weaker M-induced HR than AvrM.

Further to the work of Rahman (2016), all the effector proteins were shown here to be expressed in tobacco leaves to similar levels as demonstrated by Western blot analysis. Therefore, any difference in HR could not be attributed to differences in protein expression, and rather by the recognition of the effector by the M protein.

In a related but parallel study, the effect of light on the strength of the HR was assessed in the M/AvrM interaction assay. The M-AvrM induced HR was inhibited when tobacco leaves were pre-treated with darkness for 72, and 48 hours before infiltration in comparison with that of a 24 hours dark pre-treatment and the control. The effector protein showed equivalent levels of expression in the protein samples extracted from dark treated leaves and was higher than that from the control leaves. This result confirms reports in the literature that light is required for the activation of the R-protein induced HR. A possible explanation for this is discussed.

DECLARATION

I certify that this thesis does not contain any material previously submitted for a degree or diploma in any university or institute and does not incorporate with any material previously published or written by another person except where due references have been made in the text.

Thi Danh Vu

June 2019

ACKNOWLEDGMENTS

To finish this thesis, I would like to thank:

+ Vietnam government who sponsored the scholarship and provided me with a good chance to study overseas.

+ My understanding supervisor, A/Prof Peter Alex Anderson for his time to support, explain and help me to conduct this research. Without him, I could not finish this thesis. I am highly appreciative for his help in sharing and being ready to help me answer any questions and address immediately during the time. He is an excellent supervisor, helpful and has kind characteristics.

+ All lecturers and staff (in biological sciences building) who created a pleasant environment and training for me in the necessary laboratory skills before and during the experiment process. Moreover, these lecturers in the Biological lab namely Prof Kathleen Soole, Prof David Day, Prof Colin Jenkins and Dr Yuri Shavrukov who transmitted science research spirit and useful contributions in laboratory presentations; and funny stories during morning tea every week.

+ All friends in the biological laboratory, special thanks to Alex Mc Donald, Thanh Hai Tran, Christopher Waterman, Barry Rainbird, Lauren Philp-Dutton, Crystal Sweetman, Carly Schramm, Jason Smith, Troy Miller at Flinders University and Dr. Simon William (Australian National University) who spent their time to share their experiences and knowledge to help me for 9 months while I worked in the biological lab. Your support and your time gave me more understanding about these experiments. Moreover, your advice helped me address lab problems with a good spirit.

+ The topic coordinator in Biotechnology studies, A/Prof Fiona Young, Prof Chris Franco and Dr. Ricardo Pinto Araujo and all Bachelor and Master of Biotechnology friends namely, Thi Dan Cu, Nathan Geyer, Pamela Joyce Gorriceta and Zhaolin He. You made my life more balances between study and experience.

+ All members of my family, relatives, and friends at Flinders University and Vietnam. The connection, sharing and your encouragement always created a great motivation to help me overcome and finish this course.

Lastly, I would like to thank my husband, Hoai Duc Nguyen, who is always listening, helps me relax and puts a smile on my face at all the time. Your understanding and your encouragement make my life easier and comfortable.

Thanks everyone and I hope you will always be happy and successful in life.

ABBREVIATION

A: Alanine

Dpi: day post infiltration

E: Glutamic acid

ETI: Effector-triggered immunity

HR: hypersensitive response

I: Isoleucine

K: Lysine

LB: Luria-Bertani Medium

LRR: leucine rich repeat region

MALS: multi-angled light scattering

MAMPS: microbial-associated molecular patterns

MS: Murashige and Skoog Medium

NB: Nucleotide binding

NLR: Nucleic Leucine Rich repeat region

PCD: programmed cell death

PRR: pattern recognition receptors

PTI: pathogen trigger immunity

R: Arginine

ROS: Reactive oxygen species

S: Serine

T: Threonine

TTSS: Type III secretion system

CHAPTER 1: INTRODUCTION

1.1 The effect of plant pathogens on agricultural production

Agricultural production plays a vital role in the economic growth of many countries and is necessary to support an increasing global population. A sustainable and productive agricultural production is essential now and will becoming only more important in the future. However, there are many environmental factors such as climate change, adverse soil conditions, and plant pathogens that affect this productivity. Plant diseases results in an 15% average annual loss world-wide (Schwessinger et al., 2015). An ongoing challenge for future production is that plant-pathogen co-evolution is unending, intricate and happens across many spatial and temporal scales (Ravensdale et al., 2011).

Rust fungi are obligate biotrophic pathogens; therefore, they require a living host. All rust pathogens colonise within their host plants (Tang et al., 2017) and obtain nutrients via the formation of haustoria (or feeding structures) within host cells (Catanzariti et al., 2006). Successful infection, colonisation and sporulation of rust fungi results in disease and yield losses in many plant species. Therefore, they have a significant effect on global agricultural production and food security (Periyannan et al., 2017). This problem is only exacerbated by the increasing population that places more significant stress on crop productivity. Therefore, research on decreasing the effect of plant pathogens will contribute to maintaining food security in the world and promotes the development of sustainable agricultural production.

1.2. Plant defence system

There are two mechanisms that reflect the interactions of plants and pathogens, namely microbe-associated molecular patterns (MAMPs) triggered immunity and the effector triggered immune system (Bonardi et al., 2011, Wang et al., 2017). The activity of plant resistance (R) genes relates to the effector triggered immune system.

The plant immune system is a host defence system consisting of many structures and biological processes to protect plants against diseases. In order to function normally, the immune system must detect several pathogenic agents that range from viruses to fungi, bacteria and even some insects and nematodes. Like the animal immune system, this detection process must distinguish different cells from the healthy cells of the organism. In vertebrates, the immune system can be broken down into two parts, such as the innate immune systems and the acquired immune system (McMurran et al., 2016).

The world of plant pathogens is plentiful, and they use different strategies to attack plants. Many bacterial species enter plants via stomata, hydathodes, or through wounds, then proliferate in the apoplast. Nematodes and aphids use a stylet that directly penetrates plant cells to gather nutrients. Many fungi penetrate the plant epidermis directly, whereas others enter via stomata and form haustorial feeding structures to gain nutrients (Jiang, 2011). Haustoria are shown in Figures 1.1 and 1.2. A communication system is made up of the contact of the haustorial membrane, the extracellular matrix and the host cell membrane to perform direct communication between the host and the pathogen. Also, all these plant pathogens secrete virulence substances into the plant cells to suit their growing needs (Gudesblat et al., 2009).

Unlike animals, plants do not have flexible and mobile protective cells and a fast and adaptive immune system. Instead, plants rely primarily on the passive immunological properties of each cell and the messenger substances derived from the pathogen at the infected sites (Dangl and Jones, 2001, Ausubel, 2005, Chisholm et al., 2006). These authors report on the diversity of protective proteins (R proteins), on the level of genetic polymorphism at the *R* loci that controls the interaction between plants and pathogens, as well as on the mechanism of internal responses when R proteins are activated (Dangl and Jones, 2001). They hypothesize that many of the plant's R-proteins are activated indirectly by the molecule produced and injected into the plant host cell by invading pathogen species. This protection theory assumes that R proteins indirectly recognize

infectious substances as they altered the intracellular activity of the host (Van Der Biezen and Jones, 1998). R protein sense changes in the infected plant cell due to an infectious organism in a similar way to the mechanism of self-non-self-reactive signaling in the animal's immune system (Matzinger, 2002). However, R-effector interaction is not always via an indirect recognition mechanism. In some cases, namely *Cladosporium fulvum*/Tomatoes; *Venturia inaequalis*/Apple interactions, and the the effector molecules AvrM and avrM studied here, the secreted effectors by the pathogen are directly detected by the plant R proteins (de Wit, 2016).

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The link is https://www.tandfonline.com/doi/full/10.1080/21501203.2011.605181

Figure 1A showed extracellular interfaces that effectors (black dots) move through, either the apoplastic space or xylem. Another way is to form a Hartig net that helps the fungus can uptake the nutrients from its host and secrete the effector. In the intracellular interface two plant-pathogen interactions are demonstrated: haustorium and invasive hyphae. The fungus pushes the haustorium into the plant cell and releases the effectors. In the invasive hyphae the biotrophic interfacial complex that connects the way fungal or oomycete move in the plant is illustrated.

Plants used two layers of their immune system to protect themselves against diseases. The first system is the transmembrane pattern recognition receptors (PRRs). PRR proteins bind to conserved molecular structures of microbial/pathogen-associated molecular patterns (MAMPs / PAMPs), an example being bacterial flagellin. Activation of PRR proteins leads to signalling pathways and gene expression that creates an unfavourable environment for the pathogen resulting

in disease resistance. This is known as PAMP triggered immunity or PTI. Some specialised pathogens can overcome PTI and restore a disease state by secreting molecules into the host cell to dampen PTI. These are known as effector proteins and a subset of these are interchangeable with Avirulence or Avr proteins. This is shown in the Figure 1.2 below.

The second system operates primarily within the cell and involves polymorphic R proteins (Matzinger, 2002, Dangl and Jones, 2001). A large class of these proteins are called NB-LRRs because they have nucleotide binding (NB) and leucine-rich repeat (LRR) domains. Infectious agents from different pathogens are identified by the NB-LRR protein which then becomes activated leading to a defence response. NB-LRR proteins are only effective against specific pathogenic organisms in host tissue (biotrophs or semi-biotroph) but not for organisms that break down host tissues (necrotrophy).

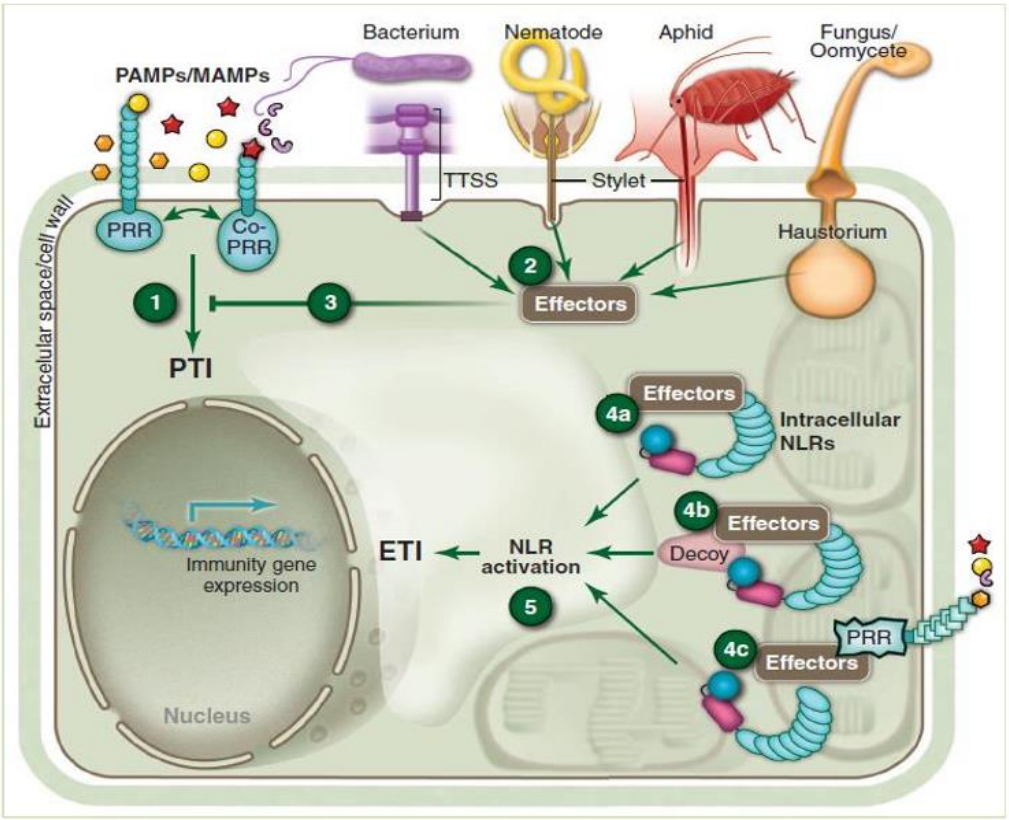

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Figure 1.2: The overview model of plant defending system *(Dangl et al., 2013).*

There are many different pathogens such as bacteria, nematodes, aphids and fungi/ oomycetes that express PAMPs (Pathogen-associated molecular patterns) and MAMPs (Microbe-associated molecular patterns) inside the hosts. Step 1: PAMPs and MAMPs are sensed by plant PRR, and PTI (pathogen triggered immunity) is activated. Step 2: In more specialised pathogens, effectors are secreted into plant cells. Step 3: Effectors block PTI to retort virulence and a disease state. Step 4: NLRs of the plant respond by detection of the pathogen effectors. This can be via; 4a: Direct interaction of NLR and effectors, 4b: The effector modifies a decoy protein that imitates an effector target. 4c: The effector-mediated modification in host virulence target. Step 5: NLR activation stimulates ETI (effector-triggered immunity) and immunity gene expression leading to hypersensitive cell death or the HR (Dangl et al., 2013).

1.3. Nucleotide binding and leucine rich repeat regions (NLRs)

NLRs played a key role in reducing pathogens via isolating the infection (Heath, 2000). NLRs activate a hypersensitive response that leads to localized programmed cell death (PCD). Nucleotide binding is considered by some as a molecular switch where pathogen effector perception leads to nucleotide exchange whereby the binding of ATP activates the protein, and the intrinsic ATPase activity restores the protein to its resting state (Tameling et al., 2006). Others believe this ATP binding; ATP hydrolysis process is regularly cycling within plant cells and the presence of the effector molecule shifts the balance towards an ATP-bound state that activates the resistance response (Bernoux et al., 2016).

The LRR is a structural motif believed to bind the effector molecule or the cellular consequence of the effector molecule and is characterized by hydrophobic leucine residues that are under diversifying selection. LRR are present in many immune receptors and provide diversity to the receptors mediating protein-protein interactions relevant to defence activation (Padmanabhan et al., 2009).

1.4. M resistance gene

The *M* flax rust resistance gene is an example of an ETI-activating NLR protein. The M protein is located at the tonoplast membrane of flax cells (Takemoto et al., 2012). It was cloned independently in the same M gene by two distinct methods: use L6 gene-derived DNA probe to analyse spontaneous M mutants and tagged with the transposon activator of maize. The *M* resistance gene is related to allelic members of the *L* locus that confers resistance to strains of flax rust. Only one gene encodes the specific *M* resistance gene. It includes two repeat regions of 147 and 149 amino acids in the C-terminal part of the leucine-rich region (Anderson et al., 1997). It confers resistance to flax rust fungus that carry the *AvrM* avirulence gene (Lawrence et al., 2010).

1.5. Flax and flax-rust disease

Flax is an important crop and grown in many countries at low temperatures. It is a good source of fibre, omega-3 fatty acid, and other medicinal and nutritional products (Kajla et al., 2015). It is a member of Linaceae family that is attacked by many rust fungi, in which *Melampsora lini*, a causal agent of flax rust, has been intensively researched and become a model plant/pathogen system for many scientists for several decades. The genetic studies of virulence of flax rust and flax rust resistance have provided an opportunity to understand the molecular biology of the plant immune system. Harold Flor introduced the well-known "gene-for-gene" hypothesis in 1956. He showed that single pairs of allelic genes decide the phenotype of avirulence or virulence in a flax rust race. A cognate set of genes in the host give the ability to resist flax rust disease (Flor, 1956).

Flax-rust disease usually presents under the leaves of plant with different spore types. In the disease plants, the leaves become yellowish and fall apart, then the shoots become dry, and the plants grow poorly and the yield decreases.

1.6. Plant pathogens

Based on the way of acquiring nutrients, pathogens can be divided into three groups:

- *Biotrophs:* use the food from a living host, for example: *Phytophthora parasitica* (leads to blight disease) and *Melampsora lini* (causes rust flax disease). Generally, biotrophs have a narrow host range, and do not kill host cells immediately after infection; instead, they let and even promote the cells to live as long as possible because they depend on the intact metabolism of the host cell to obtain nutrition and reproduce. This allows the host to establish defensive responses related to interaction between the resistance protein and effector molecules secreted into cell from the pathogen. For vegetative fungi, nutrient uptake is often via a haustorium. The dead tissue forms only after the fungus has finished reproducing (Sache and Vallavieille-Pope, 1995).

- ***Necro-trophs:*** Kill the host tissue by the secretion of toxins and then use the food from the dead or dying tissues, for example: *Botrytis cinerea, Alternaria brassicicola* and *Rhizoctonia solani*. They often have a bigger host range than that of biotrophic pathogens. The host has insufficient time to establish a defensive response from an activated resistance protein. Tissue surrounding the infection can produce resistance reactions with diffusers from the lesion (Hawksworth and Mueller, 2005, Horbach et al., 2011).

- ***Semi-biotrophs:*** Some organisms have a mixed biotrophic/necrotrophic interaction, for example *Pseudomonas syringae*. Bacteria invade via mechanical injury or stomata, obtain nutrients via a biotrophic interaction and then release toxins that kill the host tissue prior to sporulation (Horbach et al., 2011).

Figure 1.2 shows that bacteria, nematodes, aphids and fungi can secrete effector proteins into the plant cells. These effector proteins inhibit PTI and if unnoticed by the plant restore a diseased state. If, however the effectors are recognised they activate ETI. For instance, the bacteria *P. syringae* can grow on *Arabidopsis thaliana* because it delivers the effector AvrPto that inhibits PTI by interfering the signal of the receptor, FLS2 that is caused by flg22 (Xiang et al., 2008).

In terms of biotrophic growth, the hypersensitive response isolates the growth of the pathogen and is representative of resistance. In contrast, hypersensitive cell death is an advantage for the

development of necrotrophic pathogens. Thus, the resistant mechanism (based in R resistance and hypersensitive response) is different between biotrophs and necro-trophs.

- Hypersensitive response: The hypersensitive response is present in the second layer of the plant immune system (Hou et al., 2011). It helps plants immobile pathogen growth by localized cell death. In term of the mechanism of the hypersensitive response, it is activated when the cell plant detects the presence of a pathogen by the effector molecules it secretes. The pathogen will secret a recognizable effector (known as an avirulence factor) that interacts directly or indirectly with a R gene product and activates a HR in the host plant. However, if the resistance gene cannot identify the presence of the effector (known now as a virulence factor), the pathogen will use the nutrient and the synthesis machinery of the plant to grow and colonize the host. In this research, the M resistance protein recognizes the avirulence protein AvrM-A and not that coded by the virulence allele, *avrM*.

1.7. The fungal effector *AvrM* and avrM

The AvrM effector protein is secreted by the rust fungal pathogen (*Melampsora lini*). The AvrM effector from flax rust is a small secreted protein that is recognized by the M resistance protein in flax. The *AvrM* effector locus includes five avirulence alleles (*AvrM* A-E) and one virulence allele, *avrM* (Catanzariti et al., 2010). AvrM-A elicits a strong HR in M containing plants and exists as a stable homodimer in solution (Ve et al, 2011). It forms a unique pocket that is negatively charged at the dimer interface. However, avrM does not have this same region and is not recognised by the M protein (Ve, 2011). Crystal structures of AvrM and avrM show that the C-terminal domain of AvrM-A (residues 103-343) and avrM (residues 46-280) formed crystallographic dimers. However, based on the attributes of the dimer interface and experimental programs such as the quaternary prediction program PISA, size-exclusion chromatography and multi-angled light scattering (MALS), only AvrM-A forms stable dimers in solution, whereas avrM exists in a monomer-dimer equilibrium (Williams, unpublished data). In the region of residues 108-343 in AvrM-A there is 96% sequence similarity to *avrM*

(Figure 1.4) with 13 polymorphic residues in a deviation of helices α8 and α11 in the coiled-coil region (Rahman, M. 2016).

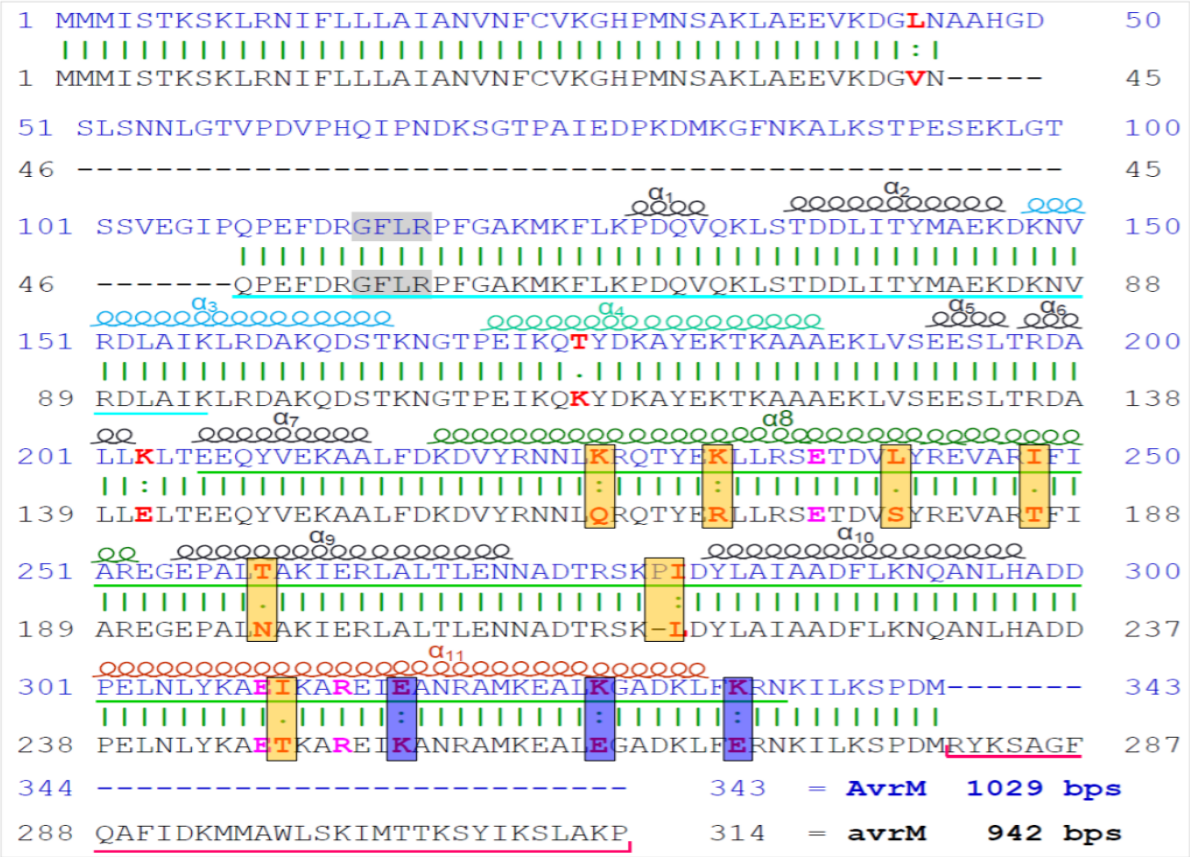

Figure 1.3: The sequences of AvrM and avrM.

Figure 1.3 showed the sequence of AvrM (above-blue colour) and avrM (below- black colour) with 343 and 314 amino acids respectively. The first sequence of AvrM and avrM (amino acid positions 1 to 50) play an essential role in protein trafficking. Amino acids from position 45 to 107 in AvrM are deleted in avrM. Polymorphic amino acids between AvrM and avrM are presented in red. The residues in purple are not related to the recognition of the M resistance protein, whereas those in the yellow boxes control the recognition of the M protein. The pink residues play an essential role in determining the charge of the central pocket in AvrM-A and in M recognition. These amino acids are located in α8 to α11 helices (Rahman, 2016).

In order to determine which of the polymorphic residues control the interaction between AvrM-A and M, Rahman (2016) designed many mutations in both polymorphic and non-polymorphic residues in AvrM-A and *avrM* and tested their ability to elicit a HR in M-containing transgenic

tobacco. In Table 1 below the position and chemical features of the polymorphic residues between *AvrM-A* and *avrM* are shown. Their charges are different and are also presented in distinct colours with yellow (hydrophobic residues), blue (positively charged residues), red (negatively charged residues) and green lines (indicating the important region recognised by the M protein to signal HR: 206-315 in AvrM-A and 144-272 in avrM) (Rahman, 2016). Based on crucial residues in Table 1, Rahman designed many mutants in both AvrM-A and *avrM* in order to understand clearly the structure and function of individual residue in controlling M recognition. The changes for single polymorphic residues had no impact on the recognition of M protein as determined cell death induction by agro-infiltration in M containing tobacco (Ve et al., 2013).

Table 1: The position and chemical features of the polymorphic residues between AvrM and *avrM*

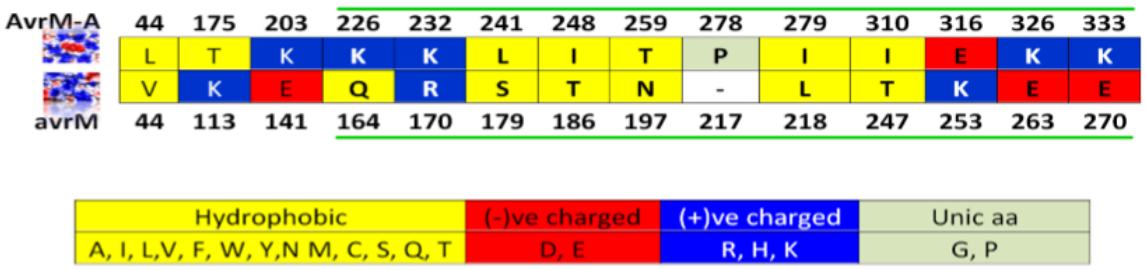

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Table 1 shows the list and the position of the different amino acids between AvrM and avrM. The list of amino acid in AvrM-A are at position 44, 175, 203, 226, 232, 241, 248, 259, 278, 279, 310, 316, 326 and 333 corresponding to at position 44, 113, 141, 164, 170, 179, 186, 197, 217, 218, 247, 253, 263 and 270 in avrM. The yellow colour showed the hydrophobic amino acids; red have a negative charge, blue have a positive charge, (-) show no amino acid at that position. Amino acid positions underlined in green play an important role in recognition of M resistance protein.

By analysing the characteristic, comparison and position of residue, Rahman, (2016) could knock-out M recognition in AvrM-A and gain the function of recognition in *avrM*. Also, he was able to

show that the neutralisation of the charged pocket in AvrM-A (by E237A+E309A) lost recognition with M *in planta* and lost protein-protein interaction in vivo with the M protein as demonstrated by the yeast-2-hybrid assay (unpublished data). Furthermore, the multiple mutants of avrM$^{R170K+S179L+T247I}$ and avrM$^{R170K+S179L+T247I+\Delta L217PI}$ existed as stable dimers in the solution that were recognizable by M *in planta* (Williams, unpublished data). The alanine substitutions at E237 and E309 of AvrM-A were sufficient to knock out recognition and interaction. Collectively, these data point to the role of AvrM-A dimerization in M recognition and thus provide one of the first clear demonstrations of how fungal effector molecules may evolve to avoid activation of the resistance response in their host plants and point towards a potential role of fungal effectors in manipulating the cells of their host plant.

One of the significant limitations in the work of Rahman 2016, is that only in several of the AvrM-A and *avrM* mutants was their expression *in planta* demonstrated by western blot analysis. It is evident in the case where M recognition leads to a HR, that the effector must be expressed, however in cases where recognition does not occur, it is unclear if that is because the effector is unrecognisable by the M protein or is not expressed. To address this limitation, a thorough investigation of the expression of all AvrM-A and *avrM* mutants *in planta* was required. This is the primary aim of this project and will be conducted by Agro-infiltration, protein extraction, SDS-PAGE separation of protein and immunoblot analysis using an anti-haemagglutinin tag engineered onto the N terminus of the effector protein studied in this project.

Figure 1.4 below shows the secondary structure of AvrM-A (left) and avrM (right). The zoom in shows the central pocket of the dimer of AvrM-A and avrM showing the amino acids located in the interface of the dimer namely E237, E309, R313, E316 in AvrM-A and E175, E246, R250 and K253 in avrM. The bottom images reveal the charge in the central pocket: red colours (negative charge) in AvrM-A and blue ones (positive charge) in avrM.

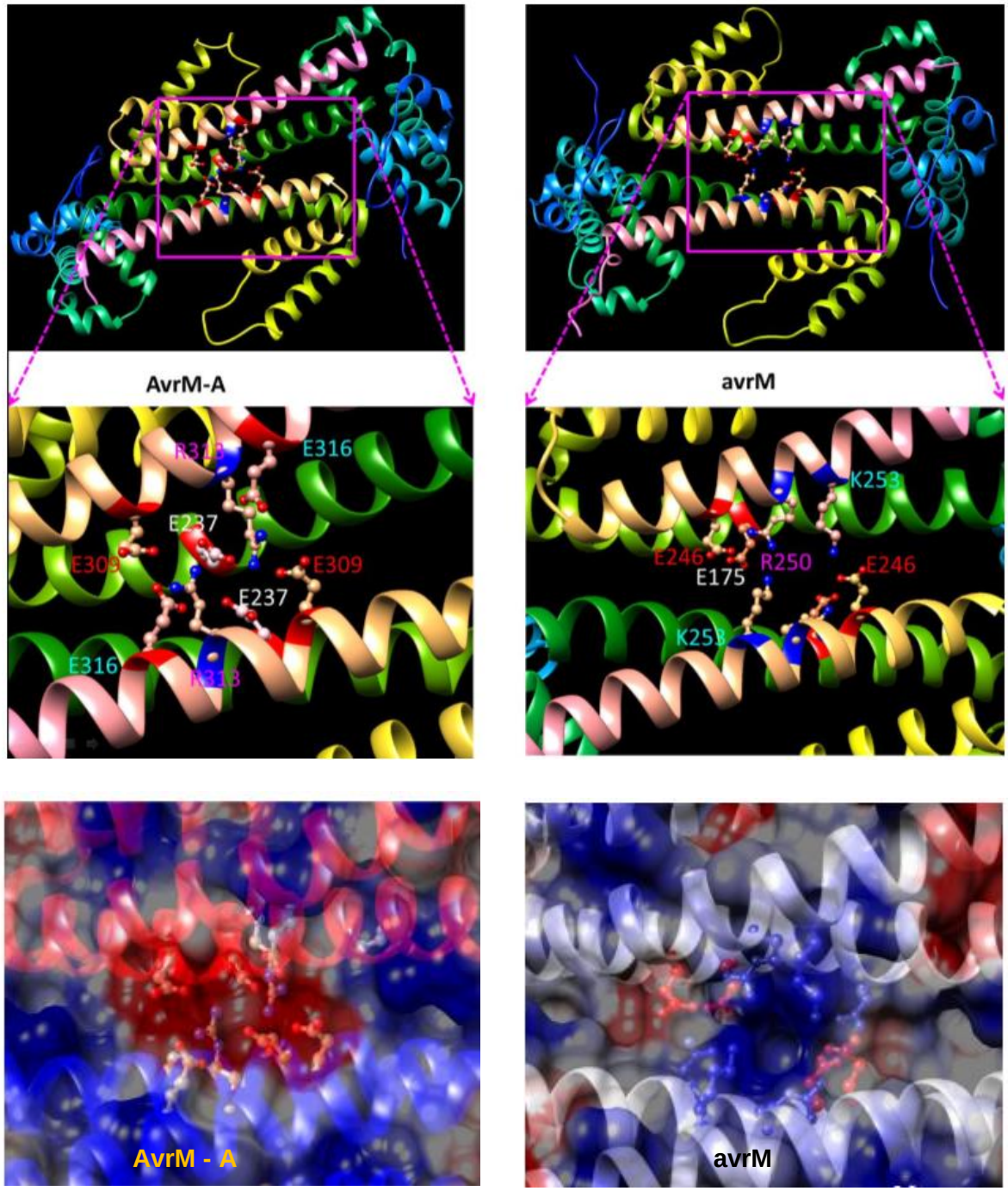


Figure 1.4: Secondary Structures of AvrM-A and avrM showing amino acid residues related to form the central pocket in AvrM. AvrM-A has negative charge and the corresponding region in avrM has a positive charge.

1.8. The effect of light to hypersensitive respond

As a parallel study to the analysis of the AvrM/M interaction, the role of light in the activation of the AvrM/M-induced HR was investigated.

1.8.1. The role of light in plant growth and development

Light is a crucial factor for the growth and developmental processes of a plant (Mustilli and Bowler, 1997, Karpinski et al., 2003). It is undeniable that the light affects the temperature, humidity, and therefore, the light influences directly and indirectly many biosynthetic processes of the plants. One of the remarkable examples of relation of plant and light is that 2,500 genes of *A. thaliana* are controlled by the photoreceptors (Gyula et al., 2003). Also, light adjusts the morphology and regulates genes related to phototropism in sunflower, *Helianthus annuus* (Vandenbrink et al., 2014).

1.8.2. The importance of light to hypersensitive response

There are many previous researches showed that light is related to transfer and activity of many biological mechanisms in plants. It is stressed that light is not only a vital component of the plant immune system against pathogen attack but also necessary for the activation of the defence genes and hypersensitive respond (Abbink et al., 2002, Mateo et al., 2004, Weldon, 2015). In term of molecular biology, light receptors are in the chloroplast and the nucleus of plant cell. It has been shown that a light-sensing network and the oxygen-evolving complex in photosystem II is linked to the plant defence mechanisms (Genoud et al., 2002, Abbink et al., 2002). For example, the avirulent pathogen *P. syringe* elicited a dampened resistance response in Arabidopsis plants that grew in limited light and in complete darkness (Zeier et al., 2004).

The presence of light relates to the second layer in the plant immune system that leads to the HR in resistance plants. The duration of light exposure is linked to the activation of this plant protection mechanism. The reason of the relationship of light and HR is still unclear. It can be related to the biosynthesis process of salicylic acid and jasmonic acids that are important secondary messengers in the plant defence response (Griebel and Zeier, 2008, Weldon, 2015). In

the expression of plant related to HR, there were many hypothesises that explained the response inside plant cell lead to the HR. Firstly, in the research of Poor et al, 2007 showed that the overexpression of high levels of endogenous salicylic acid (SA) along with HRT (a resistance gene to Turnip crinkle virus in Arabidopsis) leads to resistance. SA is needed for the plant growth and is a crucial role in HR and the activation of resistance systems. Moreover, SA destroys cell membranes led to oxidative degradation of lipids. The SA-mediated signalling pathways and the reactive oxygen species (ROS) are also dependent on light (Poór et al., 2017). For example, in tobacco mosaic virus resistance, N gene-mediated HR required light and light play an essential role in defence signalling (Chandra-Shekara et al., 2006). In the research of tomatoes also showed that the cell death presented at 1mM SA and the HR is delayed in the dark condition. Furthermore, there are some other factors were also affected by the light such as the NADPH, and systemic acquired resistance. It is stressed that the activity of NADPH oxidase and SIRBOH1 gene (encoding an NADPH oxidase subunit) in light condition were higher than that of dark condition (Poór et al., 2017). Also, plants reduced systemic acquired resistance (SAR) and lesion formation against avirulent pathogens; The leaves are reduced the burst and lesion formation in the dark (Poór et al., 2019). Therefore, based on the research presented above, the research presented here aims to investigate the effect of the HR process into the interaction between the pathogen and host in the absence and presence of light.

1.9. Hypothesis and aims

1.9.1. Hypotheses

- That all AvrM-A and *avrM* mutants are expressed *in planta* to similar levels.
- The hypersensitive response of *Nicotiana tabacum* will be delayed in dark condition.

1.9.2. Aims

- To confirm the expression of the recombinant effector proteins by immuno-blot analysis by Agro-infiltrate all mutants of *AvrM* and *avrM* into leaves of *Nicotiana tabacum*.
- Beyond this initial experimental aim, the effect of light and dark on the development of the HR will be investigated.

CHAPTER 2: MATERIAL AND METHODS

2. Material and Methods

2.1. Material

2.1.1. Plant materials

***Nicotiana tabacum* seed (W38 containing the *M* gene)**

Transgenic *Nicotiana tabacum* plants (cultivar W38) contained the *M* flax resistant gene under the control of its native promoter (designated from now on as W38: *M*) was also provided by Jeff Ellis (CSIRO Canberra).

Nicotiana benthamiana: Seed used for protein expression, and was provided by Dr. Ian Dry CSIRO, Adelaide.

2.1.2. Lists of gene mutation constructs in *AvrM* and *avrM*

+ The flax rust effector genes AvrM and avrM were supplied by Peter N.Dodds, CSIRO, Canberra.

+ The list of gene constructs was provided by Rahman, (2016):

Table 2.1: Gene mutation constructs (^{p}: polymorphic residue)

Mutants in avrM		Mutants in AvrM		
avrM:	S179L + T247I	+ **AvrM-A:**	E237A	E237A+R313A
	R170K + T247I		E309A	E309A+R313A
	R170K + S179L		R313A	E237A+E309A
	a3: R170K + S170L +T247I		E237A+E309A+R313A	
	R170K + S170L +T247I +K253E	**+ AvrM-A^{p}**	L241I+I310T	
	R170K + S170L +T247I +T186I		K232R+I310T	
	R170K + S170L +T247I +N197T		K232R+L241S	
	R170K + S170L +T247I +ΔL218PI		K232R+L241S+I310T	

2.1.3. Bacterial strains

In this research, *Escherichia coli* (DH10B) and *Agrobacterium* (GV3101) containing genes of interest were used for propagation of plasmids and transformation of genes into W38: *M,* respectively.

2.1.4. Cultures and antibiotic

LB and MS media were used to incubate *Agrobacterium tumefaciens (*that contained plasmids) and *Nicotiana tabacum*. It is sterilized by autoclave at 121^0C for 15 minutes. The composition of culture media is as follow:

+ LB: Tryptone 10 g/l; yeast extract 5 g/l, milli Q water 1l (pH 7)

+ LB agar: Tryptone 10 g/l; yeast extract 5 g/l, agar 12 g/l; milli Q water 1l

+ MS media: 4.3 g/l MS salt; 30 g/l sucrose; 50 mg/l spectinomycin; agar 8 mg/l (pH 5.8)

The cultures will be supplied the different specific antibiotics follow in Table 2.2.

Table 2.2: The cultures and antibiotics

Name	**Cultures**	**Antibiotics**
pEG201	LB	Kanamycin 50 µg/ml
E. coli (DH10B)	LB	
GV3101 *(A. tumefaciens*)	LB	Kanamycin 50 µg/ml, Rifampicin 25 µg/ml Gentamycin 50 µg/ml.

2.2. Methods

2.2.1. Electrocompetent bacterial cells

The preparation of electrocompetent cells was done according to the protocol of a laboratory manual Cold Spring Harbor (Sambrook et al., 1989b). The work flow of process are as follows: 100 ml of an overnight LB culture were incubated in the incubator at a suitable temperature (37^0C for *E. coli* and 28^0C for *A. tumefaciens* (GV3101)). The optical density at 600nm was measured,

and the culture stopped at an OD or less than 1 and cooled on ice. Cells were pelleted by centrifugation at 3500 x g, 15 minutes at 4^0C. Cells were resuspended with ice-cold water and centrifuge at 3500 x g, 15 minutes at 4^0C. This step was repeated two times. After that, the pellet was resuspended with 10% glycerol, centrifuge at 3500 x g, 15 minutes at 4^0C, and the supernatant carefully discarded, and the pellet resuspended in a small volume of glycerol (as small as possible). Finally, the harvested competent bacterial cells were put in 20µl aliquots and quickly put in liquid nitrogen and stored at -80^0C.

2.2.2. Electroporation to transfer plasmid to *E. coli*

The pulse (40msec) of high voltage electricity (12,500 V/cm) is used to transfer plasmid DNA to *E. coli*. Plasmid DNAs (1 µl of 100 ng/µl) was mixed with 20 µl aliquot of electrocompetent cells. A control aliquot was used without DNA. Electroporation conditions followed those of the manufacturer of the Cell Porator (life technologies, Inc). When electroporation process was finished, cells were mixed with 200 µl of LB medium, incubated at appropriate temperature and time (37^0C, 30 minutes for *E. coli* and 28^0C, 45 minutes for *A. tumefaciens*). The next step was to incubate in LB agar with suitable antibiotics.

2.2.3. Preparing transgenic tabacum plant samples

The seeds of wildtype and transgenic tabacum were sterilized by ethanol 99% (15 seconds), bleach 10% (10 minutes) and then rinsed 3 times (3X) with sterile water. They were germinated on MS agar plates supplemented with 50 ug/ml spectinomycin and put in the condition with 23^0C, 12 light hours and 12 dark hours. Spectinomycin was used as the selective agent in the generation of the *M* - containing transgenic tobacco (Jeff Ellis; personal communication). After one week, green plants that contained *M* rust resistant gene were selected and transferred to the plots that contained autoclave sterilized soil. These plants were grown at 23^0C with the same rate of light and dark, 12 hours and 12 hours respectively.

2.2.4. Infiltration

Agroinfiltration was an easy and effective way to recombine delivery mutants that contain effector protein into plant cells (Norkunas et al., 2018).

A. tumefaciens (GV3103) containing *AvrM-A* or *avrM* were incubated in LB liquid supplemented with 50 µg/ml kanamycin, 50 µg/ml gentamycin and 25 µg/ml rifampicin for 48 hours. Harvested agrobacterium cultures, were centrifuged and measured OD_{600} in 10mM MES buffer (pH 5.6). Then, put 10mM MgCl2 and 200 µM aceto-syringe to the collect culture to prepare for infiltration (Krasileva et al., 2010, Williams et al., 2011). Incubated the bacterial suspension for 4 hours at room temperature.

Selected the fourth leaves of 4-5 weeks old plants (both W38: *M* and *N. benthamiana*) to use for infiltration. Next, created a small nick in the leaf by a scalpel blade, used a 1ml needleless syringe containing the *Agrobacterium* suspension. Slowly put light pressure to the lower side of the leaf (Ma et al., 2012). Finally, put the plant at 23^0C and balance the light and dark.

2.2.5. Protein extraction

Collected the leaves after infiltrating one day by a cork borer and store in the -80^0C. Ground the samples by a motorised pestle at 4^oC. Put 110µL of 3X Laemmli buffer (0.24M Tris-Cl pH 6.8, 6 % SDS (w/v), 30% glycerol (w/v), 0.006% bromophenol blue, 16% β-mercapethanol, 5M urea) to the sample, after that mix totally the sample and 3X Laemmli buffer by using vortex in short time at high speed. Then, the samples were heated at 97^0C for 5 minutes. Next, centrifuged at 10,000 x g in 10 minutes at room temperature. Lastly, collected the supernatants and ready for loading in SDS-PAGE.

2.2.6. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A 15% acrylamide gel (components were present in Appendix 1) was used to separate the protein the samples. Loading the supernatants (see Sector 2.2.5) to the gel. Set up 170V in 60 minutes. Pre-stained ladder present in each gel to know the size exactly in each protein. Two gels were

run at the same time, then, one gel was used for Coomassie blue, and another one was the immunoblot analysis.

For Coomassie blue, the gel was put in fix solution (acetic acid 10 % (v/v), ethanol 40 % (v/v)) for 30 minutes, after that incubate with Coomassie (Fixing solution + coomassie brilliant blue R-250 0.1% (w/v)) overnight and wash Coomassie with DH_2O, all of the stages at room temperature. Visualized in the machine a Bio-Rad Gel Doc^{TM} EZ imaging system.

For immunoblot analysis, transferred the gel to the nitrocellulose blotting membrane at 60V for 90 minutes in the cold room condition (4^0C). After that incubated with block buffer (20 mM Tris pH 7.4, 0.1 % tween (v/v), 150 mM NaCl, 5 % (w/v) skim milk powder) for 1 hour, then incubated with primary antibody (mouse anti-HA (clone B12; Covance, Emeryville, CA, USA), 1:5,000 dilution). Moreover, washed with fresh blotto (the same components with the block buffer) then incubated with secondary antibody (the horseradish peroxidase conjugated goat anti-mouse (Rockland, Gilbertsville, PA), 1:10,000 dilution) and washed three times and 10 minutes/time in the shaker at 80 rpm with TSB-T buffer (20 mM Tris pH 7.4, 0.1 % tween (v/v), 150 mM NaCl). Incubated with Chemiluminescence reagents that provided by Clarity ^{TM} Western ECL substrate of BIO-RAD. Completely, the blot is visualized in the Bio-Rad ChemiDoc^{TM} MP system. After that, the blot also incubated with Ponceau stain for 10 minutes on the shaker at 60 rpm, washed with sterile water and visualized in the Bio-Rad ChemiDoc^{TM} MP system to see the Rubisco protein.

2.2.7. Plasmid purification

Plasmid purification followed the Wizard plus SV minipreps DNA purification system (Brisco et al., 1996). Electrophoresis 1% (W/v agarose) was used to check the purification of the plasmid (Sambrook et al., 1989a).

2.2.8. DNA sequencing

Before sending the sample for sequencing, plasmid DNA was extracted from Agrobacterium cells using the Wizard® Plus SV Minipreps DNA Purification system (Promega). The DNA sample was collected and stored at -80^{0}C. After that, the plasmid DNA was transferred to *E. coli* by heat shock transformation. A colony was picked and cultured overnight. Plasmid DNA was extracted from the cultured cells using the Wizard® Plus SV Minipreps DNA Purification system (Promega) and a sample sent for DNA sequencing according to the recommendations of the Australian Genome Research Facility (AGRF). The sequences of primers were presented in Appendix 6.

2.2.9. Protein Quantitation

The amount of protein loaded in each well was measured by the Coomassie stain of RuBisCo protein using ImageJ (Davarinejad, 2017). Data was collected in each well by ImageJ and analysed to Excel to build a chart.

2.2.10. Methods for light experiment

The 6-week-old W38: *M* plants were put in a chamber without light for different periods of time (72, 48, and 24 hours) before infiltrating with Agrobacterium (strain GV3101) containing the *AvrM* gene. Following infiltration, plants were kept in darkness and tissue samples were collected 24 hours after infiltration. Two controls were tested, one plant kept in 12 hours light and 12 hours dark regime for the entire experiment, and those plants maintained in 12 hours light 12 hours dark, but then kept in darkness post infiltration. All samples were collected 24 hours after infiltration. Recording of the HR and protein extraction, SDS-PAGE and Western Blot with an anti-HA primary antibody followed the methods outlined in sections 2.2.6, 2.3, 2.4, 2.5 of Chapter 2.

CHAPTER 3: RESIDUE MUTANTS IN *AvrM* AND *avrM*

3.1. Introduction

The war between pathogens and plants has evolved over millions of years (Chakraborty and Newton, 2011). The pathogens transformed themselves to avoid the recognition of the plant immune system (Jones and Dangl, 2006). In order to understand the interaction of plant defence systems and effector molecules from the fungus *in planta*, we conducted an assay of the HR in M-containing transgenic tobacco leaves after Agro-infiltration of 19 different mutants in both effector protein AvrM and avrM. Firstly, we conducted a time course experiment to know the suitable time to collect the samples to have the highest AvrM protein content. The results show that the time point at 20 and 22 hours after infiltrating is a good time for the accumulation of effector protein in W38: *M* and the start time can see the HR is 18 hours. After 30 hours, little effector protein was present. Furthermore, the presence of 19 mutants of both the AvrM and the avrM *in planta* will be described to show the essential amino acid in AvrM and avrM that can knock in and knock out the M recognition of plant.

3.2. Results

3.2.1. Selection of W38: *M* for the experiment

The W38: *M* seed was selected and generated on the MS agar plates containing spectinomycin with 50µgL-1 concentration. After two weeks, the seed had germinated. There were two kinds of plants from the same original seed in which the bleached plants were the non-transgenic plants and was removed whereas the green plant was the resistant plants. Next, the green plant was selected and transferred to small soil plots. After 4 to 6 weeks transferring in the soil condition, the green plants were used as an assay for HR with infiltrated effector protein *in planta*.

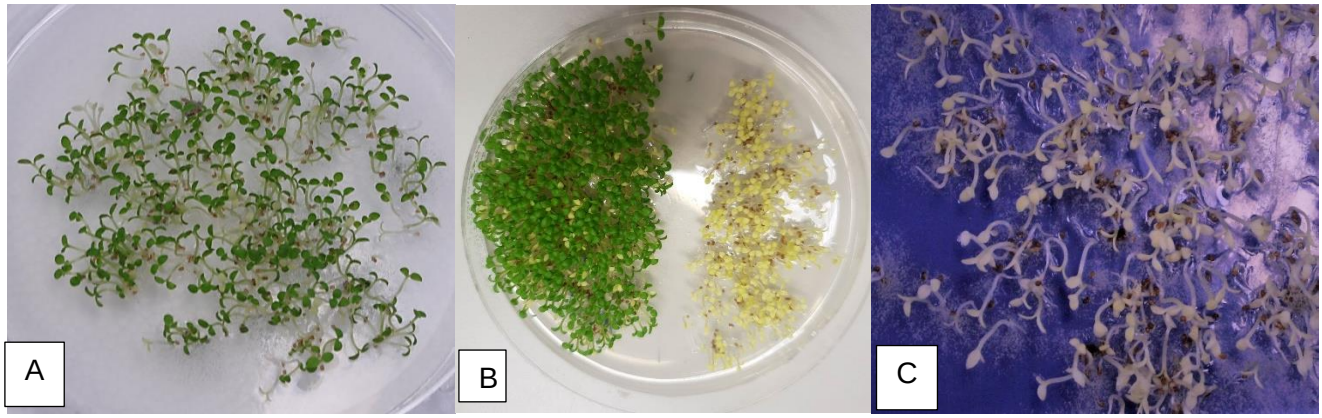


Figure 3.1. Screening of control and W38: ***M. The seeds were cleaned and geminated in the agar plates containing the MS culture supply with Spectinomycin 50mgL-1, after that these plates were put in the chamber with 12 hours light and 12 hours dark (followed sector 2.2.3). After one week, the seedings of the W38: M in (A); the*** **W38:** ***M (green colour - left) and control (white colour – right) in (B) and the control in (C).***

N. benthamiana seed was sown directly into soil. These plants could use for infiltration 5 to 6 weeks after germinating. To prevent the development of the harmful fungus in soil, the autoclaved soil was used in all stages.

Both the resistant plants (W38: *M*) and non-transgenic plants (*N. benthamiana*) were put in the same conditions in the chamber with 23^0C and 12 hours dark/12 hours light.

3.2.2. Time course of effector protein expression

The purpose of this experiment was to determine the suitable time to see the HR and compare with AvrM protein expression *in planta*. From the observed results shown in Figure 3.2 the HR was visible from 18 hours post infiltration. In general, the leaves did not change from the time 0 to 12 hours, however, after that from 18 to 48 hours have seen the HR and the level of the HR have grown along with the last of the time.

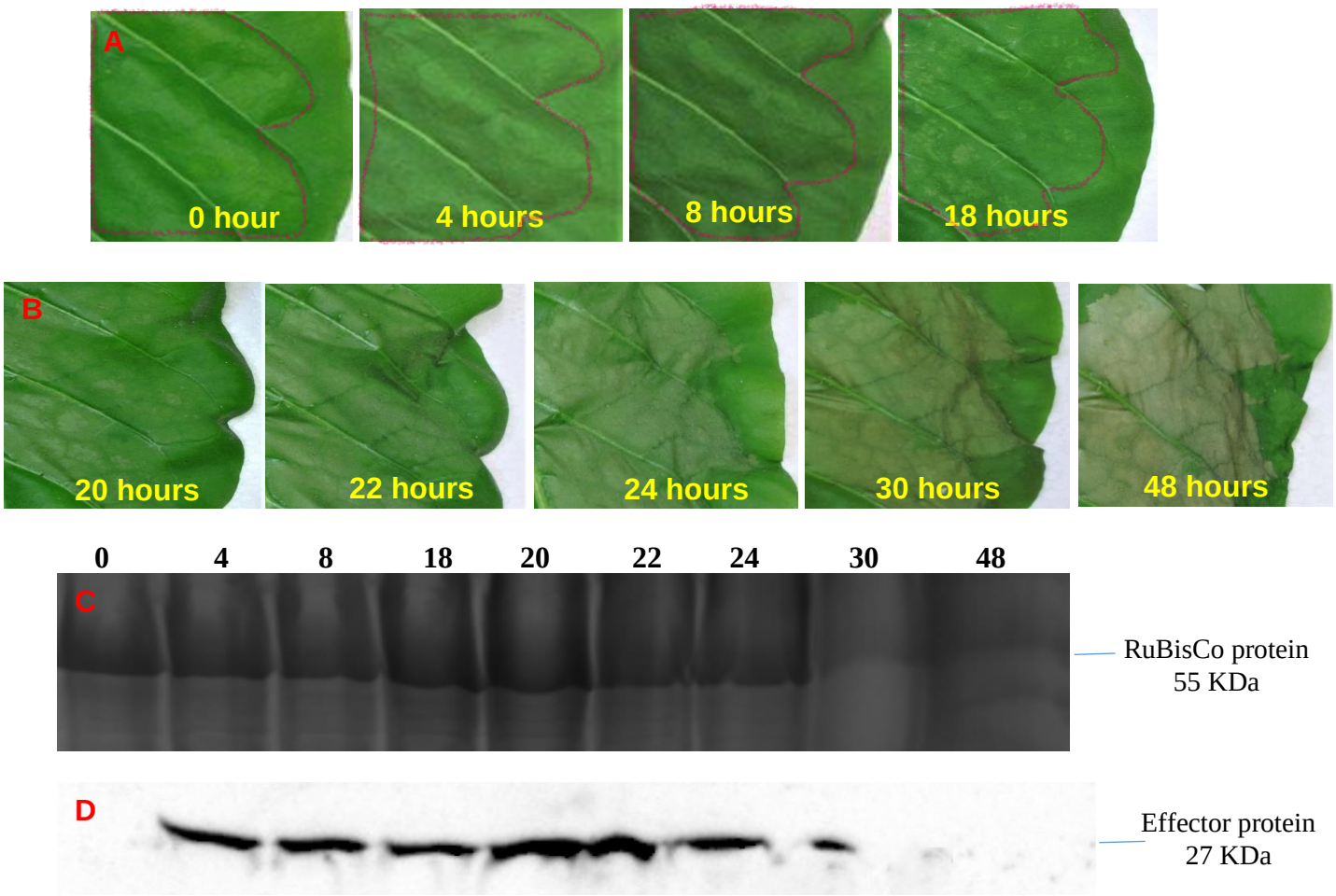


Figure 3.2. Time course experiment for AvrM effector expression at different time points.

6-8 weeks-old W38: M were infiltrated with Agrobacterium containing the AvrM effector at a concentration of OD_{600}nm = 1. Before and after infiltrating the plants were grown in the chamber with 12 hours light, 12 hours dark and 23^0C. A + B: Leaves after infiltrating follow the time course at 0, 4, 8, 18, 20, 22, 24, 30 and 48 hours; C: The leaf tissue at these times was collected and put at -80^0C, followed by protein extraction, SDS-PAGE and incubated overnight with Coomassie Blue. D: Protein expression of time course leaf tissue samples, run SDS-PAGE and analysed by Western Blot with using the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies for primary and secondary antibodies, respectively. The red line shows the infiltrated sectors.

The time at 20 and 22 hours after infiltrating had the highest effector protein and combined with the gel results in the Coomassie blue showed that at the time point 20 hours, the amount of

RuBisCo protein was the highest. Therefore, 20 hours after infiltrating was selected for collecting the samples in the next experiments.

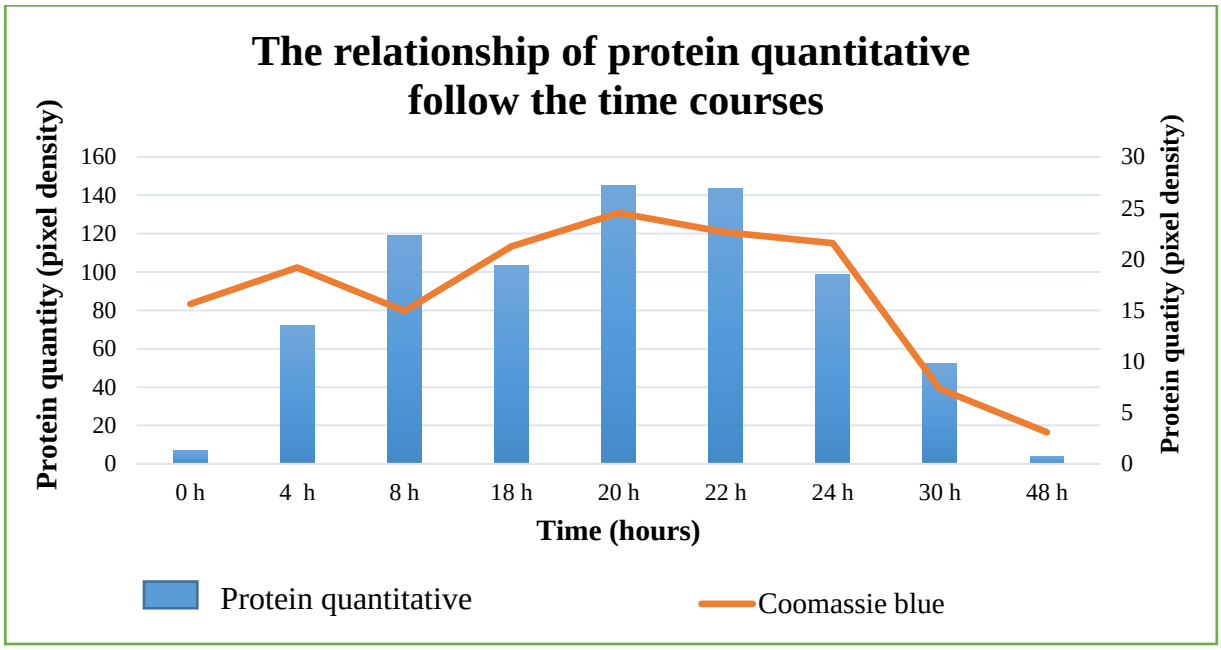


Figure 3.3: The relationship of effector protein and the time course.

The protein quantity was created by ImageJ method (section 2.2.10). The blue column showed the protein level of effector protein, whereas the orange line was RuBisCo protein.

Figure 3.3 showed that effector protein was expressed at detectable levels after 4 hours and increased gradually through to 24 hours after which the HR was clearly visible time.

3.2.3. The activity of AvrM, avrM and GV3101 in the HR

This work aims to see the change in the HR and the effector protein expression in the leaves. After 20 hours post infiltration, samples were collected, protein extracted, and SDS-PAGE and Western Blot analysis was performed. This experiment showed that the effector protein AvrM results in the full HR, whereas avrM and GV3101 did not. The AvrM and avrM proteins were detectable while not immune-reactive proteins could be seen in the GV3101 infiltrated and un-infiltrated leaves. The photo had taken four dpi, three replications *in planta* and four replications in the Western Blot. The results are presented in Figure 3.4.

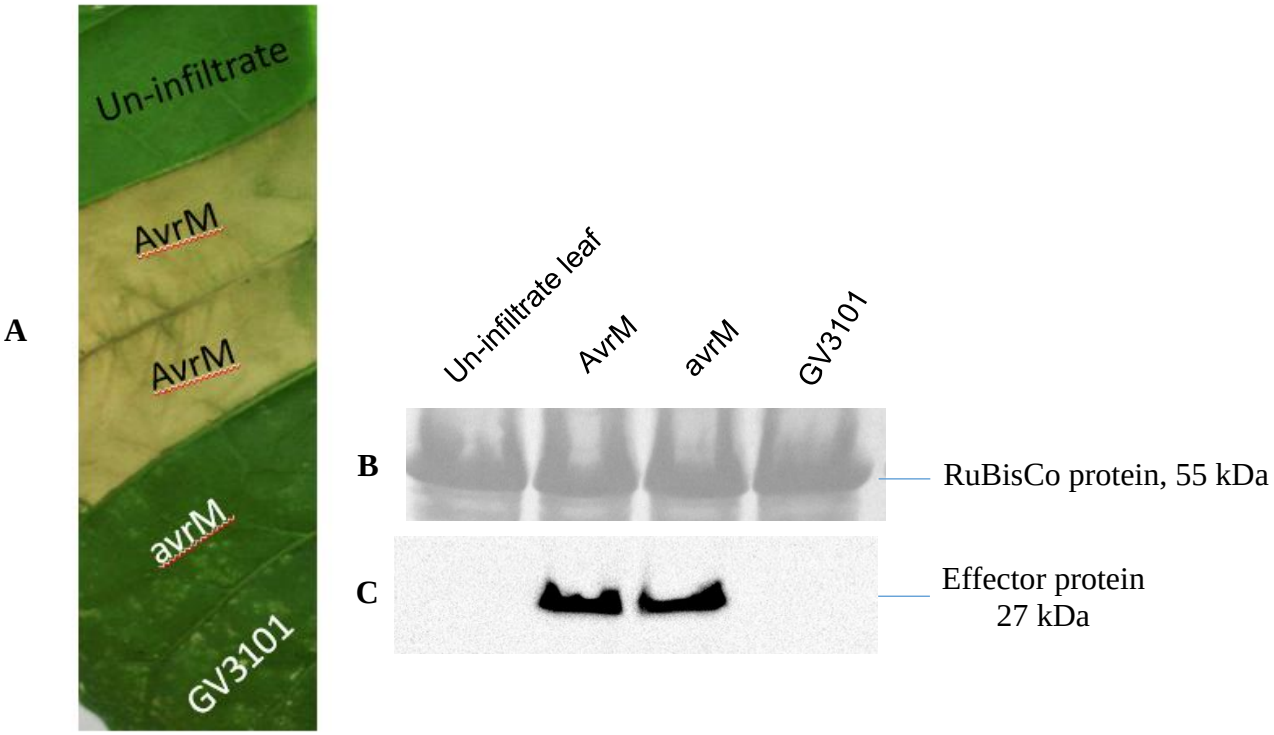


Figure 3.4: The activity of un-infiltrate leaf, *AvrM*, *avrM* and GV3101 and their proteins in the Blot.

(A): 6-8 weeks old of W38: M were infiltrated with Agrobacterium contains effector protein AvrM, avrM and GV3101 at concentration 1M. (B) RuBisCo protein content and (C) effector protein content of total protein extracts determined by ponceau staining and anti-HA immuno labelling with the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies, respectively.

3.2.4. The recognition of M resistant protein to non-polymorphic mutant residues in *AvrM*

Based on the crystal structure of the AvrM, the amino acids, namely E237, E309 and R313, are in the interface of the dimer and play an essential role in M recognition. The combination of these amino acids created a negative charge at a pocket of the dimer interface of the AvrM effector. Rahman (2016) showed that M recognition could be knocked out if the central cleft of the AvrM dimer was changed from a negative to a neutral charge (Alanine(A)). The results showed that single mutants E237A, E309A and R313A, and double mutants AvrM E237A + E309A,

E237A+R313A and E309A + R313A had a strong HR indicative of M recognition, whereas the triple mutant AvrM E237A + E309A + R313A had partially lost M recognition. The detail of this result is presented in Figure 3.5 below. The photo has taken five days after infiltration. The experiment was repeated five times *in planta* and four times by Western Blot.

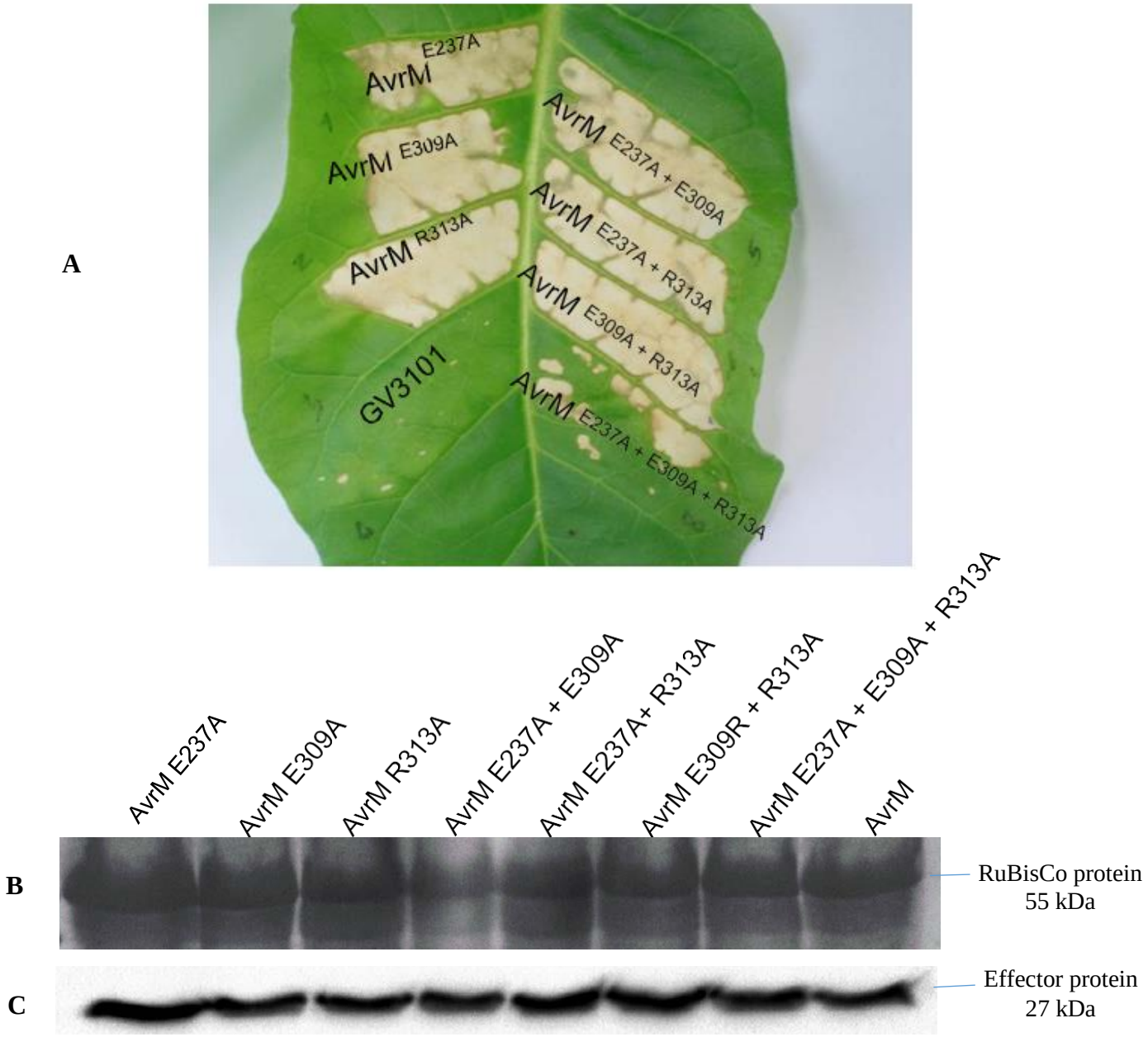


Figure 3.5: The *AvrM* mutants *in planta* and Western Blot

(A) The leaf of* W38: *M was infiltrated with Agrobacterium contains AvrM mutants namely, AvrM E237A, AvrM E309A, AvrM R313A, AvrM E237A + E309A, AvrM E237A + R313A, AvrM E309A + R313A, AvrM E237A + E309A + R313A and GV3101. (B)

RuBisCo protein content and (C) AvrM mutants protein content of isolated protein extracts from infiltrated leaf tissue in N. benthamiana was determined by ponceau staining and immune labelling with the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies, respectively.

The result after infiltrating in W38: *M* leaves showed that single and double mutants AvrM E237A, E309A, R313A, E237A + E309A, E237A+ R313A, E309R + R313A had the fully HR while only the mutant AvrM E237A + E309A + R313A has a loss of HR with M resistance protein. However, all mutants had the equivalent protein in the blot. These results are almost consistent with those of Rahman (2016) except that the double mutant (AvrM E237A + E309A) in Rahman's hands showed a partial loss of M recognition. In order to check if this was a result of a labelling error of the Agrobacterium stock on the part of Rahman, plasmid DNA extraction and sequence analysis was conducted to confirm the sequence. Appendix 2 showed the sequence of the AvrM E237A + E309A mutant and shows that the labelling of the tube was correct.

In order to check if the M recognition of the AvrM E237A + E309A mutant was compromised, infiltration was done at lower concentration of Agrobacterium and compared with that of AvrM containing Agrobacteria. These results are shown in Fig 3.6. It is clear from these results that M recognition by AvrM E237A + E309A cannot be differentiated from that of AvrM and this represents a difference between this study and that of Rahman (2016). This significance of this result will be discussed in Section 4.2.

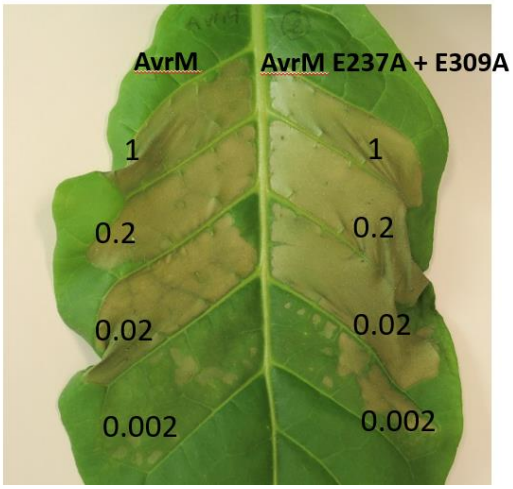


Figure 3.6: The dilution of AvrM E237A + E309A.

The leaf of* W38: *M (8 weeks old) was infiltrated with Agrobacterium contains AvrM E237A + E309A at distinct concentrations 1, 0.2, 0.02 and 0.002 OD at 600nm. The photo was taken four days post infiltration. Expression of AvrM and AvrM E237A + E309A protein showed full HR in the leaves at an Agrobacterium concentration of Od 600nm of 1.0, 0.2 and 0.02, whereas those effector proteins revealed weak and partial HR at a concentration of 0.002 OD at 600nm.

3.2.5. The recognition of combined mutants in *avrM by the M resistant protein*

The M resistant protein did not recognise avrM, and the single mutants of avrM whereby polymorphic residues of AvrM and avrM where changed from their avrM amino acid to the amino acid in AvrM could not knock-in M recognition. However, when the amino acid residues in *avrM* at the positions R170, S179 and T247 where changed in combination (avrM R170K+S179L and avrM R170K + T247I) a partial HR was observed. Moreover, the double mutant avrM S179L + T247I, the triple mutants avrM R170K+ S179L + T247I and the four combined mutant observed a full HR. Therefore, the double mutant, avrM S179L + T247I was crucial in knocking in the M recognition. The experiment was done five times *in planta* and four times by Western Blot. The photo shown in Figure 3.7 was taken five days post infiltration.

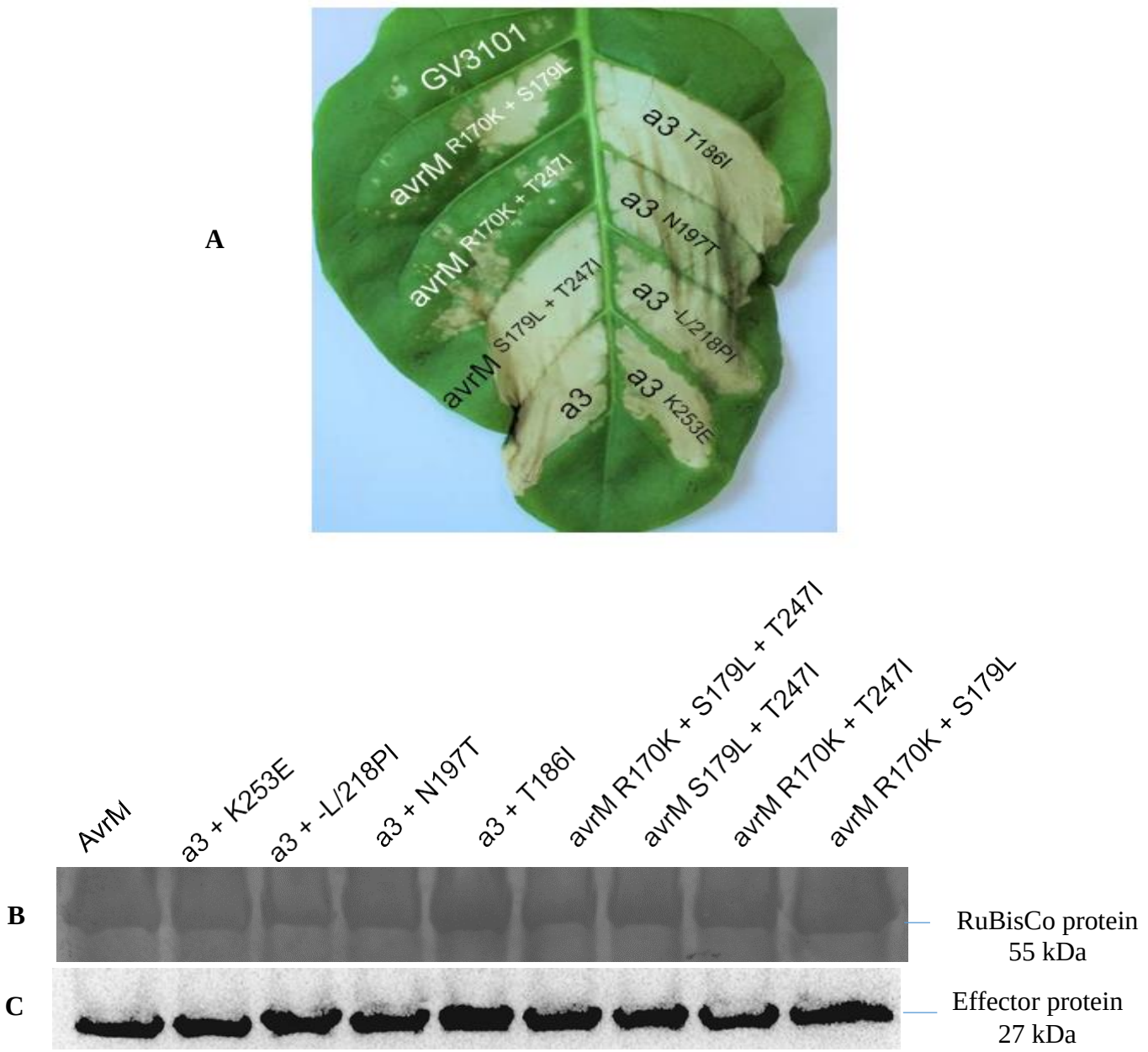


Figure 3.7: The *avrM* mutants *in planta* and Western Blot

(A) The leaf of W38: M was infiltrated with Agrobacterium contains avrM mutants namely, avrM R170K+S179L, avrM R170K + T247I, avrM S179L + T247I, avrM R170K+ S179L + T247I (a3), avrM a3+ T186I, avrM a3+ N197T, avrM a3 + -L/218PI, avrM a3 + K253E and GV3101. (B) RuBisCo protein content and (C) avrM mutants protein content of isolated protein extracts from infiltrated leaf tissue in Nicotiana Benthamian was determined by ponceau staining and immune labelling with the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies, respectively.

In term of effector protein expression, all mutants showed an equivalent level of expression, and the level of the RuBisCo protein demonstrated that equivalent levels of total protein was extracted and loaded on the gel.

3.2.6. The recognition of M resistant protein to polymorphic mutant residues in AvrM

To test if the amino acids in AvrM at positions K232, L241, and I310 (analogous to the avrM residues R170, S179 + T247) could knock out M recognition, the reciprocal substitution were made in AvrM. As seen in Figure 3.8, both double and triple mutants could not knock out M recognition *in planta*. Relative to the level of RuBisCo protein, the level of effector protein expression of each mutant in these leaves was approximately equivalent. The experiment was done five times *in planta* and four times by Western Blot. The photo shown in Figure 3.8 was taken four days after infiltration.

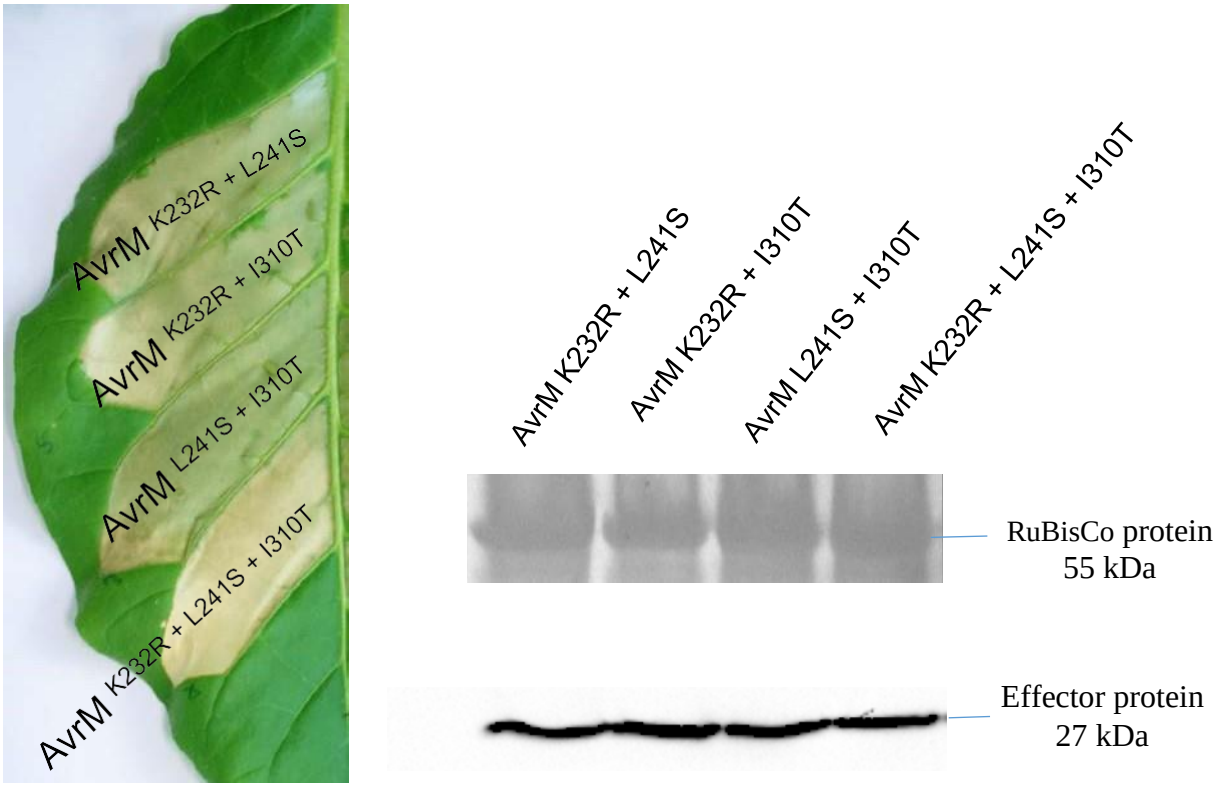


Figure 3.8: The reciprocal mutants of the *AvrM in planta* and Western Blot.

(A) The leaf of W38: M was infiltrated with Agrobacterium contains AvrM mutants namely, AvrM K232R+L241S, AvrM K232R + I310T, AvrM L241S + I310T and AvrM K232R + L241S + I310T. (B) RuBisCo protein content and (C) AvrM mutants protein content of isolated protein extracts from infiltrated leaf tissue in N. benthamiana was determined by ponceau staining and immune labelling with the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies, respectively.

These mutants in AvrM K232R+L241S, AvrM K232R+ I310T, and AvrM L241S + I310T showed a full HR. Nevertheless, the triple mutants AvrM K232R + L241S + I310T displayed the highest HR in comparison with other mutants. The RuBisCo protein had the equivalent level of all samples; therefore, the levels of each mutant effector protein was approximately the same.

Dilution

In order to compare the level of the HR *in planta* between AvrM and other mutants, avrM S179L + T247I, avrM R170K + S179L + T247I and AvrM K232R + L241S + I310T. The solution of these infiltrations was diluted in four distinct concentrations 1, 0.2, 0.02 and 0.002 OD at 600nm. This experiment was conducted three times and these photos taken four post-infiltration, and the results are presented in Figure 3.9.

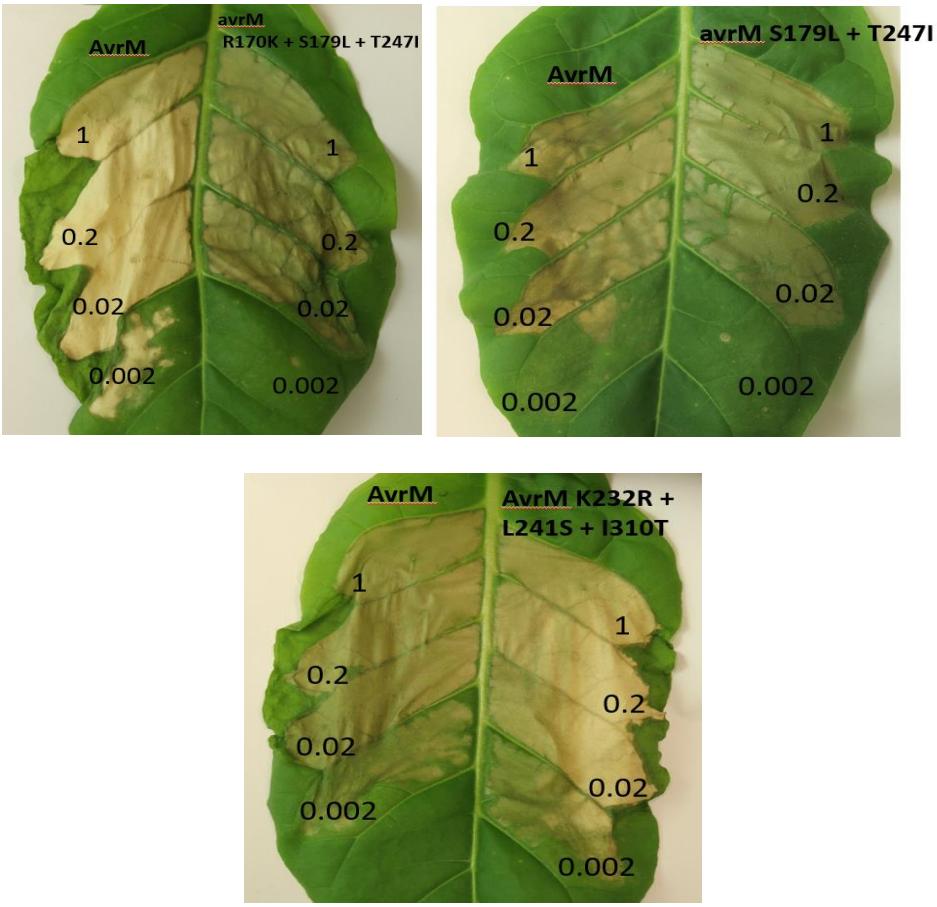


Figure 3.9: The dilution of the infiltrated concentration some mutants avrM R170K + S179L + T247I, avrM S179L + T247I, and AvrM K232R+ L241S + I310T in comparison with the *AvrM*.

The leaf of W38: M was infiltrated with Agrobacterium contains AvrM, avrM S179L + T247I, avrM R170K + S179S + T247I and AvrM K232R+ L241S + I310T at the different concentration of infiltration solution, 0.002, 0.02, 0.2 and 1 respectively. In the infiltrated leaf of AvrM and mutant avrM R170K + S179L + T247I and avrM S179L + T247I a full HR was observed at the concentration of 1.0, 0.2, and 0.02 OD at 600nm; while partial HR found in the infiltrated sector of AvrM and no HR in the mutant avrM R170K + S179L + T247I and avrM S179L + T247I at the concentration 0.002 at 600 ODnm. In the infiltrated leaf of AvrM and the mutant AvrM K232R + L241S + I310T the same level of HR was seen at all these concentrations.

There is not difference between AvrM and any of these mutants except for avrM R170K + S179S + T247I mutant that show a significantly weaker response than that of AvrM at 0.2, 0.02 and 0.002 OD600nm.

CHAPTER 4: DISCUSSION

4.1. Introduction

The plant immune system has two layers to help the plant defend itself against pathogen invasion. In the first layer, the activation of the plant immune system is based on many biological processes inside the plant cell to create an unfavourable environment for the pathogen. In the second layer, the plant can identify the pathogen by the effector proteins that it secretes and reacts to it by programming the death of the infected cell. This identification and response is orchestrated by plant R proteins, namely NLR proteins (Burdett, 2018). Additionally, adenosine triphosphate is exchanged within the NLR protein leading to activation and defence gene induction (Franchi et al., 2009). Ultimately, the response culminates in many cases in hypersensitive cell death, or HR. In some conditions, the functions of NLRs work in pairs to mediate immune recognition (Eitas and Dangl, 2010). An example of this is RPS4 and RRS1 that confer resistance in *Arabidopsis thaliana* to *Pseudomonas syringe* 4 and *Ralstonia solanacearum* 1, respectively (Yuan et al., 2011). These genes were required to recognise AvrRps4 and PopP2 (bacterial effectors). Also, the combination of NLR gene pairs helps rice resist *Magnaporthe oryzae* (Yuan et al., 2011).

In terms of the flax rust fungus, Motuir Rahman's study (2016) showed that single mutants in the flax rust effector proteins, AvrM and avrM, could not knock in or knock out the recognition of the M resistance protein. However, the combination of two or more mutants provided the right signals to activate the M protein and lead to HR *in planta*. From this study, crucial polymorphic and non-polymorphic amino acid residues in AvrM were revealed that control M recognition. In the research presented here, I have shown that single mutants on non-polymorphic AvrM residues, E237A, E309A, and R313A and all combinations of double mutants were recognised by M resistance protein. However, the triple mutant (AvrM E237A + E309A + R313A) can knock down the recognition of the M protein. Conversely, the combination in avrM of several polymorphic amino acid residues could induce M recognition, in particular, avrM R170K + S179L and avrM R170K + T247I induced partial HR, and avrM S179L + T247I induced full HR. It can be

concluded from this work, that the amino acids at the positions 179 and 247 in avrM play an essential role in the recognition of M resistant protein. The combination of two mutants in avrM with one or two other mutants led to full restoration of M protein induced HR. What was surprising was that the reciprocal changes in AvrM in positions 232, 241 and 310 could not knock down M recognition. In term of Rubisco protein that is the most abundance protein in a plant, in fact it is the most abundant protein on the planet. It consists of 50% or more of all protein in the leaves of a plant (Ma et al., 2009). Therefore, when extracting total protein and loading on SDS-polyacrylamide gel, the RuBisCo protein was used as a protein loading control. In the research presented here, the amount of Rubisco protein was equivalent in most of samples.

4.2. The role of non-polymorphic mutant residues in AvrM in recognition by the M resistance protein.

The plant-pathogen interface is diverse, and many specialised pathogens secrete effectors-proteins and metabolites to modulate plant physiology in order to infect and colonize the host. Nevertheless, the presence of secreted effectors also activated intracellular plant immune receptors, especially the notable NLR proteins that helped plant fight off the pathogens (Białas et al., 2017). The P (flax rust resistance protein) recognised by AvrP and AvrP123 (flax rust effector protein) was controlled by polymorphic amino acid located close to the C-terminus of the protein; and AvrP and AvrP123 were bonded to three zinc atoms (Farah, 2018).

In the previous study by Rahman (2016) the M resistance protein could recognize the effector AvrM by a negatively charge in the central pocket that it possesses at the interface of the AvrM homo-dimer. The negative charge was found in the AvrM, but not the avrM, structure (Ve et al, 2011) and by changing the charge of the pocket, recognition by the M resistance protein was decreased or lost. This negative charge is formed by three amino acid residues at the position E237, E309 and R313 in the interface of the AvrM.

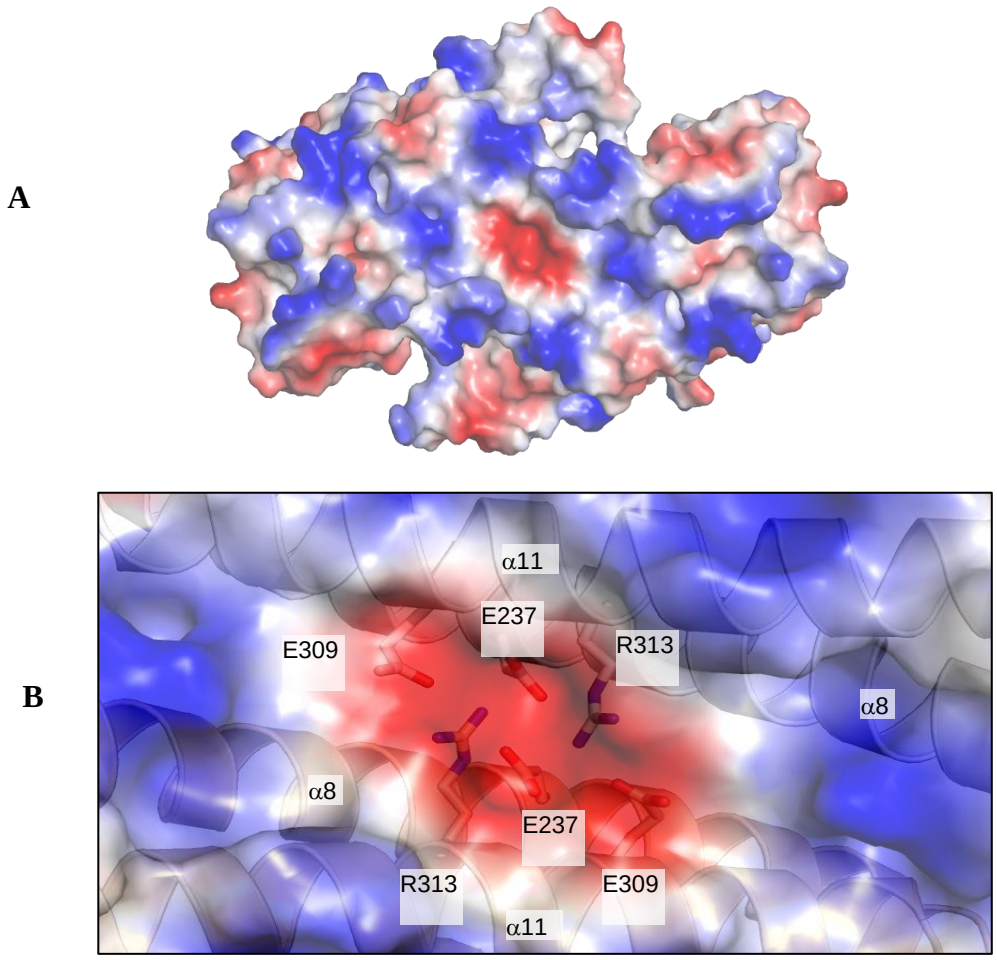


Figure 4.1: The dimer structures of AvrM-A.

(A) Crystal structure of AvrM-A showed the negatived charge in the central pocket of the dimer. (B) The negative surface patch in zoomed revealved the position of amino acid at position E237, E309 and R313 in the interface of the dimer of AvrM-A (Rahman, 2016).

When it comes to the functions in the AvrM molecule, E (Glutamic acid) 237 is located in the α8 helix that is in a vital position to form the central pocket of the AvrM-A dimer. The E brings acidic, polar and negative charge properties whereas the amino acid R (Arginine) 313 possesses basic, polar and positive charge. The central pocket of AvrM-A has two negative and one positive charges; therefore, its central clef will have an overall negative charge. In Rahman's study the single and double mutants (E237A+R313A and E309A+R313A) induced HR, whereas the E237A+R309A and triple mutant combination did not.

In this study, only a triple mutant AvrM E237A + E309A + R313A could knock out M recognition whereas the other single and double mutants (in particular AvrM E237A + E309A) could not. This

difference between my study and that of Rahman (2016), specifically that of the AvrM E237A + E309A double mutant, could be explained by the insensitivity and inherent variability in the agro-infiltration HR assay. Different aged leaves and even leaf positions can lead to slight differences in the HR assay (Rahman; personal communication). Regardless, both the data presented here and that of Rahman (2016) indicate that the central charged pocket in AvrM is important in M recognition. The amino acid glutamic acid (E) consists of only around 2.7% of the total amino acids in *AvrM*, nevertheless it is a genuinely functional amino acid that is important in many signalling and metabolically active proteins (Brosnan and Brosnan, 2013). The amino acid R (Arginine) is a member of a positively charged guanidium group and plays a crucial role in translocation properties of peptides (Pantos et al., 2008). In contrast to E and R, A (Alanine) possesses the simple structure that is the second simplest of all amino acid and it is rarely involved in protein function. Therefore, when amino acid at position E237, E309 and R313 transfer to Alanine, may lead to the break of many functional bonds inside the central pocket of the AvrM that prevent M recognition and activation. The Western blot data presented in this study confirms that levels of AvrM protein expression of all mutants are equivalent and therefore differences in proteins levels cannot explain difference in HR.

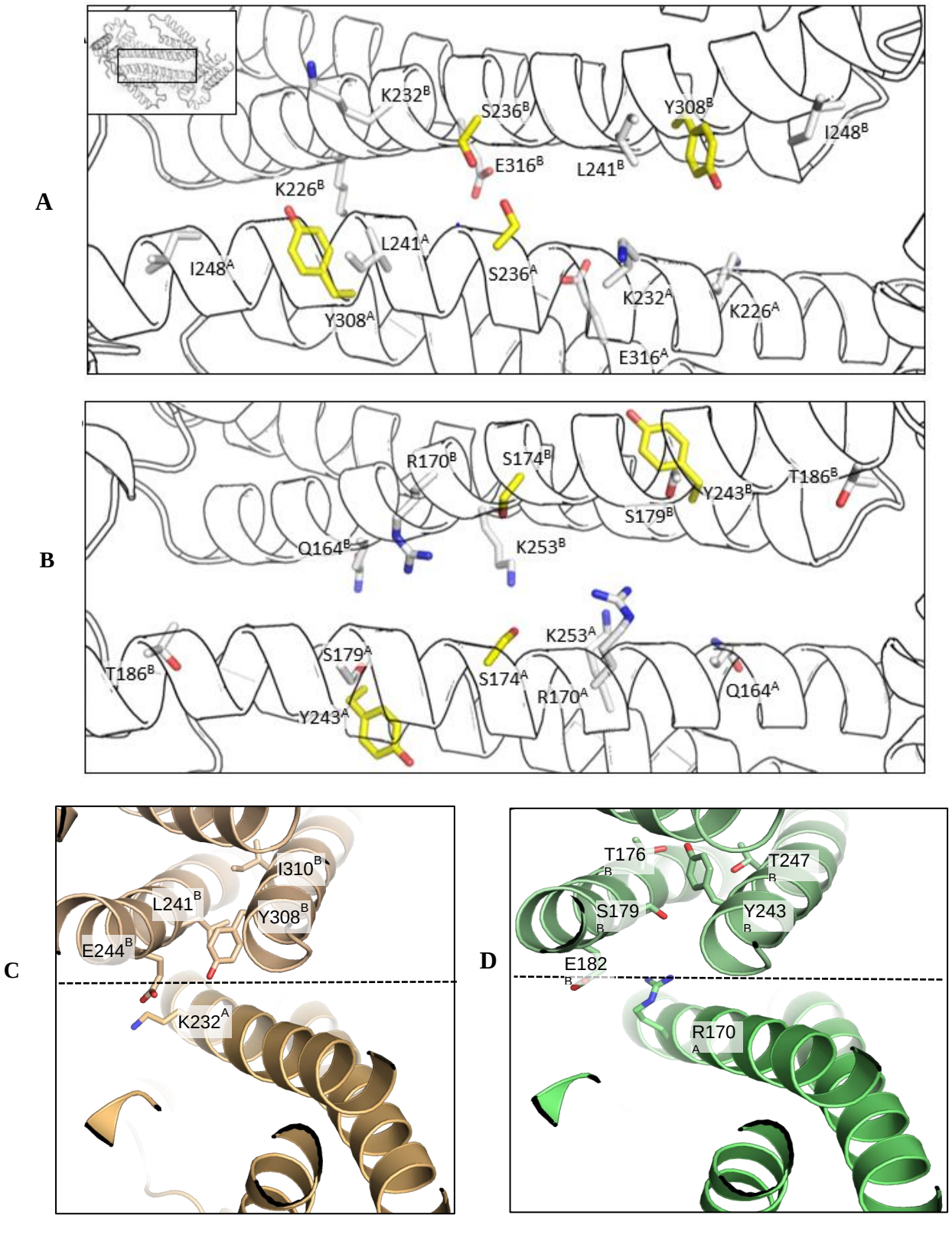
4.3. The combined mutants in *avrM*

The avrM R170K + S179L and avrM R170K + T247I are present in the α8 helix. The changing of the amino acid residues in the mutants avrM R170K + S179L and avrM R170K + T247I lead to the partial HR. The amino acid in position 170 of avrM is R (Arginine) that has similar properties as K (Lysine) found at the same position in AvrM. However, the amino acid in positions 179S and 247T of avrM, compared with 241L and 310I in AvrM have different properties (nucleophilic to hydrophobic – Appendix 3). Changing the two amino acids in avrM S179L + T247I, by the combination of two hydrophilic amino acids can activate the recognition of M protein. The combination of S179L + T247I in *avrM* showed full HR. Therefore, these amino acid residues play an essential role in the recognition of M protein.

The combined triple mutant (avrM R170K + S179L + T247I) and the quadruple mutants (avrM R170K + S179L + T247I + T186I; avrM R170K + S179L + T247I + N197I; avrM R170K + S179L + T247I + ▲L/218PI, and avrM R170K + S179L + T247I + K253E) increase the hydrophobicity of the dimer interface. Thus, the hydrophobic and acidic groups in these mutants combined and changed the charge of the *avrM*. Because of this reason, the M protein can recognise these mutants leading to full HR *in planta*.

Based on the structure of avrM shown in Figure 4.2 (B+D), the side chain of Y243 (tyrosine) was pointing inwards into the avrM monomer structure and not outwards into the dimer interface in the AvrM structure.

In comparison with Rahman's results, mutants of avrM (R170K + S179L and R170K + T247I) did not show any HR, whereas they showed partial HR in this study. All of the other mutants in *avrM* observed partial or full HR in Rahman's study, whereas they showed full HR in this research. The reason for these differences may be due to variation in the age of the leaves and the position of the leave sector used for infiltration, as discussed above.



"Reused with permission" ***(Simon William, unpublished data).***

Figure 4.2: The dimerization crystal structure of *AvrM* and the *avrM*. *(A-B) The comparison in the interface in the structure of AvrM-A (A) and avrM (B). These crucial amino acid*

residues were highlight in A and B. These amino acids had red colours showed the negative charge whereas that of the blue colours were positive charged. Amino acid (Y308) had yellow ring colours participate in the AvrM- interaction; however, that was not presented in that of avrM (Y243). (C-D) Two promoters of AvrM-A (C) and avrM (D) were separated by the dotted line. These amino acid residues in the interface of AvrM- A namely K232, E244, Y308, I310 and L241 and that of avrM such as R170, S179, T176, T247 and Y243 (Simon William, unpublished data).

4.4. The recognition of M resistance protein to polymorphic mutant residues in *AvrM*

Based on the crystal structure of the *AvrM*, the amino acid residues K232R, L241S and I310T located in the α8 and α11 helices are likely to be important in securing the dimerization of AvrM. Changing the amino acid residues in avrM to their AvrM counterparts could knock in M recognition whereas the reciprocal changes in AvrM could not knock out or knock down M recognition. Based on the Figure 4.2 (A), it can only be concluded that other polymorphic residues in AvrM (namely at positions K226, I248, E316, S236, and Y308) are sufficient to provide compensatory structural support for AvrM dimerization and M protein recognition.

The change of S (Serine), T (Threonine) and R (Arginine) in AvrM are likely to provide structural support to the α11 helix to present the Tyrosine ring to the dimer interface. This may increase the link between amino acids in the vicinity of this tyrosine side chain and help stabilize the AvrM dimerization. As shown in Figure 4.2 (A and C), the dimeric AvrM molecule has ring structure at position Y308. This ring structure may form a bond with K226. It is also possible that the E316 and S236 in adjoining protomers interact because of the orientation of the helices that is stabilised by the polymorphic residues mentioned above. Collectively, these amino acid residues help the M resistance protein recognise these effectors and signal the HR.

4.5. Conclusion

Determining the crucial amino acid residues in a fungal effector protein that control NLR protein recognition is essential to help our understanding of the plant immune system. In this research, I have studied the interaction between the effector protein AvrM, avrM and the M resistance protein in *N. tabacum* and achieved some positive results.

Firstly, single mutants in the *AvrM* cannot change the recognition of the M resistance protein, and it did not affect the HR. However, double, triple or more combined mutants could knock in and knock out M recognition because the change of two or more amino acid residues in important position leading to a change in the side chains and structures as well as the charge of the protein molecules. Secondly, the changing charged and combined triple mutants in the central pocket of the AvrM can knock out M recognition.

Also, double to four polymorphic residues mutants in *avrM* can change the HR in which the double mutant avrM S179L + T247I observed full restoration of HR, and other three and four mutants showed the full HR as well.

Lastly, the mutants in the non-polymorphic residues *AvrM* at position E237, E309 and R313 had the opposite effect of knocking out M recognition. The molecular weight of the amino acids does not have a significant difference and all of mutants and the AvrM and avrM were expressed at equivalent levels *in planta*. Collectively, these data indicate that key polymorphic residues between AvrM and avrM are important in the orientation of the α8 and α11 helices in order to stabilise AvrM dimer formation and the presentation of the charged pocket at the dimer interface that is important in M recognition.

4.6. Future direction

The expression of the level of the HR in leaves reflect the recognition ability of the plant immune system; However, the changing of molecular structure of these mutants are only predicted. Therefore, if we can do the depth studies about the molecular crystal analysis and the interaction

of amino acid residues of the mutants, we will have more accurate conclusions about the link between them.

Beside amino acids that were position at E237, E309 and R313 in AvrM, it is stressed that amino acid at the position Y308 may also be essential because of the ring structure and its link with other amino acid residues. Therefore, an important next experiment would be to create this new mutant in this position and see the HR elicited in M transgenic tobacco. This will help us obtain more understanding about the crucial amino acids in this effector protein.

Understanding the structure of effector protein opens the key to understand how effector/NLR interaction takes place. This knowledge is critical if we are to generate new genetically modified crops in the future. Rust diseases present in many stable crops, and for this reason, experimenting with other rust diseases, such those that infect wheat and rice will translate this fundamental knowledge into improving world food security in the future.

CHAPTER 5: THE LIGHT AND HYPERSENSITIVE RESPONSE

5.1. Introduction

In plants there is a balance between photosynthesis and respiration to help the plant to grow and develop. Moreover, the plant immune system works to naturally detect and respond to pathogen attack. When the AvrM/M interaction was performed in plants pre-treated with darkness, the development of the HR was compromised. Therefore, light plays an essential role in the host-pathogen interaction and light is crucial in the plant defence response. Despite a link between light and the development of the HR reported in the literature (discussed below), a detailed understanding of the role of light in the second layer of plant immune systems is limited and further research is necessary (Karpinski et al., 2003). In this study, by putting plants in the dark for a pre-treatment or 48 to 72 hours before infiltrating with *AvrM*-containing Agrobacteria, delayed the HR in comparison with the control plants.

5.2. Results

The results for the HR assay showed that the pre-treatments of plants for 72 and 48 hours in darkness before infiltrating were different to that of 24 hours dark pre-treatment, and the two controls; dark after infiltration (Dark A24h) and the constant 12 hours light and 12 hours dark treatment. In the plants pre-treated with three days in darkness before infiltrating, the hypersensitive respond presented only in small areas in the leaf whereas in the two days pre-treatment, the hypersensitive respond demonstrated a higher level of the HR than that of three days pre-treatment. However, 1day pre-treatment and dark after infiltrating and the other control (plants put in 12 hours light and 12 hours dark before and after infiltration) showed a full developed HR. The experiment was done four times *in planta* and three times by Western blot. The photo has taken 24 hours after infiltration.

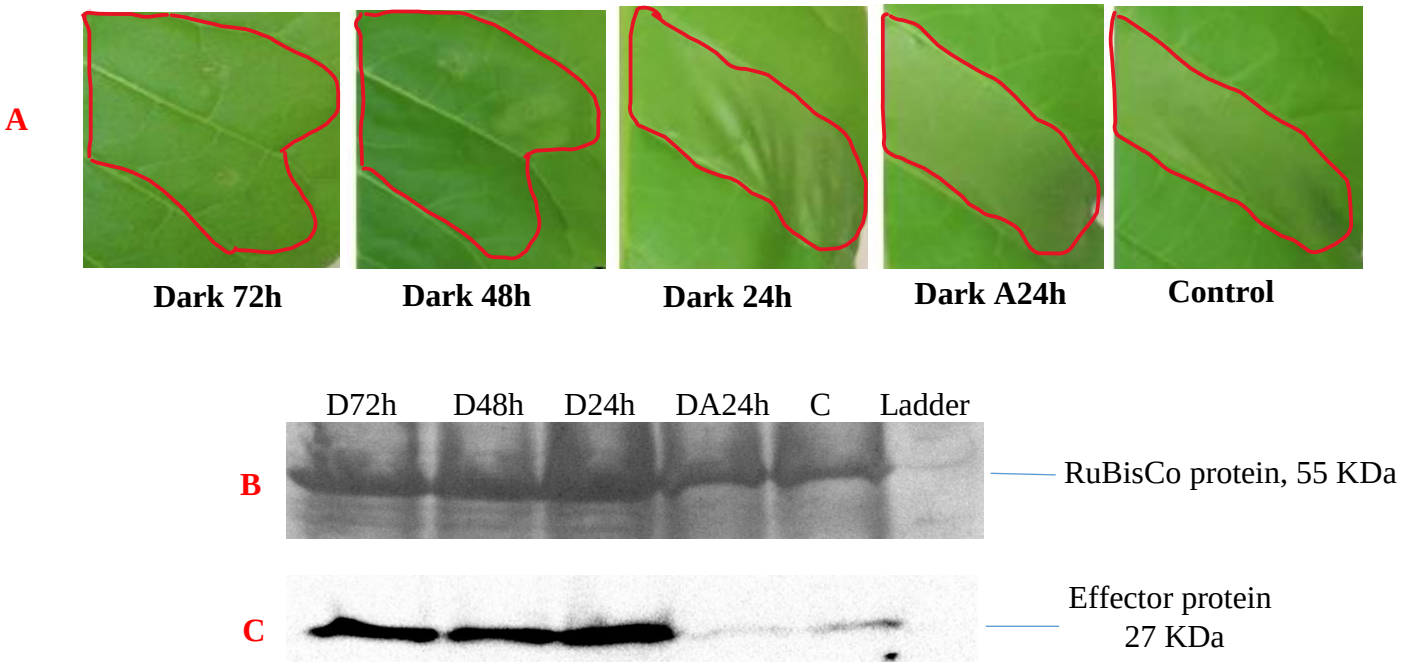


Figure 5.1: The development of HR in W38: M is dependent on prior light exposure. (A) N. tabacum plants were grown in the dark for 72 hours (D72h), 48 hours (D48h), or 24 hours (D24h) prior to infiltration with Agrobacterium containing the* AvrM *effector gene. After infiltration all plants were kept in the dark for a further 24 hours, except for the control (C) that was returned to normal 12 hours light:12 hours dark growth conditions. (B) RuBisCo protein content and (C) AvrM protein content of isolated protein extracts from infiltrated leaf tissue was determined by ponceau staining and immuno-labelling with the mouse anti-HA antibodies and horseradish peroxidase conjugated goat anti-mouse, respectively. The infiltrated sector is outlined in red.

In terms of AvrM protein expression, the 72, 48 and 24 hours dark pre-treatment had higher levels of protein compared to the Dark A24h and control treatment. This is interesting because the 24 hours dark pre-treatment led to more AvrM protein but also complete M triggered HR, whereas the 72 and 48 hours dark pre-treatment had similar levels of AvrM accumulation 24 hour after infiltration, but showed very limited HR.

5.3. Discussion

In a previous study the auto active Maize resistance gene mutant, Rp1-D21, relies on temperature and light for phenotype expression (Negeri et al., 2013). Moreover, light also related to the presence of biotic stress responses (Mühlenbock et al., 2008, Kangasjärvi et al., 2012). There are many reasons related to the delayed HR in the darks; it could be the insufficiency of ATP or ROS generated from photosynthesis or another product of the light reaction. This is discussed as follows.

The formation of HR could be related to the process of ATP formation in plant cells. Based on Figure 5.1 the longer the pre-treatment in the dark, the lower of HR in the leaves. The level of HR also inversely proportional to the amount of the protein in the blot. The lower or higher of light reaction will affect to the regeneration of molecules such as $NADP^+$, ADP and Pi; Thus, the photosynthetic electron transport will be limited, leading to less of the reactive molecules mentioned above (Kim et al., 2008, Rott et al., 2011). The AvrM effector protein interacts directly with the M resistance protein as determined by yeast 2-hydrod analysis (Catanzariti et al, 2011) but because of the low levels of ATP derived from photosynthesis, there may be less activation of the resistance protein. In order to activate the HR, plant NLRs need to rearrangement their structure and this requires a combination of the activating elicitor and ATP (Bentham et al., 2016). In other words, the interaction of AvrM and M was less than that in stable light conditions. Thus, HR is reduced or prevented by starving the plant of light. The HR phenotype of the leaf is also tightly connected with the AvrM protein levels in the leaf. Apart from the HR decreased in the dark, the protein in the blot was higher in all the dark pre-treated leaves. This suggests that given sufficient photosynthetically derived substrates, only a small amount of AvrM is required to trigger M-mediated HR. When photosynthetic substrate is limiting, more AvrM is required to activate the HR.

The second hypothesis of this delay HR in the dark could come from the absence of ROS. Many previous researchers showed that ROS is related directly to many biological mechanisms of plants. Firstly, it is undeniable that ROS takes part in many essential processes such as immune defence activation, uptake nutrient and cell differentiation (Weinberger, 2007, Roe and Barbeau, 2014, Cieślar-Pobuda et al., 2017). It has become apparent that ROS, produced in the chloroplast, is crucial in the execution of localised programmed cell death (Zurbriggen et al., 2010, Floryszak-Wieczorek and Arasimowicz-Jelonek, 2016). Under stress conditions, ROS was maintained at a low level and considered as a signalling molecule that controls programmed cell death (Dietz et al., 2016). Therefore, insufficient ROS levels in the dark could lead to a dampened HR.

In this study, we can see at the point time dark after infiltration 24 hours observed the highest protein and full HR. This could be at this point time the plants accumulated enough ATP and essential compounds that provide nutrient for the development of the effector protein and then HR to help plant protect themselves avoid the pathogen attraction.

5.4. Conclusion

From these results it is clear that the HR elicited in W38: *M* by M/AvrM interaction is dependent on light. This study showed that the HR could be prevented by prolonged dark treatment prior to infiltration. It is interesting that the amount of effector protein in the leaves seem to be equivalent between pre-treatment 1, 2 and 3 days in the dark; However, the observation *in planta* were different. The 1-day pre-treatment showed full HR while other treatment 2 and 3 days observed weak and no HR, respectively. In comparison with the control, they revealed the full HR and low level of effector protein.

5.5. Further work

Light affects many processes in plants, and one of these is plant-pathogen interaction. In this study I have shown the HR phenotype of plant leaf in relation to effector protein accumulation. However, the biochemical link between photosynthetic activity and/or other photo receptors in

plants and the activation of the HR mediated by NLRs is still unclear. As we continue to understand more about how NLRs are activated and the role of downstream proteins, we will hopefully find out why light is required for expression of the HR. This knowledge may help to improve resistance in our crop plants in the future that help shape and engineer new and novel forms of disease resistance.

In the next experiments, I would like to suggest measuring the amount of ATP by a luminescent-based assay to compare the ATP production in the light and dark. Moreover, try to measure the effect of light on the HR in a shorter daylength, such as 8 hours light and 16 hours dark.

APPENDIXS

Appendix 1: Sodium dodecyl sulphate polyacrylamide gel preparation

15% Resolving gel

No. of gel	1	2	3	4	5	6	7	8
Acrylamide	1.875	3.750	5.625	7.500	9.375	11.250	13.125	15.000
Mili-Q H_2O	1.798	3.595	5.393	7.190	8.988	10.785	12.583	14.380
1.5M Tris HCl pH 8.8	1.250	2.500	3.750	5.000	6.250	7.500	8.750	10.000
10% SDS	0.050	0.100	0.150	0.200	0.250	0.300	0.350	0.400
APS	0.025	0.050	0.075	0.100	0.100	0.175	0.175	0.200
TEMED	0.003	0.005	0.008	0.010	0.013	0.018	0.018	0.020

Stacking Gel

No. of gel	1	2	3	4	5	6	7	8
Acrylamide	0.2500	0.500	0.750	1.000	1.250	1.500	1.750	2.000
Mili-Q H_2O	1.585	3.170	4.755	6.340	7.925	9.510	11.095	12.68
1.5M Tris HCl pH 6.8	0.625	1.250	1.875	2.500	3.125	3.750	4.375	5.000
10% SDS	0.025	0.050	0.075	0.100	0.125	0.150	0.175	0.200
APS	0.0125	0.025	0.0375	0.050	0.063	0.075	0.088	0.100
TEMED	0.0025	0.005	0.0075	0.01	0.013	0.015	0.018	0.020

Appendix 2: The sequence of the AvrM E237A + E309A mutant

```
AvrM E237A + E309A   1   CAACCAGAATTTGACAGAGGATTCCTTAGACCTTTTGGAGCAAAAATGAAATTCCTCAAG   60
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                  1   CAACCAGAATTTGACAGAGGATTCCTTAGACCTTTTGGAGCAAAAATGAAATTCCTCAAG   60

AvrM E237A+ E309A   61   CCGGACCAAGTTCAGAAACTTTCTACAGATGATCTCATCACATACATGGCAGAAAAAGAT   120
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                 61   CCGGACCAAGTTCAGAAACTTTCTACAGATGATCTCATCACATACATGGCAGAAAAAGAT   120

AvrM E237A + E309A 121   AAAAATGTACGAGACCTGGCCATCAAACTTCGCGATGCCAAACAGGACTCCACAAAAAAT   180
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                121   AAAAATGTACGAGACCTGGCCATCAAACTTCGCGATGCCAAACAGGACTCCACAAAAAAT   180

AvrM E237A + E309A 181   GGTACACCGGAAATCAAACAAACGTACGACAAGGCTTATGAAAAAACCAAGGCGGCTGCT   240
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                181   GGTACACCGGAAATCAAACAAACGTACGACAAGGCTTATGAAAAAACCAAGGCGGCTGCT   240

AvrM E237A + E309A 241   GAAAAGCTGGTCTCGGAAGAATCACTCACACGAGACGCGCTCTTGAAGCTCACCGAGGAG   300
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                241   GAAAAGCTGGTCTCGGAAGAATCACTCACACGAGACGCGCTCTTGAAGCTCACCGAGGAG   300

AvrM E237A + E309A 301   CAATATGTAGAAAAAGCAGCATTATTTGACAAAGATGTGTATCGAAACAATCTCAAGAGG   360
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                301   CAATATGTAGAAAAAGCAGCATTATTTGACAAAGATGTGTATCGAAACAATCTCAAGAGG   360

AvrM E237A + E309A 361   CAAACTTATGAAAAACTTCTACGTTCCGCTACGGATGTTTTGTATAGGGAGGTTGCTAGA   420
                         |||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||
AvrM                361   CAAACTTATGAAAAACTTCTACGTTCCGAGACGGATGTTTTGTATAGGGAGGTTGCTAGA   420

AvrM E237A + E309A 421   ATATTCATCGCCAGGGAGGGTGAACCGGCGTTAACGGCGAAGATCGAACGCTTAGCTCTG   480
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                421   ATATTCATCGCCAGGGAGGGTGAACCGGCGTTAACGGCGAAGATCGAACGCTTAGCTCTG   480

AvrM E237A + E309A 481   ACTTTGGAAAACAACGCAGACACCCGAAGCAAACCAATTGATTACCTTGCTATCGCTGCG   540
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                481   ACTTTGGAAAACAACGCAGACACCCGAAGCAAACCAATTGATTACCTTGCTATCGCTGCG   540

AvrM E237A + E309A 541   GACTTCCTCAAAAACCAAGCAAACCTCCATGCAGACGATCCAGAGTTGAATTTGTATAAG   600
                         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                541   GACTTCCTCAAAAACCAAGCAAACCTCCATGCAGACGATCCAGAGTTGAATTTGTATAAG   600

AvrM E237A + E309A 601   GCTGCTATCAAGGCGCGTGAAATTGAAGCCAACAGAGCTATGAAAGAAGCTTTGAAAGGT   660
                         ||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                601   GCTGAGATCAAGGCGCGTGAAATTGAAGCCAACAGAGCTATGAAAGAAGCTTTGAAAGGT   660

AvrM E237A + E309A 661   GCTGATAAACTATTCAAACGCAACAAGATATTGAAATCTCCAGACAT   707
                         ||||||||||||||||||||||||||||||||||||||||||||||||
AvrM                661   GCTGATAAACTATTCAAACGCAACAAGATATTGAAATCTCCAGACAT   707
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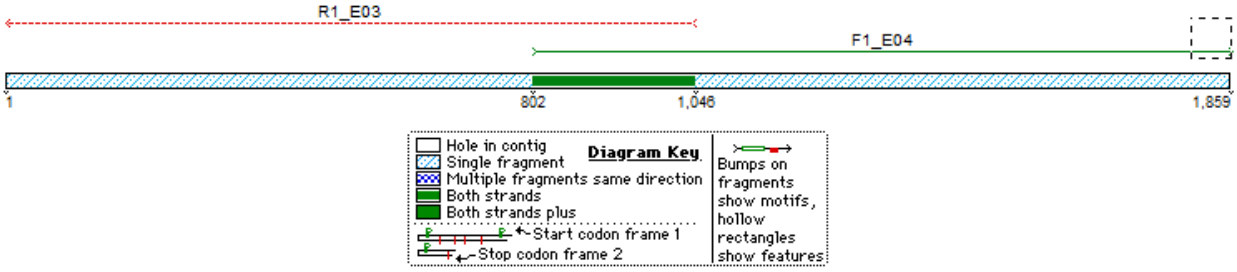
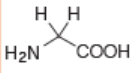
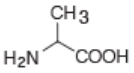


Appendix 3: The structure and properties of amino acids

SMALL

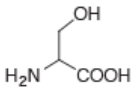


Glycine (Gly, G)
MW: 75.07

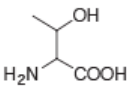


Alanine (Ala, A)
MW: 89.09

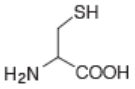
NUCLEOPHILIC



Serine (Ser, S)
MW: 105.09, pK_a ~ 16

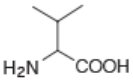


Threonine (Thr, T)
MW: 119.1, pK_a ~ 16

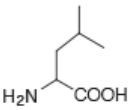


Cysteine (Cys, C)
MW: 121.2, pK_a = 8.18

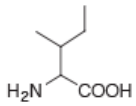
HYDROPHOBIC



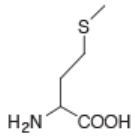
Valine (Val, V)
MW: 117.1



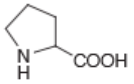
Leucine (Leu, L)
MW: 131.2



Isoleucine (Ile, I)
MW: 131.2

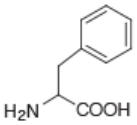


Methionine (Met, M)
MW: 149.2

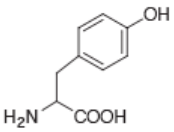


Proline (Pro, P)
MW: 115.1

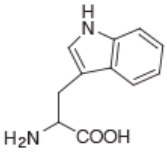
AROMATIC



Phenylalanine (Phe, F)
MW: 165.2

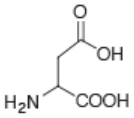


Tyrosine (Tyr, Y)
MW: 181.2, pK_a = 10.46

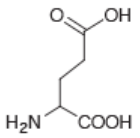


Tryptophan (Trp, W)
MW: 204.2

ACIDIC

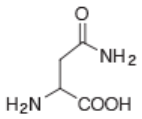


Aspartic Acid (Asp, D)
MW: 133.1, pK_a = 3.9

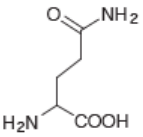


Glutamic Acid (Glu, E)
MW: 147.1, pK_a = 4.07

AMIDE

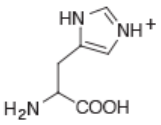


Asparagine (Asn, N)
MW: 132.1

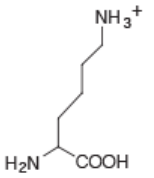


Glutamine (Gln, Q)
MW: 146.1

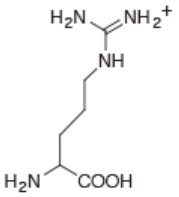
BASIC



Histidine (His, H)
MW: 155.2, pK_a = 6.04



Lysine (Lys, K)
MW: 146.2, pK_a = 10.79



Arginine (Arg, R)
MW: 174.2, pK_a = 12.48

APPENDIX 4: THE *PONCEAU* OF THE BLOT

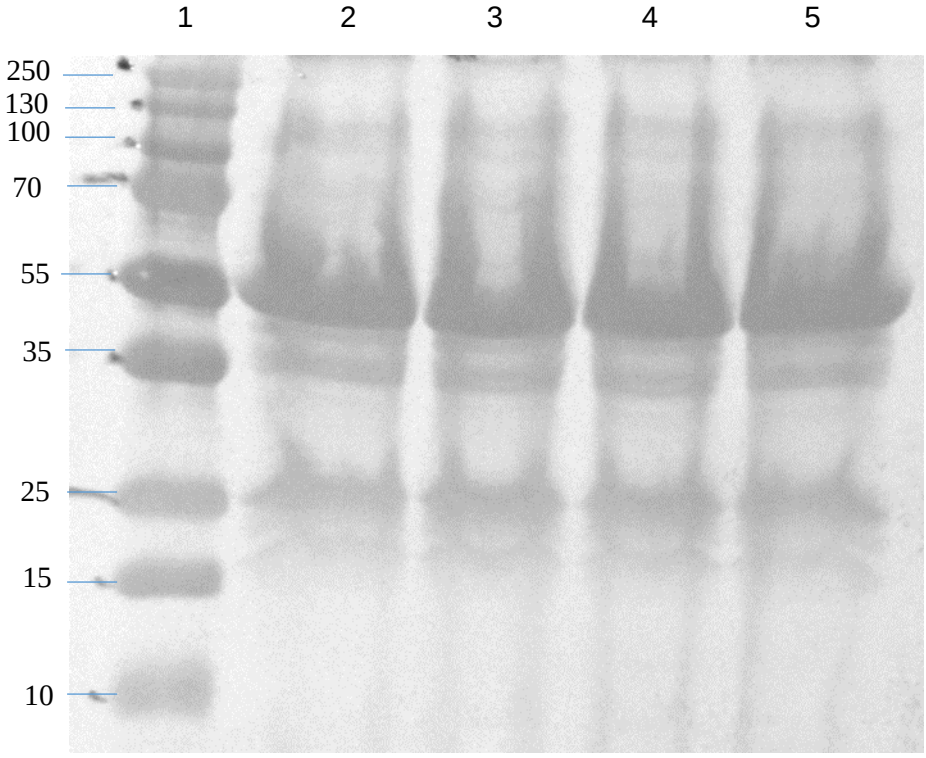


Figure 1: The Blot of AvrM mutants in polymorphic residues

The leaf tissue was extracted following the protocol 2.2.5 and 2.2.6, after that run in the SDS-PAGE and transferred onto the nitrocellulose membrane. The effector protein content of total protein extracts determined by ponceau staining and anti-HA immuno labelling with the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies, respectively. The membrane showed the amount of Rubisco protein in four samples of tissues of un-infiltrated, AvrM, avrM and GV3101. The unstained protein ladder neb was used in the well 1 that considered as a ladder.

The order in the blot are as follows

1. Ladder
2. AvrM K232R + L241S
3. AvrM K232R + I310T
4. AvrM L241S + I310T
5. AvrM K232R + L241S + I310T

APPENDIX 5:

THE BLOT AFTER INCUBATING WITH CHEMILUMINESCENCE REAGENTS

1 2 3 4 5

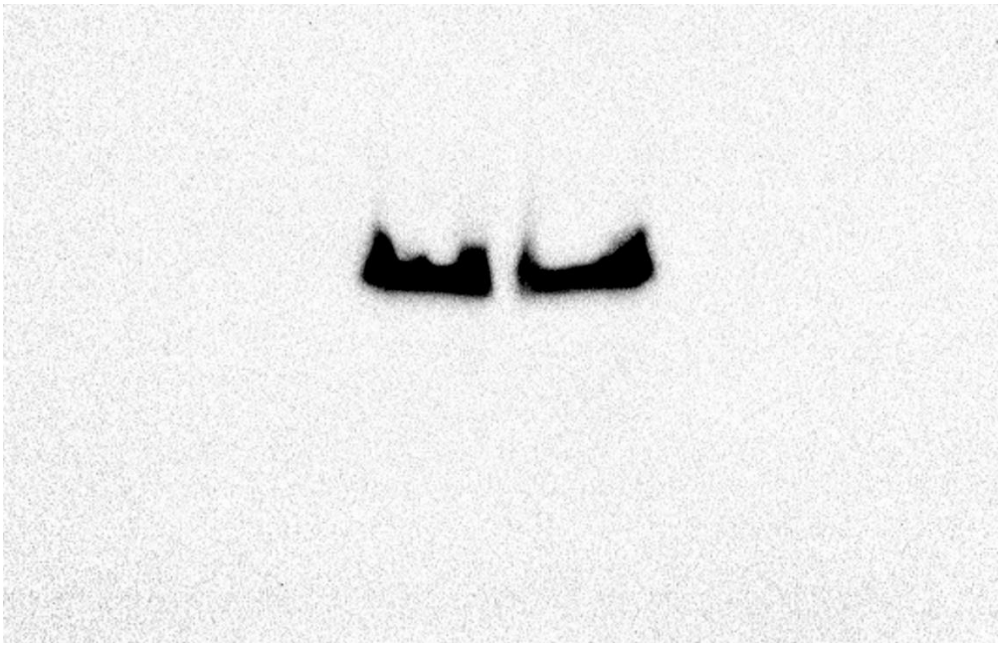


Figure 2: The presence of Effector protein in AvrM and avrM tissue leaves

Effector protein was extracted from the infiltrated leaf tissue in N. benthamiana after infiltration 24 hours. The order in the gel were as follow: well 1: ladder, well 2: un-infiltrated leaf; well 3: AvrM effector protein; well 4: avrM effector protein; well 5: GV3101. The effector protein determined by ponceau staining and immune labelling with the horseradish peroxidase conjugated goat anti-mouse and mouse anti-HA antibodies, respectively. After incubating with Chemiluminescence reagents and visualizing in the Bio-Rad ChemiDoc^{TM} MP system, the Effector protein presented in the tissue of AvrM and avrM samples while there was not have effector protein in the samples of ladder, un-infiltrated and GV3101.

APPENDIX 6: THE SEQUENCES OF FORWARD AND REVERSED PRIMER OF AvrM E237A + E309A MUTANTS

Forward primer:

AvrM K226Q GTATCGAAACAATCTCCAGAGGCAAACTTATG

Reverse primer:

AvrM E316K AGCTCTGTTGGCTTTAATTTCACGCGCC

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